COMPLEX COPPER COMPOUNDS WITH PENTAAMINOTETRAZOLE ARE THE NEW CHALLENGE IN TREATMENT AND PREVENTION OF FREE-RADICAL CONDITIONS

Irina Shugalei, Mikhail Ilyushin, Veronika Sokolova, Nadejda Dubjago, Irina Bachurina, Alexander Garabadzhiu*

St. Petersburg State University of Technology (Technical University)
26Moskovskyave., St. Petersburg, 190013 Russia,
Tel.+79643628922, e-mail: shugalei@mail.ruand gar-54@mail.ru

Abstract: The present article provides route of synthesis of complex copper compounds with 1.5-pentamethyltetrazole as a ligand. These compounds are considered promising antioxidants that can find application in treatment and prevention of "free radical" conditions caused by a variety of reasons, e.g. harmful environmental factors. The obtained compounds were rated by their antioxidant potential; the ability to inhibit lipid peroxidation and SOD-like activity thereof were assessed by independent experimental techniques.

Keywords: complex compounds with 1.5-pentamethyltetrazole as a ligand, antioxidants, lipid peroxidation, SOD-like activity

Introduction

Human body needs oxygen to enable active performance of its organs and systems. All major metabolic processes are based on oxidation-reduction reactions. In the mitochondria molecules of oxygen are not converted to water completely, up to 5% form highly reactive free radicals [1]. The products of free radical reactions are highly toxic oxygen containing radicals and peroxidates known as reactive oxygen species, hereinafter referred to as ROS [2]. These highly reactive ROS interact with a wide range of biomolecules [3-6]. To maintain desired level of free radical pool at the adverse environment is the critical issue of the day [7-10].

Non-specific immunity comes from the mechanism of free radical formation [11, 12]. Phagocytosis causes multiple increase of free radical amount in the phagocytizing cells and 20-fold oxygen uptake boost [13].

However, overactivation of free radical oxidation reactions presents typical pathological process that occurs during various medical conditions [14] and damaging influences. It is proved that various disease processes have free radical mechanism, e.g. shocks, atherosclerosis, brain circulation failure, peripheral circulation failure, coronary circulation failure [15], diabetes mellitis and diabetic angiopathy [16], rheumatoid, inflammatory and degenerative diseases of locomotor system, eye lesions, pneumopathy, oncopathology, thermal injuries, various toxicoses, oncological diseases [17, 18], reperfusion injuries [19-22]. Overproduction of free radicals induced by harmful factors is the general reason of premature aging [23, 24].

Human body has natural protective mechanisms from free radical loads. Multi-component antioxidant defense system includes enzymes, vitamins, low molecular weight species [25-27].

Huge loads of xenobiotics drop upon human bodies in our anthropogenic age. The body can not clear the whole amount of extra free radicals, which results in mismatch between the substances inducing free radical formation(prooxidants) and substances lowering the level of radical metabolites – antioxidants (AOs). This mistmatch leads to oxidative stress [28-30]. Hence, natural antioxidant defense system requires continuous supply of specific compounds to maintain its effectiveness [31].

These substances vary over a wide range; they have different structures and derive from different origin. Their classific concepts have been established. Currently we recognize the following groups of antioxidants:

1. Antiradical agents:
   - 1.1. Endogenous substances: vitamin E, vitamin C, vitamin A, carotene (provitamin A), ubiquinone, lycopene;
   - 1.2. Synthetic chemicals: ionol (dibunole), emoxipine, probucol (fenbutol), dimethyl sulfoxide (dimexide), olifen (hypoxene);
   - 1.3. Antioxidant enzymes and activators: superoxide dismutase (Erisod. Ergotein), sodium selenite;
   - 1.4. Free radical formation blockers: Allopurinol (Milurit), antihypoxants.

Complex compounds as antioxidants

Despite the wide range of existing drugs regulating free radical reactions within the body there is a constant search for new preparations. A recent trial explores complex compounds as antioxidants [34-37]. Currently complex compounds with nitrous ligands are considered promising antioxidants [38-40]. It is established that metal complexes

with antioxidants have a significant effect on longevity of animals in acute hypoxic states. There is evidence of distinct anti-hypoxic properties of compounds with cupric complexing agent [41-43].

In this work we have obtained new copper complexes with polynitrous ligand 1.5-pentamethyl tetrazole (corazole): copper {II) bis-(1.5-pentamethyl tetrazole) perchlorate $[Cu(PMT)_2]*(ClO_4)_2$; copper {II) tetra-(1,5-pentamethyl tetrazole) chloride $[Cu(PMT)Cl_2$, copper {II) 1,5-pentamethylene tetrazoleascorbate $[Cu(PMT)_{1/2}]*$(HAsc) and studied their antioxidant properties.

The antioxidant properties of compounds are assessed by their ability to react with active oxygen species (AOS): H_2O_2; OH^-; O_2^-. Traditional antioxidants react with all of these ROS, but the speed of interaction depends mainly on AO structure [44]. Various AO agents react mostly with one of oxygen radicals. Various AO agents react mostly with one specific oxygen radical. The specified complex compounds will probably interact with O_2^- by accepting one electron, which leads to the termination of biomolecule radical oxidation.

We also assessed antioxidant potential of corazole (1,5-pentamethylene tetrazole) considering its significant biological properties: it is known that corazole acts as a free radical scavenger.

To make the picture complete we used several evaluation methods considering that in the standard conditions a number of AOS are generated simultaneously with their further mutual transformation.

Estimation of antioxidant potential using lipid peroxidation model

An antioxidant potential of any agent is routinely assessed by its ability to inhibit lipid peroxidation [45].

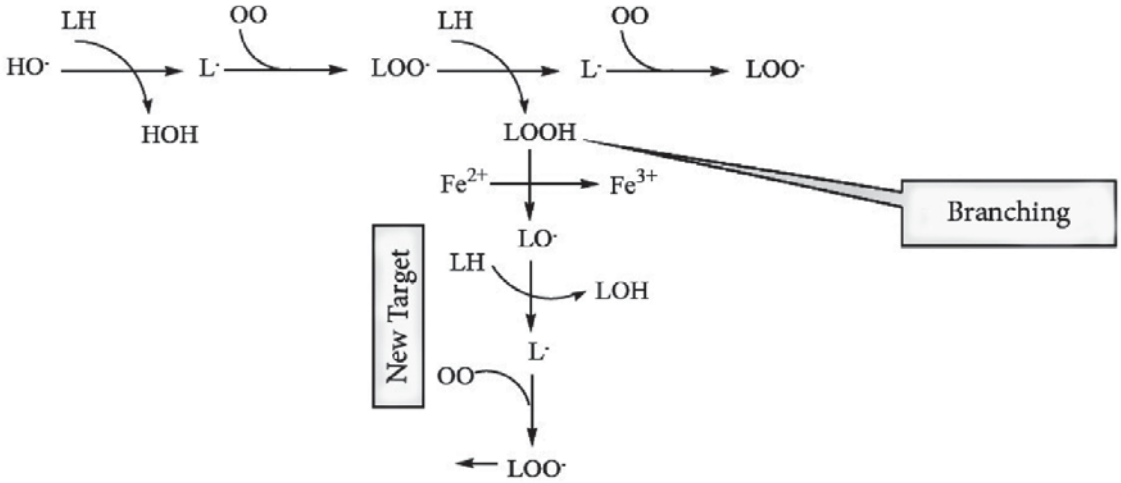


Fig. 1. Schematic diagram of lipid peroxidation

Lipids serve as one of the main substrates for free radical reactions. Among them molecules of polyunsaturated fatty acids (FA) and lipid components of very little density lipoproteins and low density lipoproteins (VLDL and LDL, respectively) stand out. The result of such interaction is complex free radical process referred to as lipid peroxidation (LP) [45-47].

Actually the process of lipid peroxidation is thoroughly studied by means of chemical kinetics and is classified as chain radical reaction [45]. General diagram of this process is given in the picture above.

This process is accompanied by slight chemiluminescence [48]. If the agent reduces chemiluminescence intensity then it is considered to have antioxidant properties.

The more chemiluminescence fades upon administration of an agent, the higher its antioxidant properties are. Studies were carried out in standard systems. A suspension of yolk liposomes in buffer solution or normal saline is a one of these systems. We have chosen a suspension of liposomes in the phosphate buffer (pH=7.4). Cystamine ($NH_2CH_2CH_2SH$) known for its scavenging and chemiluminescence reducing properties was used as a standard [50]. Compounds with thiol fragments in their chemical structure are known as effective antioxidants [51, 52].

In case of chemiluminescence fading upon the administration of ligand (1,5-pentamethyl tetrazole) we can speak of its antioxidant properties. If chemiluminescence suppression upon administration of tested compound achieves the same level of cystamine, such compound can be considered as promising antioxidants. Data for chemiluminescence suppression is summarized in Table 1.

Two criteria were used to assess chemiluminescence suppression:

- Light sum decrease;
- Reduction of peak intensity.

Table 1

Comparison of compounds in respect of their ability to quench chemiluminescence at concentration $5x10^{-6}$ M in the liposome suspension at 20^0C, pH=7.4, c($FeSO_4$)=$2.4x10^{-3}$ M

No. of compound	Compound	Light Sum, AU	Light Sum Decrease, %	Peak Intensity, AU	Decrease of Peak Intensity, %
	Control	116±3	0	109±3	0
I	$[Cu(PMT)_2](ClO_4)_2$	67±3	42	106±3	3
II	$[Cu(PMT)_4](ClO_4)_2$	108±3	7	144±3	Increase of 32
III	$[Cu(PMT)](Cl)_2$	51±3	56	107±2	2
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	84±2	27	123±2	Increase of 13
V	PMT	84±2	27	130±2	Increase of 19
VI	KHAsc	96±2	17	138±2	Increase of 27
VII	$CuSO_4*5H_2O$	85±2	27	48±2	56

It should be noted that there is no symbasis in the alteration of light sum and peak intensity values. However, light sum is the most often used value for antioxidant activity evaluation. Such choice of parameter for antioxidant activity evaluation is reasonable because the process of free radical formation during chain reaction in the biological system can have an erratic character. A short-term acceleration of free radical reactionsis possible, but overall amount of radicals produced during specified time can be less. Taking light sum as a characteristic of lipid peroxidation intensity and with reference to the results shown in Table 1 all studied compounds **I-VII** are antioxidants. Administration of corazole (PMT) in the internal sphere leads to an increase of antioxidant activity. The number of coordinated corazole molecules has an impact onchemiluminescence suppression for compounds **I** and **II**. Upon administration of two corazole molecules in the internal complex sphere chemiluminescence is reduced by 42%, showing significant antioxidant activity. Administration of two more corazole moleculesresults in almost complete quenching of antioxidant properties, as evidenced by recovery of chemiluminescence up to control level.

Study of SOD-like activity of complexes

There is a negligible effect of anion on antioxidant activity of the complex (compounds III, I). However, introducing of a reducing agent into external sphere, e.g. ascorbic acid (HAsc) significantly reduces antioxidant properties. We can't clearly determine correlation between antioxidant activity and nature of anion, since the number of corazole molecules coordinated in the internal complex sphere changes depending on the anion. In this regard it is interesting to study SOD-like activity of specified compounds.

This approach was chosen due to strong realization of the following processes in vivo [53, 54]:

$$Me^{n+} + O_2 \rightarrow Me^{(n+1)+} + O_2^{\cdot-} \quad (1)$$
$$Me^{n+} + O_2^{\cdot-} \rightarrow Me^{(n-1)+} + O_2 \quad (2)$$

Catalyzing ability of compounds of such form:

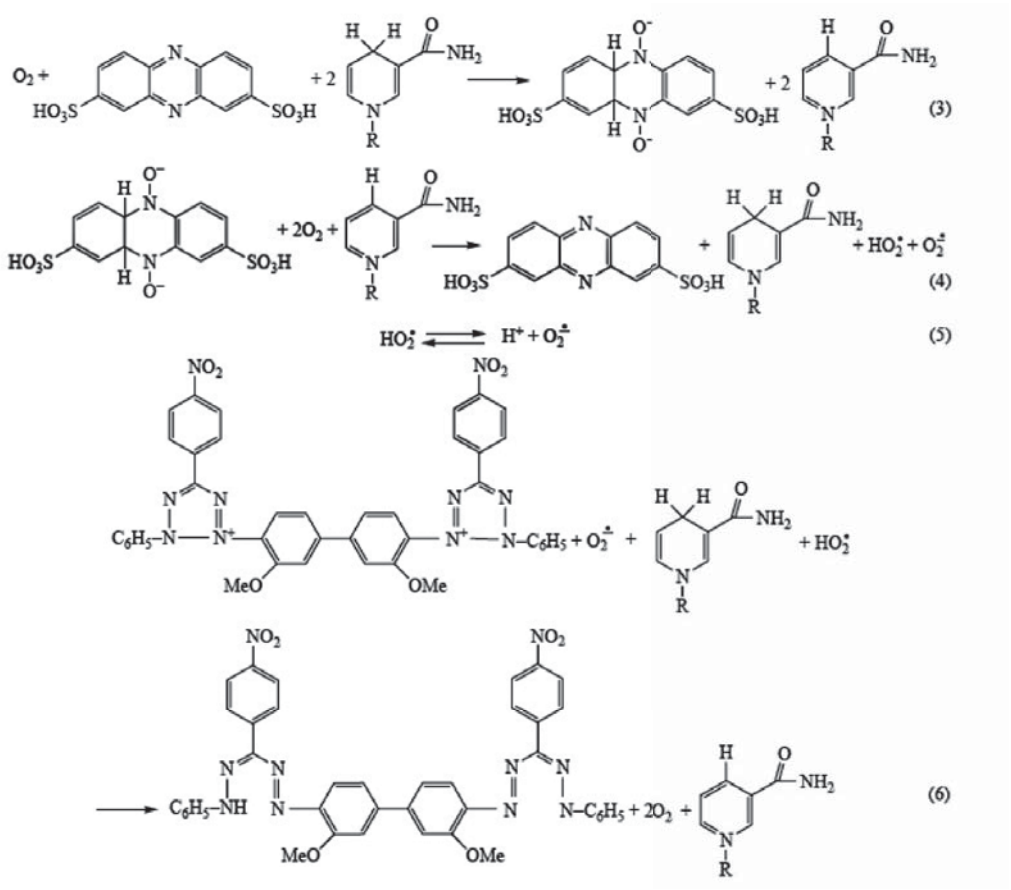
$$2O_2^{\cdot-} + 2H^+ = H_2O_2 + O_2,$$

or any other way to clear superoxide anion radical is called SOD-like activity [55], since this process *in vivo* is catalyzed by specific enzyme called superoxide dismutase (SOD) [56, 57].

Complex compounds can change superoxide anion radical level in biological systems [58]. SOD-like activity was assessed by the ability of compounds to inhibit rate of tetrazolium reduction to formazan in the standard conditions. Equations of test system reactions with paranitrobluetetrazolium, reduced nicotineamide-adenine dinucleotide ($MADH_2$), phenazinemethasulfate (PMS) are shown below (eq.(3)-(6).Products of inhibition of paranitrobluetetrazolium reduction to formazan induced by synthesized corazole complexes are shown in Table 2.

Standard method of superoxide dismutase activity determination requires presence of EDTA in the reaction medium [59].

Since EDTA is a strong complexing agent able to substitute polynitrogen ligands in the internal complex sphere [60], we decided to study its potential effect on the obtained results [61]. Similar experiments were carried out in the EDTA-free system (Table 3).



Out of the analysis of Tables 2 and 3 it can be concluded that:

(1) During the study of SOD-like activity it was found that copper sulfate **VII** has a pronounced ability for inhibition of paranitrobluetetrazolium reduction to formazan. The explored coordination compounds **I-IV** show similar activity, while free ligand and salts in the absence of copper cation don't have any antioxidant activity. Removal of $O_2^{\cdot-}$ apparently depends on the presence of copper.

(2) Copper reduction reaction is as follows:

$Cu^{2+} + O_2^{\cdot-} \rightarrow Cu^{+} + O_2$.

Table 2

Results of SOD-like activity assessment of synthesized complexes in the presence of EDTA[a]

No of compound	Compound	Optical density, D	Quenching, %
	Control	0.268±0.02	0
I	$[Cu(PMT)_2](ClO_4)_2$	0.229±0.02	16±3
II	$[Cu(PMT)_4](ClO_4)_2$	0.223±0.02	17±3
III	$[Cu(PMT)](Cl)_2$	0.183±0.02	32±3
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	0.199±0.02	26±4
V	PMT	0.242±0.01	10±5
VI	KHAsc	0.241±0.01	10±1
VII	$CuSO_4*5H_2O$	0.167±0.02	38±4

[a] EDTA – ethylenediaminetetraacetate

Table 3

Results of SOD-like activity assessment of synthesized complexes in the absence of EDTA[a]

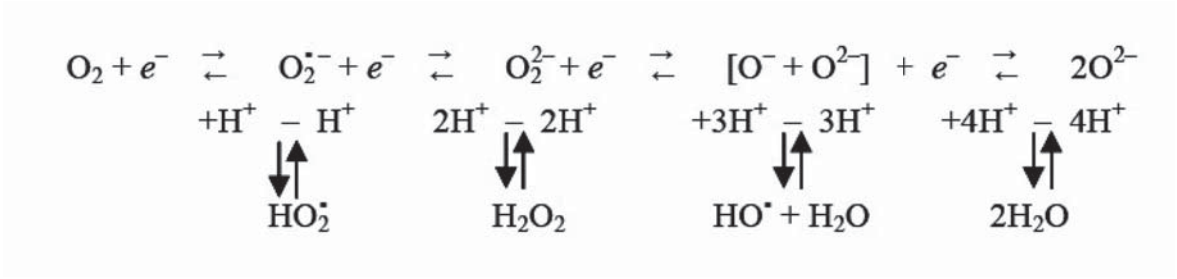
No of compound	Compound	Optical density, D	Quenching, %
	Control	0.180±0.02	0
I	$[Cu(PMT)_2](ClO_4)_2$	0.109±0.02	39±3
II	$[Cu(PMT)_4](ClO_4)_2$	0.106±0.02	41±3

III	$[Cu(PMT)](Cl)_2$	0.090±0.02	50±3
IV	$[Cu(PMT)_{1/2}](HAsc)_2$	0.094±0.02	48±5
V	PMT	0.167±0.01	7±1
VI	KHAsc	0.179±0.01	1±4
VII	$CuSO_4*SH_2O$	0.110±0.02	39±2

(3) Introduction of EDTA does not have a significant effect on the ability of copper compounds to interact with superoxide anion radical ($O_2^{\cdot-}$). However, when introduced in the system, it coordinates with copper ion and substitutescorazole from compounds **I** and **II** either in part or in whole, or enters into the internal sphere of complexes **III** and **IV,** increasing coordination number to four. Probably it causes inhibition of SOD-like activity of compounds **I**, **II**, **III** and **IV** in the EDTA medium relative to SOD-like activity of these compounds in the EDTA-free medium.

(4) In the EDTA system an external sphere anion affects the ability of compounds to exhibit SOD-like activity. The most decrease of reactivity was noted when perchlorate ion was used as an anion (compounds I and II).

In two independent tests the same compound $[Cu(PMT)]Cl_2$ demonstrated significant antioxidant activity. It follows that chemiluminescence quenching is probably associated withthe interruption of successive oxygen reduction chain:



(ROO· equivalent) (ROOH equivalent)

by means of O_2^- clearance. In this case hydroxyl radical – an active initiating agent of lipid peroxidation – is not produced, resulting in chemiluminescence decrease.

Conclusion

Compound $[Cu(PMT)]Cl_2$ can be characterized as a potential and promising antioxidant by virtue of chemiluminescencequenching and SOD-like activity.

Experimental part

General procedure of synthesis of the complex compounds

The synthesis of complexes is done according to the following reaction:

$M(An)x + nPMT \leftrightarrow [M(PMT)_n](An)_x$,
where M=Cu(II); An=Cl^-, ClO_4; n= 1, 2, 4.

Aqueous solution of salt is stirred at room temperature and spiked with estimated amount of PMT. Reaction mass is then heated to 60^0C and incubated at this temperature during 3 hours. After cooling to 17^0C precipitated residue is filtered, washed with 20 ml of ethyl acetate and recrystallized from ethyl alcohol.

Synthesis of copper (II) bis-(1,5-pentamethylene tetrazole) perchlorate $[Cu(PMT)_2](ClO_4)_2$

Yield 60%.IR spectra, ν, cm^{-1}: 2860 [$\nu_s(CH_2)$]; 1925 [ν_{as} (CH_2)]; 1470 (scissor CH_2); 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N-N); 1100-800 (Tz). NMR spectra ^1H (DMSO0-d_6), δ, ppm: 4.41 [CH_2N^1)]; 2.92 [$CH_2(C^5)$]; 1.81, 1.70, 1.57 (CH_2-CH_2-CH_2). Found, %: C 34.99; H 5.1; N 27.49. $C_{12}H_{32}Cl_3*CoN_{12}O_{12.}$ Calculated, %: C 35.4; H 5.95; N 27.5.

Synthesis of copper (II) tetrakis-(1,5-pentamethylene tetrazole) perchlorate $[Cu(PMT)_4](ClO_4)_2$

Yield was 0.61 g of light blue product (60% of theoretical). IR spectra ν, cm^{-1}: 2860 [$\nu_s(CH_2)$]; 1925 [ν_{as} (CH_2)]; 1470 (scissor CH_2); 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N-N); 1100-800 (Tz). NMR spectra

^{1}H (DMSO0-d_6), δ, ppm: 4.41 [CH_2N^1)]; 2.92 [$CH_2(C^5)$]; 1.81, 1.70, 1.57 (CH_2-CH_2-CH_2). Found, %: C 34.99; H 5.1; N 27.49. $C_{12}H_{32}Cl_3$*$CoN_{12}O_{12.}$ Calculated, %: C 35.4; H 5.95; N 27.5.

If isopropyl alcohol was used as a solvent, the yield of final product was bigger. Yield was 0.81 g of light blue product (80% of theoretical). IR spectra ν, cm^{-1}: 2860 [ν_s{CH_2)]; 1925 [ν_{as} (CH_2)]; 1470 (scissor CH_2); 1454 (N=N); 1352, 1337 (C=N); 1282, 1268 (N-N); 1100-800 (Tz). NMR spectra ^{1}H (DMSO0-d_6), δ, ppm: 4.41 [CH_2N^1)]; 2.92 [$CH_2(C^5)$]; 1.81, 1.70, 1.57 (CH_2-CH_2-CH_2). Found, %: C 35.2; H 5.2; N 27.5.

Synthesis of copper (II) 1,5-pentamethylene tetrazole[Cu(PMT)]Cl_2

Yield was 0.83 g (83% of theoretical). Calculated, %: C 21.21, H 5.31, N 16.49. $CuC_6H_{18}N_4O_4Cl_2$. Found, %: C 21.17; H 5.37; N 16.13. IR spectra, cm^{-1}: 3163 [$\nu_{as}(H_2O)$]; 1629 (δ(H_2O)]; 2908 [$\nu_s(CH_2)$]; 1477 [$\nu_s(CH_2)$]; 2943, 1535, 1421, 968 (Tetr).

PMT is an off-the-shelf product manufactured by ACROS ORGANICS, purity 98%, melting point 58.5^{0}C.

SOD-like activity measurement

We used spectrophotometry to study SOD-like activity. The following chemicals were applied during our study:

(1) Paranitrobluetetrazolium solution: 100 mg of anhydrous substance were dissolved in 50 ml of distilled water on hot water bath, filtered and stored in the dark (it is light sensitive).
(2) Phenasinemethosulfate(FMS): 1 mg was dissolved in 100 ml of distilled water and stored in the dark (it is light sensitive).
(3) Nicotineamide dinucleotide phosphate ($NADH_2$) solution: 6 mg of anhydrous substance were dissolved in 3 ml of tris-EDTA buffer (pH=8.0) and 1.8 ml of distilled water, stored in the dark (it is light sensitive).
(4) Phosphate buffer, pH=7.8.

Reaction mixture has the following composition:

(a) 0.2 ml of EDTA solution (ethylenediaminetetraacetate);
(b) 0.1 ml of gelatin solution;
(c) 0.5 ml of paranitrobluetetrazolium;
(d) 0.1 ml of FMS (phenasinemethosulfate);
(e) 2.0 ml of phosphate solution.

We did a following procedure to study the relationship between complexes and reduction of paranitrobluetetrazolium in formazan. We made two control and test sample concurrently. In the control sample were successively introduced 2.0 ml of buffer; 0.5 ml of tetrazolium solution; 0.1 ml of FMS solution; 0.2 ml of distilled water; 0.2 ml of $NADH_2$. After the introduction of $NADH_2$ we switched on a stop watch and put the samples in thermostat under dark-room conditions. Optical density of the solution was measured in 10 minutes at λ=540 nm in comparison with buffer solution. Test sample was prepared similarly, only that 0.05-0.2 ml of studied complex solution was added instead of distilled water, and diluted to volume with distilled water. Paranitrobluetetrazolium was being reduced to formazan both in control and test solutions. The effect of preparations on the reduction of paranitrobluetetrazolium to formazan was estimated by comparison of optical densities of control and test solutions. Each measurement was repeated 3-5 times.

The study of antioxidant activity by chemiluminescence quenching was carried out in standard conditions in the suspension of yolk liposomes in phosphate buffer solution with pH=7.4. Cystamine was used as a standard.

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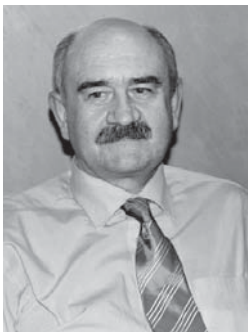

Professor Alexander Vasilievich Garabadzhiu is a Moldovan-born Russian chemist. His research interests are biopharmaceuticals, ecological biotechnology, biomedicine, bio-organic chemistry. Currently he is the Head of the Department of Technology of Microbiological synthesis and vice rector for scientific work of the St. Petersburg State Institute of Technology (Technical University).

Author of over 200 scientific works, 3 monographs, 4 books, more than 20 patents. He guidedand mentored numerous PhD students and2 Doctors of Science (habilitations). Acts as a member of the editorial boards of journals "General chemistry" and "Scientific instrumentation" of the Russian Academy of Sciences, the chief editor of the journal "Ecological chemistry".

Professor Alexander VasilievichGarabadzhiu is a laureate of the Prize of the Government of the Russian Federation in 2006 in the field of science and technology. His duties are also related to the membership of the Interdepartmental Council of the Ministry of Education and Science of the Russian Federation on awarding the Prizes of Russian Government in science and technology (section of life sciences).