Chemical and Biological Kinetics *New Horizons*

Volume 2: Chemical Kinetics

In commemoration of Professor N.M. Emanuel's 90th Anniversary

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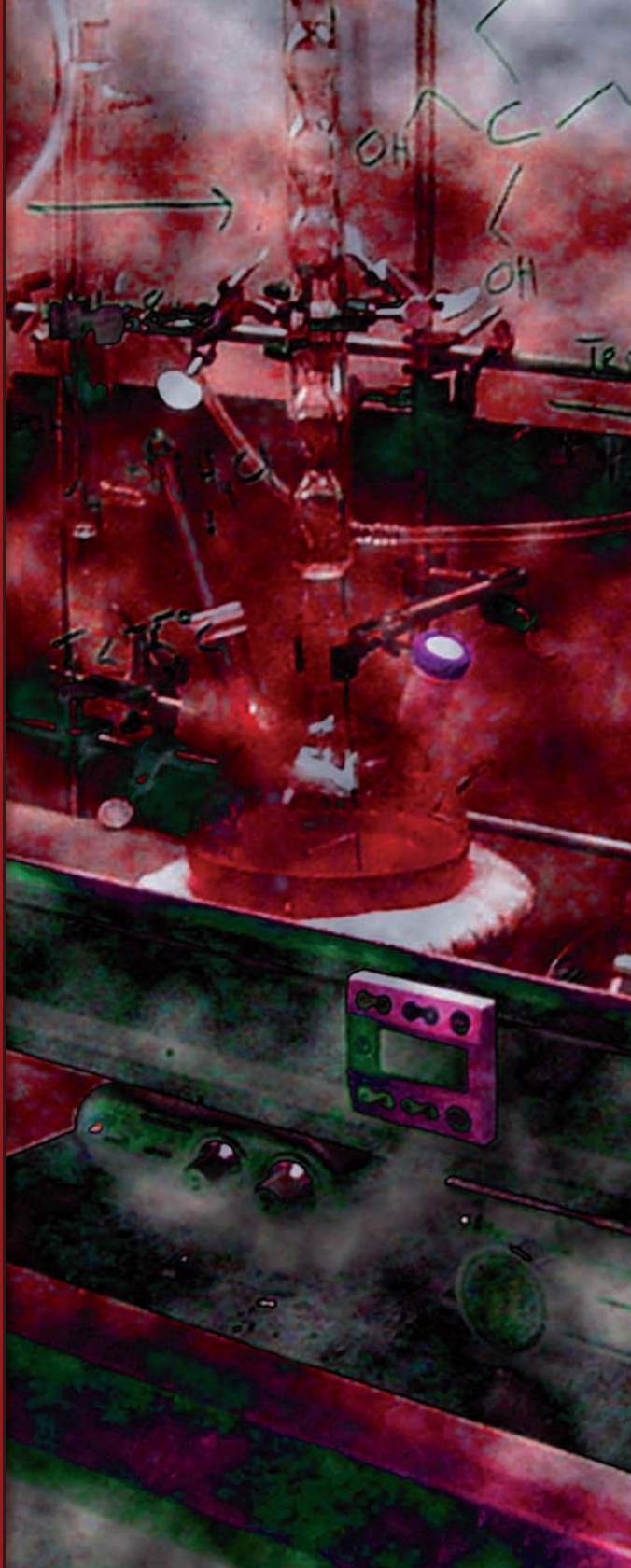

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In commemoration of Professor N.M. Emanuel's 90th Anniversary

Edited by E.B. Burlakova, S.D. Varfolomeev

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INTRODUCTION

The scientific legacy of Academician N.M. Emanuel is very wide and variable. He had no doubt that chemical kinetics should be the key point in understanding of many processes proceeding in the nature. In the article "Kinetics of some biological processes", included in the collection "Chemical kinetics and chain reactions", devoted to the 70th anniversary of Academician N.N. Semenov, N.M. Emanuel wrote that "Kinetics as the science about the laws and mechanisms of various process development in the nature obtains practically unlimited perspectives for theoretical studies and practical applications in biology".

The present book represents a good illustration of this idea. Many students and followers of N.M. Emanuel have implemented and are implementing their investigations being convinced in admiring abilities of the chemico-kinetic approach to the study of complex biochemical and biological phenomena.

In this book, devoted to the memory of Academician N.M. Emanuel, we have collected the articles by investigators of Emanuel's school, implemented in different fields of biological chemistry and medicine.

Studying the mechanisms of oxidative processes in chemical transformation of molecules (see volume I of this Collection), N.M. Emanuel has naturally passed to investigations of oxidative free-radical processes in biological systems. He and colleagues clearly recognized the great role of oxidative processes in the living systems, which is only now understood by representatives of other scientific directions.

Multiple processes of direct DNA degradation, oxidation of lipids, radiation damage and aging directly depend on the rate and mechanisms of oxidative free-radical processes. The cell must protect itself from the oxidative stress and, therefore, free-radical processes oxidative processes must be strictly controlled. These ideas have led N.M. Emanuel and his followers to the understanding of the important role of oxidative free-radical process inhibitors. Basing on this understanding, they have created the concept of bioantioxidants. In this book, the modern level of understanding of this problem is in various concerns discussed in the articles by E.B. Burlakova, N.G. Khrapova, A.V. Alessenko, N.P. Pal'mina, L.D. Smirnov and M.A. Ostrovsky *et al.*, N.N.Zoz, I.S. Morozova and A.M. Serebryanyi. It should be noted that the fundamental understanding of the problem and the reaction mechanisms has led to the

development of many drugs and protective agents, which are now widely applied in clinics. Unfortunately, this is not a tradition of our chemical practice.

When we are talking about kinetics of the processes in biosystems, we realize that kinetic components of biosystems are enzymes. The define rates and directions of biochemical reactions. N.M. Emanuel rose interest in the society of chemists to enzymes and enzymatic catalysis. A large group of scientists working in the field of chemical grounds of biocatalysis devote themselves to Emanuel's school. For many years, the Real Member of the Academy of Sciences of the USSR I.V. Berezin worked with N.M. Emanuel at the Cathedral of chemical kinetics of Chemical Faculty, Moscow State University. In this book, the mentioned direction is discussed in the works by S.D. Varfolomeev, N.F. Kazanskaya, N.N. Ugarova, V.N. Luzikov, and B.I. Kurganov.

The bright and difficult period in the life of N.M. Emanuel was associated with the initiation of cancer biochemistry investigations and synthesis of antitumor chemotherapeutic agents in the Institute of Chemical Physics, AS USSR. The followers of N.M. Emanuel have achieved a great success in this field. In the book this topic is discussed in articles by L.B. Gorbacheva, N.P. Konovalova, L.A. Ostrovskaya, and D.B. Korman *et al.*

Many factors damaging the cell, its organelles and molecular structure s are of the free-radical origin. In particular, radiochemical and radiobiological processes are of the highest importance in this connection. This is reflected in reviews by L.N. Shishkina and E.B. Burlakova, M.K. Pulatova et al., P.A. Kuzurman and V.A. Sharpatyi. The investigations by S.V. Vasil'eva should also be included in this list.

As is known, N.M. Emanuel was also interested in molecular aspects of gerontology and established several unique research groups in this direction in the Institute of Chemical Physics. The modern level of development of this branch is represented in works by I.N. Todorov, A.M. Olovnikov, L.K. Obukhova, D.I. Izmailov, and A.S. Solovieva.

This year we celebrate the 90th anniversary of N.M. Emanuel. In 1994, on the basis of the Department on Kinetics of Chemical and Biological Processes, Institute of Biochemical Physics, Named after N.M. Emanuel, was established. Many scholars and followers of N.M. Emanuel are working in it. The viability and dynamic development of Institute of Biochemical Physics is Our teacher's best memory.

Professor S.D. Varfolomeev
Professor E.B. Burlakova

BIOANTIOXIDANTS: YESTERDAY, TODAY, TOMORROW

E.B. Burlakova

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CHAPTER I. "YESTERDAY"

In 1954, the book by Prof. B.N. Tarusov (Biological Department, Moscow State University) "Fundamentals of Biological Action of Radioactive Radiation" [1], which caused the indelible impression on me. The author – the famous Soviet biophysicist, the biophysics cathedral leader at the Biological and Soil Department, MSU – came up with a hypothesis that the regularities of radiation disease development are cased by the induction of branched chain reaction of fat oxidation in cellular covers (membranes), which products are toxic for the cell.

For long time I dreamed to work in the field of the radiation disease mechanisms, but nobody dealt with them at the Chemical Department. At that time, two parts of my interests were united – chain reactions, which were the main subject of the investigations of chemical kinetics cathedral at Chemical Department of the Moscow State University, headed by the Nobel Prize Laureate, Academician N.N. Semenov, wherefrom I have graduated, and radiation disease, which was of interest for cathedral of biophysics. I've got a permission to defend the diploma in biology. My diploma was supervised by Academician N.M. Emanuel, Professors B.G. Dzantiev and G.B. Sergeev. This work was aimed at elucidation of the question about the products formed at irradiation of lipids, which may be toxic for bioobjects. For the model substrate of radiolytic oxidation of fat the natural fat of codfish liver – fish liver oil. Fat was radiated, after which its composition was determined, and then oxidized. It has been shown that even under high-dose radiation no determinable amounts of specific new products are formed, and toxicity of irradiated fat depends on its oxidation degree only. The higher the fat oxidation degree was and the greater amount of deep oxidation products were formed (aldehydes, ketones, peroxy acid, etc.), the higher toxicity of fat was. At equal oxidation degree, toxicity of both radiated and non-radiated fat was also equal [2 – 4].

The main radiation effect was reduced to degradation of natural antioxidants present in fat. Generally speaking, this result (decreasing amount of antioxidants under radiation) was not unexpected, because it had been already detected at radiation of various fats *in vitro*.

However, we were interested in the result of this work in the viewpoint of its urgency for radiation reactions. Simultaneous studies of the toxic effect from radiated fat for plant and animal objects allowed a conclusion that toxicity of radiated fat was related no to occurrence of any new oxidation products, but simply to acceleration of radiated fat oxidation, induced by degradation of natural antioxidants present in fats. The following practical conclusion is also doubtless: if we provide animals with antioxidants before radiation, this will delay processes, related to acceleration of oxidation of lipids and occurrence of oxidized toxic products. Therefore, compounds would be injected, which may serve as antioxidants degraded under radiation. Firstly, we injected natural antioxidant tocopherol to animals before the radiation impact, which increased the average lifetime if radiated animals. Then by analogy with the works on fat protection from oxidative damage, we injected nontoxic synthetic antioxidants, used in the food industry [4].

The principal meaning of this solution related not only to the unique technique of synthetic antioxidant injection to animals to protect them from radiation impact (though it was the new work in radiobiology), but also with injection of *synthetic* antioxidants to animals. E.g. we strongly believed that independently of particular structure of the antioxidant, and the origin of it (synthetic or natural), the most urgent was its ability to react with free radicals. We also suggested that this ability, which we determined in model experiments, may be preserved and displayed after antioxidant injection to animals.

To some extent, these experiments confirmed the ideas of B.N. Tarusov about the important role of chain (free-radical) reactions in lipids of radiated animals for radiation damage development.

In 1960, N.M. Emanuel suggested that not only radicals from lipids, but also radicals from other biochemical components of the cell (DNA, protein, polysaccharides, etc.), untypical of it in norm and formed under radiation impact, may induce multiple damages in the cell, which finally induce its death.

It has been shown that similar to radicals fro lipids, radicals occurring under radiation of DNA and proteins may enter exchange reactions with

antioxidants, which result in free valence transfer from biopolymer radical to antioxidant molecule. Hence, antioxidant radical $[InH]^{\bullet}$ is formed [5-8]:

$$R^{\bullet}_{biol} + HIn \rightarrow RH + In^{\bullet}.$$

In 1960, an important role of free radicals, untypical of the cell in norm, in development of other diseases and the possibility of decelerating these free-radical reaction and cause therapeutic effect with the help of synthetic inhibitors of radical processes was suggested [9 – 11]. Such suggestion could be made only by physicochemists, first of all, chemists–kinetics of the Semenov–Emanuel school who attach much importance not only to (and not much to) variations in the reagent composition, but, to a greater extent, to their physicochemical properties, when the same result may be obtained by different compositions of the components, but possessing the same physicochemical property, currently, the ability to react with free radicals. Therefore, synthetic compounds of different structure than natural antioxidants may substitute them in reactions with free radicals.

In 1960ies, a new direction – chemistry and biology of antioxidants – began active development in Institute of Chemical Physics (Moscow). The important task to clear out if biological activity of antioxidants depends on their properties as radical reaction inhibitors was arisen before scientists in the Institute. Nontoxic antioxidants of various structures were purposefully synthesized. Basically, they were derivatives of shielded phenols and oxy-derivatives of heterocyclic hydrocarbons [12, 13]. The presence of homological sequences of antioxidant derivatives allowed determination of the structure – activity relation and selection of the most effective and the lowest toxic compounds.

In experiments *in vivo*, a relation between radioprotector activity of synthetic antioxidants to their antiradical properties are determined [14, 15]. Kinetic studies of native antioxidants – vitamins, were implemented; their parameters as inhibitors of radical processes are determined [16, 17]. Khrapova *et al.* have used chemoluminescence method adapted to the study of bioantioxidants in lipids; it was also applied to the study of synergism and antagonism of synthetic and natural antioxidants, and the entire antioxidant system in the membrane lipids were characterized [18].

All the above-mentioned works were generally devoted to the study of antioxidants in lipid components of the cell. However, antiradical activity of water-soluble natural and synthetic inhibitors was also estimated on specially

developed photochemiluminescent models, and exchange reactions of such antioxidants with peptide radicals occurring under UV-radiation impact were studied [19, 20]. Special attention was also paid to broadening of the activities on the study of the antioxidant role in development of one disease or another and a possibility of antioxidant prophilactic or therapeutic effect.

For example, in the studies by N.M. Emanuel and O.S. Frankfurt, for the first time in the world, the anticarcinogenic effect of antioxidant dibutyl oxytoluene [21].

The regularities of antioxidant activity (AOA) change of lipids in organs and tissues in carcinogenesis process were studied [22]. The stage type of antioxidant activity variation during carcinogenesis, induced by benzopyrrene, *ortho*-aminoazotoluene and γ-radiation was found. At the initial stage of toxic influence of a carcinogen and induction of tumor cells AOA decreases, then increases, reaches the norm and exceeds it at the stage of transition from diffusion to focal hyperplasia. Synthetic antioxidants act differently with respect to concentration and time of injection [23]. Therefore, at the first stage of carcinogenesis protective effect is produced by the same doses, which increase AOA, whereas at the late stages these doses may accelerate carcinogenesis and increase the number of occurring induced tumors. The late stages require injection of large amounts of antioxidants, which may cause the opposite effect – decrease AOA [24].

Multiple works are devoted to the study of free-radical regularities at tumor growth and antitumor action of antioxidants [25 – 28]. It has been found that tumor process proceeds at the increased level of antioxidants, and AO manifest their antitumor action in high doses only, when they do not increase the antioxidant activity of organs and tissues, but, vice versa, decrease it and manifest themselves as prooxidants. Interestingly, there is a general regularity for various antitumor drugs: the lower they decrease AOA, the more effective they are [29].

It should be noted that many scientists in the Institute of Chemical Physics have studied antioxidant actions in the case of radiation disease. The radioprotector effect of compounds was caused in accordance with their antioxidant activity [15]. Analogous data were obtained in experiments on animals and model radiated solutions of DNA, proteins and lipids [30 – 32].

A new direction, established in 1970, was the application of antioxidants to gerontology. Kinetic investigations of model aging reactions, the study of antioxidants' variations with age, theoretical considerations of the aging process, special experimental studies of antioxidants as geroprotectors

have proved that this new directions is important both theoretically and practically [33 – 37]. It is very strange that the use of antioxidants in gerontology is presented as a new idea, ascribed to other people, instead of those who are working in this field more than 40 years.

Besides the study of antioxidants in treatment of one disease or another, their role in normal physiological processes is now actively investigated. Palmina *et al.* have studied the role of antioxidants in the cellular proliferation indicating that all effects increasing the antioxidant activity intensify proliferation, whereas the effects decreasing the antioxidant activity inhibit proliferation [38 – 40].

Alessenko *et al.* studied the influence of antioxidants on activity of genetic apparatus [41 – 43]. They have shown that the ability of bioantioxidants to effect on the composition of lipids of cells may lead to activity variation of lipid-dependent synthesis proteins and DNA repair, and affect chromatin activity.

Thus, in the late 1970ies, the role of antioxidants in normal cell metabolism was studied actively. It has been concluded that AO represent the universal modifiers of the composition, structure and functional activity of membranes and that many regularities of their influence on the cellular metabolism can be explained from this viewpoint [44, 45]. There has been discovered physicochemical system of cellular metabolism control by membranes, based, on the one hand, on the interrelation between the rate of peroxide oxidation of lipids in membranes and, on the other hand, variation in the membrane lipid composition and their oxidation ability [46, 47].

Basing on the data on parameters of this system, it is possible to transfer a cell, an organ or an organism from one metabolic state to another with the help of antioxidants.

In 1970ies, antioxidants have found application in cardiology, oncology, treatment of neurodegenerative and many other classes of diseases [48 – 50]. Antioxidants were also intensively studied as compounds for plant growing and agriculture as growth intensifiers, prophylactic and therapeutic drugs for cattle and poultry [51 – 55].

Thus, the main conclusions in the works by the scientists of the Institute of Chemical Physics, made in 1970ies, were the following:

1. Nontoxic inhibitors of radical processes – antioxidants, possess a broad spectrum of biological activity;

2. Biological effectiveness of antioxidants correlates with their antioxidant properties;
3. With respect of the dose, antioxidants may both increase (in low doses) and decrease the antioxidant activity;
4. The activity of antioxidants in treatment of any disease depends on time of drug injection, because the disease development may be accompanied by the stage-by-stage change of the antioxidant activity. A drug will be effective only when it is injected in a low dose at the stage of decreased AOA, or in a high dose at the stage of increased AOA.

Of course, it could not proceed in the different way: many scientists – biologists, medics, and even chemists and physicists – have strictly argued against these initial works on antioxidants and free-radical reactions in the living systems. Despite any proofs, the vitalistic tendencies were very strong: no one may even think that synthetic antioxidants are able to substitute their natural analogues. "Free-radical reactions may not develop in a living organism, too", – the opponents stated, "because they are uncontrollable, which is impossible for the living organism, where everything is precisely regulated". Moreover, according to ideas of that time, the membrane structure was so that the valence transmission from one molecule to another was strictly prohibited, because the lipoproteid model of the membrane indicated separation of lipids by protein molecules. The important reason against the inhibitors of radical reactions and radical processes proceeding in the organisms was the absence of specific enzymes regulating these reactions. The opinion was commonly accepted that the antioxidant function of even tocopherol, for example, was the secondary effect of its activity, valuable only for *in vitro* processes, but being absolutely insignificant in the life of biological objects. This idea was supported by the observations that tocopherol deficiency (E-avitaminosis) could not be completely eliminated with the help of synthetic antioxidants [56, 57]. Finally, it has been suggested that the works, in which concentration of peroxides isolated from lipids of organs and tissues, have measured not the real amounts of free-radical reaction products *in vivo*, but the amounts formed during isolation.

All objections and scepticism were neglected with time. The development of biochemistry and biophysics of antioxidants strictly confirmed the proper direction [58, 59].

The pages of history should be concluded by the statement that the works in the field of free-radical biology, implemented by Soviet and Russian

scientists, were the pioneer steps, and many data obtained in those investigations are still urgent and valuable.

CHAPTER II. "TODAY"

In recent 50 years, passed since the beginning of the systematic investigations of the role of radicals and antioxidants in norm, under the effect of environmental damaging factors on the organism, at pathological process development in the organism, multiple works on various aspects of this problem were published. Actually, a new branch of science – free-radical biology – was established. It would be naive to think that a single review may give a comprehensive description of the results obtained during a half of the century, specifically taking into account that free-radical biology was developing at the exponent-increasing rate with time. Therefore, we limit the current discussion by considering tendencies in the development of antioxidant direction in Institutes of Chemical Physics and Biochemical Physics, the fundamentals of which were established by N.M. Emanuel, and sharply define the list of questions resolved already after 1984.

Despite the fact that at present antioxidants are considered in relation to their various applications, we have to turn back to *bioantioxidant* term.

By bioantioxidants the substances are meant, which in model free-radical processes of oxidation act as reaction inhibitors and preserve this property at their injection to a living organism (cell culture or so). Disturbance of even a single postulate does not allow calling the substances bioantioxidants (BAO). Despite the fact that antioxidant activity of lipids may also be increased with the help of substances, which are synergists to natural antioxidants or transform to antioxidants during metabolism, according to our definition, bioantioxidants must possess the ability to inhibit the oxidative free-radical process in the model reactions. This property allows forecasting of their biological effect spectrum and implementation of directed synthesis of compounds.

At present, the following ways of antioxidant influence on the cellular metabolism are considered:

1. The interaction between bioantioxidants and free radicals of various origin;

2. Bioantioxidant incorporation in the membrane structure, the change of functional activity of the membrane associated with the change of its viscous properties (yielding);
3. Direct influence of bioantioxidants on the activity of membrane proteins: enzymes, receptors;
4. The influence of bioantioxidants on genetic apparatus of the cell, including gene expression;
5. The influence of bioantioxidants on regulatory systems of the cells and is transferred to its metabolism, as a whole.

For all these ways, the following question is principal: if the activity of antioxidant action by one way or another is related to its ability to decelerate free-radical oxidation reactions or different antioxidants interact specifically with any targets and, hence, their antiradical properties for this type of activity are insignificant.

According to our point of view, in this case, not the activity of bioantioxidant, but the influence of a definite substance on one structure or another on a definite function should be discussed. Such conformity of the effect of different compounds, united by the presence of antiradical properties, on the target allows consideration of this way as typical of bioantioxidants.

It is still urgent, which physicochemical characteristics define their biological activity. We have paid most attention to determination of antiradical activity (the constant of radical exchange reaction with inhibitors – k_7, and antioxidant effectiveness ε – the value practically taking into account the consumption of antioxidants and radicals, formed from them, in secondary reactions) and the reaction $In^{\bullet}$ + RH rate constant, which characterizes participation of radicals and inhibitors in the chain propagation reaction.

The important moment is the model selection, in which these constants can be adequately calculated. The most important result of these works was determination of the following regularity: antioxidants injected to the organism must possess the antiradical activity giving k_7C product of injected exogenic inhibitor comparable with k_7C product of natural endogenic one, where C is the antioxidant concentration [60, 61].

If k_7C product of exogenic antioxidant was smaller than for endogenic one, free-radical reactions both *in vitro* and *in vivo*, first, consumed the self rather than injected antioxidant. Comparative study of these characteristics show that in processes with sharply increasing quantity of active radicals, which first could interact with any components of the cell at high reaction

rates, we are incapable of protecting the cell components with synthetic inhibitors, because they possess k_7C lower than for the cell components (due to low concentration or k_7).

Table 1

Impossibility of antioxidant protection from hydroxyl radicals *in vivo*
Characteristics of hydroxyl radical reaction with cell components [62]

Substrate	Rate constant, $M^{-1}s^{-1}$	Concentration in the cell, M	$k_7C \cdot 10^7$, s^{-1}
Amino acid proteins	10^{10}	0.01 (estim.)	10
Arginine	$2.1 \cdot 10^9$		
Methionine	$5.1 \cdot 10^9$		
Phenylalanine	$3.5 \cdot 10^9$		
Tryptophan	$8.5 \cdot 10^9$		
DNA	$10^9 - 10^{10}$	0.005	1 – 5
Nucleic bases			
Adenine	$3 \cdot 10^9$		
Cytosine	$2.9 \cdot 10^9$		
Guanine	$1.05 \cdot 10^{10}$		
Thymine	$3.1 \cdot 10^9$		
Glutathione	$9 \cdot 10^9$	$5 \cdot 10^{-4}$	0.45
Exogenic antioxidants	$10^8 - 10^{10}$	$10^{-5} - 10^{-5}$	0.0001 – 1

As an example, Table 1 [62] shows the rate constants of hydroxyl radical reaction with protein amino acids and nucleic bases indicating much higher concentrations of amino acids and bases compared with injected antioxidants. Hence high rate constants of hydroxyl radical reactions with antioxidants make impossible protection of the cell components from hydroxyl radicals by particular synthetic antioxidants. High chain propagation reaction rate constants for inhibitor radicals also decrease effectiveness of antioxidants [63 – 65].

As an example, let us present the data on the correlation between AO inhibitor properties and activity antioxidant phenoxyl radicals of various structures (Table 2).

Table 2

Kinetic parameters of phenoxyl radicals of various structures
($T = (20 \pm 2)$°C, $p < 0.05$) [65]

Kinetic parameter	Number of π-bonds in substrate*	Antioxidant			
		Hydroxy-benzene	2-*tert*-butyl tyrosol	α-TP	Dibunol
$W_{cons} \cdot 10^{-5}$, M/s	0	2.3	10.5	8.0	15.0
	1	–	–	7.0	14.0
	2	1.7	6.7	5.0	13.5
	4	–	–	0.5	7.5
$k_{9,eff} \cdot 10^3$, $M^{-1}s^{-1}$	0	0.52 ± 0.02	2.38 ± 0.01	0.94 ± 0.02	3.4 ± 0.03
$W_{reg} \cdot 10^{-5}$, M/s	1	–	–	2.0	1.0
	2	7.0	4.9	5.0	1.0
	4	–	–	7.5	6.4
$k_{10} \cdot 10^2$, $M^{-1}s^{-1}$	1	–	–	0.20 ± 0.04	0.09 ± 0.01
	2	0.69 ± 0.02	0.48 ± 0.03	0.49 ± 0.01	0.09 ± 0.01
	4	–	–	0.74 ± 0.12	0.64 ± 0.04

Notes: $C_{substr} = C_{AO} = 8 \cdot 10^{-3}$ mol/l.

The confidence parameter p was determined by the Student criterion at

$$f = n_1 + n_2 - 2,$$

where f is the first function of normalized deviance; n_1 is the number of control tests; n_2 is the number of test samples. Constant values were assumed confident at $p < 0.05$.

* 0 – the substrate is absent; 1 – methyl oleate (containing one π-bond) is the substrate; 2 – linoleic acid (with two π-bonds) is the substrate; 4 – arachidonic acid (with four π-bonds) is the substrate.

The standard photolysis method was applied to estimation of the disproportionation rate constant k_9 for phenoxyl radicals of inhibitors, depended on the phenoxyl shielding degree.

For these phenoxyls, the interaction constants for inhibitor radicals and any oxidation substrate in the chain propagation process was also calculated.

The estimation of a relation between phenoxyl radical activity and total effectiveness of inhibitors as antioxidants shows that k_7 constant is not enough for correct assessment of antioxidant, and activity characteristics of the inhibitor radical products are required. These data may produce a forecasting of possible inversion of AO action *in vivo*.

Also, it should be taken into account that the reaction constants of the same inhibitors with different radicals differ significantly (by orders of magnitude) from one another (refer to Table 3) [66, 67]. Moreover, the cases are possible, when antioxidants active in relation to some kind of radicals is unable to protect cell components from other radicals, because their affinity to radicals is much higher than to injected inhibitors.

Table 3

Rate constants of interaction between inhibitors-antioxidants and biologically active radicals

Inhibitors	Radicals (rate constants, l/m·s)			
	$OH^{\bullet}$	$R^{\bullet}$, proteins	$RO_2^{\bullet}$, lipids	$O_2^{\bar{\bullet}}$, superoxide anion-radical
2-ethyl-6-methyl-3-hydroxypyridine hydrochloride	$3.3 \cdot 10^{10}$	$1.9 \cdot 10^{6}$	$9.0 \cdot 10^{5}$	26 – 4
Phenosan	$4.4 \cdot 10^{10}$	$1.2 \cdot 10^{6}$	$2.2 \cdot 10^{4}$	10 – 2
α-Tocopherol	$8 \cdot 10^{10}$		$3.4 \cdot 10^{6}$	$4.7 \cdot 10^{5}$ (soluble form)

One more obstacle to the effective application of antioxidants is the extreme dependence of the effect on concentration. As mentioned above, in high concentrations antioxidants begin acting inversely e.g. not suppressing by intensifying free-radical reactions. This is usually induced by high activity of accumulated inhibitor radicals or preferable consumption of natural antioxidants rather than injected synthetic ones. Many of these effects will depend on the initial characteristics of free-radical processes and the initial level of antioxidants.

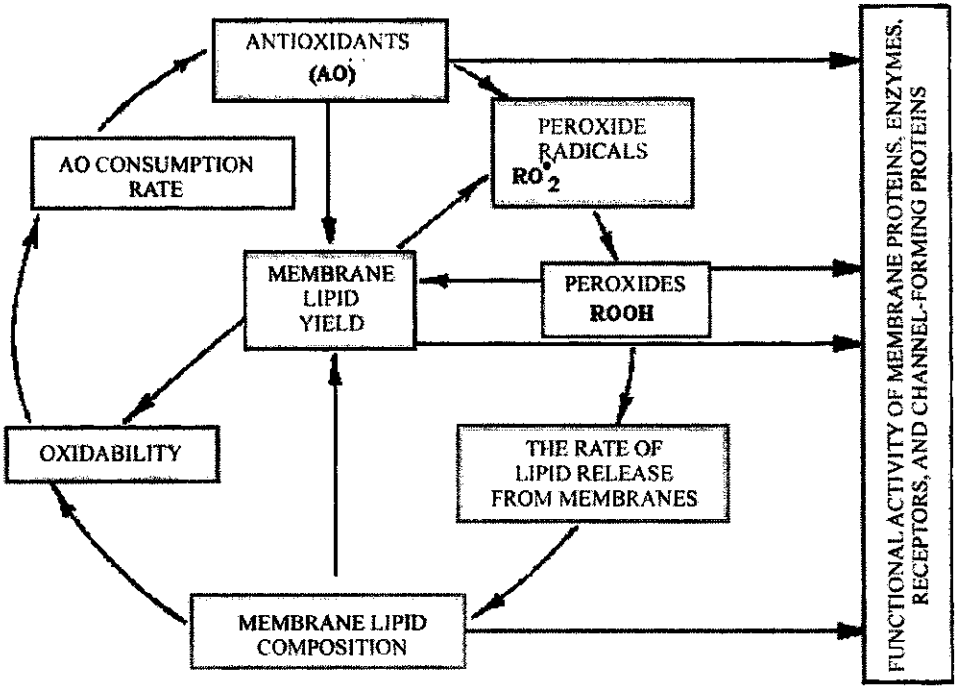

Figure 1. The scheme of peroxide oxidation of lipids control by membranes

Thus, versatility of antioxidant properties and the possibility of positive effect on proceeding of various normal and pathological states requires the knowledge of the origin of radicals, which induce pathological changes, precise time of AO injection, concentration and elementary constants of the inhibitors. Non-consideration is incorrect approach to therapeutic treatment using antioxidants may cause harm to a patient.

The question about the role and place of endogenic and exogenic antioxidants in the cell metabolism, on the one hand, and antioxidant enzymes, on the other hand, is of high importance. Simultaneously with the works on bioantioxidant application and studies of relation between biological activity and structure of antioxidants, the data on the presence of specific enzymes in the cell called antioxidant enzymes were issued. Among these enzymes, the following substances should be mentioned: superoxide dismutase and a set of thiol exchange enzymes, including glutathione peroxidase, glutathione reductase, glutathione transferase, and phospholipase, which control the rate of oxidative transformations in lipids of membranes, and which presence in the

cell is considered as the first protection barrier against "oxidative" radicals [68, 69]. As indicated in the following publications, it is incorrect to ascribe the main role in protection to any structure, because each system is required for implementing its own specific function at different stages of the oxidation process development: chain initiation, propagation, branching or termination [70].

As indicated in the study of bioantioxidant action, there is a global system of bonds between separate indices of cellular metabolism, which varied under the effect of antioxidants.

At that time, studies of the physicochemical regulatory system, which supports the level of free-radical reaction, on the one hand, and controlling the exchange of membrane lipids and antioxidant consumption rate in lipids, on the other hand, obtained further development (Figure 1) [46, 47].

Antioxidants, free radicals and peroxide oxidation products, composition of lipids, their oxidation susceptibility, and the rate of antioxidant consumption are the components of this system.

It has been shown that the antioxidant level increase causes a decrease of peroxide oxidation of lipids (POL) rate, oxidation product concentration, decrease of lipid isolation from the membranes, their enrichment with unsaturated lipids, and corresponding increase of the oxidation susceptibility of lipids. In turn, the oxidation susceptibility increases with the rate of antioxidant (AOA) consumption and to further reduction of AOA and the peroxide oxidation rate to norm. The inverse picture is observed at the decrease of AO concentration, POL rate increase, etc. The existence of such control system was observed for all studied intracellular and cellular membranes in cells of animals, plants and microorganisms. It should be taken into account that the change in the composition of lipids and their oxidation susceptibility induces changes of yielding of various membrane layers. All the above-mentioned characteristics affect the activity and kinetic characteristics of membrane-bound proteins – enzymes, receptors; therefore, a change of POL rate may cause changes in the structure and functional activity of membranes. In all membranes in norm, similar relations between parameters are observed, and the difference is observed only in relaxation times of the system (from minutes to days).

As an organism is affected by any damaging factor, the regulatory system is also changed. Firstly, long-term changes may be induced by an effect of a chronic factor, which does not break bonds in the regulatory system. Then after the end of such effect the system may return to the normal state.

Secondly, there may be cases, when damaging factor causes transition of a system to a new level of regulation and, finally, bond breaks may happen in this system, which do not allow reduction of the normal state. In the latter case, antioxidants may be useful as components of the complex therapy. Such regularities were observed in both experimental and clinical investigations.

For instance, radiation impact breaks relations between peroxide oxidation and oxidation susceptibility of lipids [71]. At the background of intensified peroxide oxidation, lipids should be saturated with hardly oxidized fractions, which would decrease the oxidation susceptibility and return the system to norm. Actually, the opposite picture is observed that the oxidation susceptibility increases and gives no opportunity to the system to return to norm. Hence, the effectiveness of antioxidants as radioprotectors decreases.

In the case of tumor growth, the system works with two bonds break, which at definite parts of the system produce the response identical to the norm [72]. During carcinogenesis, the main disturbances between the composition of lipids and the activity of membrane-bound enzymes are observed already in the early period after carcinogen injection [73]. In the case of epileptic seizures, the experiments with CM strain rats display a break of relations between changes in the amount of antioxidants and peroxide oxidation rate [74]. At the background of antioxidant activity, an increased concentration of peroxide oxidation products is observed. The same type of broken relation in the control system of peroxide oxidation may be observed under the effect of synthetic antioxidants on these rats – the system responses by increasing antioxidant activity and simultaneous increase of POL.

At the development of atherosclerosis, the system works in the normal mode at the initial stage of the disease. The injection of antioxidants to patients at this stage normalizes both peroxide oxidation and the composition of lipids. At the third stage of the process bonds are broken in the system and yet at least two different compounds should be introduced, one of which influences on POL, and another normalizes composition of lipids [75].

In this review, we will not discuss the reasons for breaks in the regulation system specific for different diseases. We just pay attention of the readers that the fact of POL rate change, its increase or decrease, is not specific for any pathological state, because in all these cases, the change in the peroxide oxidation rate is observed. Specificity is displayed only for the entire regulation system.

Table 4

Concentration of primary POL products, dienic conjugates (DC) and total nonsaturation of lipids with double bonds (DB) in blood erythrocytes of patients with AD before and after therapy

Index	Healthy (n = 11)	Patients (treated)			
		Before treatment (n = 19)	Amiridine, 2 months, (n = 6)	Gliatilin	
				3 months (n = 7)	6 months (n = 5)
DC	0.2 ± 0.02	0.628 ± 0.183	0.341 ± 0.192	0.443 ± 0.179	0.330 ± 0.156
DB: 10^{18} molecule/1 mg lipids	2.4 ± 0.1	14.6 ± 3.9	14.2 ± 2.8	12.2 ± 1.4	10.5 ±3.4

Let us consider an approach to selection of drugs on the example of neurodegenerative disease – Alzheimer's disease (AD), for the regulatory system of which bond breaks are also detected. There are several viewpoints on the etiology of Alzheimer's disease. One of them is the deficiency in cholinergic system, the logic conclusion from which is the selection of compounds among acetyl cholinesterase (ACE) inhibitors – cholinomimetics [76]. Another famous hypothesis is that Alzheimer's disease occurs due to excessive development of the oxidative stress and, consequently, the treatment should be provided with bioantioxidants.

Despite the fact that there are different viewpoints on the etiology of Alzheimer's disease, many scientists believe that the oxidative stress either as the basic reason or its main component in the neuron death is responsible for Alzheimer's disease development. We have used the same approach in the study of the role of bioantioxidants and free-radical mechanisms in Alzheimer's disease development, which was mentioned above. Hence we have studied changes in the regulatory system and considered the relation between parameters of it.

Many investigators, and we also, have determined an increase in the quantity of POL products in cerebrum membranes, as well as erythrocytes, thrombocytes and other cells both experiments modeling different aspects of Alzheimer's disease and directly in people with Alzheimer's type deletion (ATD) (Table 4).

Table 4 shows amounts of peroxide oxidation products in patients with Alzheimer's disease, as well as the change in the composition of lipids and their oxidation susceptibility. They possess relation different to norm [77], e.g. at the background of POL increase the lipids were enriched with fractions not more resistant to oxidation, but vice versa, much easier oxidizing.

Our determinations of the double bond quantity in lipids of erythrocytes in patients with Alzheimer's disease indicated possible relation of this result not only to variations in phospholipid/choleserol ratio or relation between fractions of phospholipids, but also with the change of nonsaturation of lipids (DB). The increase of double bond concentration in lipids of patient erythrocytes induced reduction of their viscous properties, which were determined in experiments with spin probes. Therefore, the relation was broken simultaneously in the "peroxide compounds–oxidation susceptibility" and "peroxide compounds–yielding of membrane lipids" units [78].

In norm an enrichment of lipids with saturated compounds or a decrease of oxidation susceptibility and viscosity increase, related to

accumulation of cholesterol in lipids, should be observed. Hence the treatment induces a dilemma. If we use antioxidants for the treatment, this may much more increase the yielding of lipids. This will cause a noticeable nonconformity between yielding and activity of membrane proteins. If patients with Alzheimer's disease receive substances increasing rigidity of membranes, the cholenergic system will become more deficient. In this direction, we have synthesized *hybrid* molecules containing fragments, responsible for inhibition of acetyl cholinesterase, antioxidant activity, and localization of molecules on the membrane [79]. In some doses, these compounds act as normalizing agents.

The study of changes in the entire regulation system but not its separate parts indicates the cases of possible monotherapy (antioxidants) application, and when a complex of therapeutic (except for antioxidants) biologically active substances should be used. To some extent, this can be fulfilled by synthesis of *hybrid molecules*.

One of the most perspective and practically important directions in modern chemistry of phenol bioantioxidants is the synthesis of *hybrid* compounds, which combine antioxidant activity with ability to structural interactions with the biosystem [80]. Among these substances, the *floating* compounds, synthesized from quaternized derivatives of dialkylaminoalkyl-substituted 2,4-, 2,6-di-*tert*-butylphenols and dialkylaminoalkyl esters of phenosan acid. These compounds have demonstrated a broad spectrum of biological activities – antimicrobial, antiviral, analgetic, etc. Also, it is found that gradual redox and solvolytic transformations induce formation of a cascade of intermediates, each possessing individual kind of activity – antioxidant, chelator, ability to incorporate in the charge transfer chain, etc. In the cascade mechanism of 2,4-, 2,6-di-*tert*-butylphenol derivatives, of importance is the tendency to formation of heterocyclic compounds, which also contribute to the total biological activity. Another currently developed direction is the synthesis of hybrids of functional di-*tert*-butylphenols and biocompatible macromolecules. In this direction, the maximal antioxidant activity of hybrid substances with broad variations of hydrophobic-hydrophilic ratios and specific structural characteristics in solutions may be reached [81].

Summing up the above-mentioned, it should be noted in the period of 1980 – 1990ies the range of investigations in relation to antioxidant application to treatment of a new spectrum of diseases; elementary constants in reactions of inhibited oxidation, specific breaks in relations between definite parameters of the regulatory system, based on the lipid oxidation, were actively

investigated; hybrid molecules of antioxidants were actively synthesized and studied both experimentally and in clinics.

CHAPTER III "TOMORROW"

The most impressive discovery made in the middle 1980ies concerns new ways of the antioxidant influence. It is related antioxidant activity in hyperlow doses (HLD) – in 10^{-13} – 10^{-12} M concentration or lower. It should be noted that such effects were determined for antioxidants of the various chemical origin: for alcohols (tocopherol), phenols and oxypyridines, and antioxidants both synthetic and natural [82].

In 1983, scientists from Institute of Biochemical Physics in cooperation with colleagues from Institute of Psychology studied the influence of antioxidants on electrical activity of an isolated neuron in Roman snail and obtained an unexpected result. The initial dose of the preparation (10^{-3} M) was active for the neuron and highly toxic, simultaneously. Therefore, we had to use solutions of lower concentration. The dose by four orders of magnitude lower than the initial one was found less toxic and more effective. Further decrease of concentration induced effect growth, it reached the maximum (at 10^{-15} M), and then (at 10^{-17} M) decreased to the control level [83].

Primarily, the observed effect was studied on antioxidants of various structures and then under the effect of a broad spectrum of external factors: antitumor and antimetastatic agents, radioprotectors, plant growth inhibitors and stimulants, neurotropic compounds of different classes, hormones, adaptogens, immunomodulators, detoxicants, and physical factors such as ionizing and non-ionizing radiation, etc. There are many levels of biological organization, at which the effect of hyperlow doses (HLD) of biologically active substances is observed – from macromolecules, cells, organs and tissues to animals, plants and even populations. This does not mean that the effect is caused by hyperlow doses of any biologically active substance on any biological object. It should be emphasized that the effect of concentrations ranged within 10^{-17} – 10^{-13} M or lower could not be associated with any structure of the substance or level of biological organization.

The results of implemented studies have suggested an idea that we are talking about principally new regularities of biological object interaction with hyperlow doses of biologically active substances rather than the features of

action of any compound or response of any biological object. Each of these substances may have specific target, amplification mechanism, and specific metabolism features. However, hyperlow doses of biologically active substances obey some general regularities [84]. Recently, more and more scientists turn to the effect of HLD. The spectrum of biological objects for investigations and the list of chemical substances and physical factors demonstrating activity in hyperlow doses are broadened.

The analysis of our results and data from the literature indicates multiple general features of effects caused by hyperlow doses of biologically active substances and low rate physical factors. These regularities concern both formal signs (dose dependencies) and biological activity indices.

This phenomenon originates from the community of critical targets, for example, cellular and subcellular membranes, as well as the reaction kinetics features, in which weak interaction are of importance. Among typical properties of HLD effect, the following should be mentioned:

- non-monotonous polymodal "dose–effect" dependence. In many cases, activity maxima are observed in definite dose ranges, separated by the so-called "dead zone" (Figure 2);
- the change of biological object sensitivity to the effect of various agents both endogenic and exogenic (the latter may be of the origin similar to HLD action or different, which induces synergic or antagonistic effect);
- kinetic paradox demonstration, specifically an ability to detect HLD effect of biologically active substances, when a cell or an organism has the same substance in concentrations by several orders of magnitude higher, as well as the influence on the receptor of the substance doses by orders of magnitude lower than dissociation constants of the ligand–receptor complex;
- the effect "sign" dependence on the initial characteristics of the object;
- "stratification" of biologically active substance properties with decreasing concentration, at which activity is preserved still , but secondary effects disappear.

General regularities of antioxidant hyperlow dose effect are most clearly observed in the study of dose dependencies. In some cases, this is bimodal dependence: the effect increases for hyperlow doses of the compounds and then decreases with the dose increase, enters the "dead zone" and then increases again. Sometimes, the dose dependence shows the "sign change"

effect. For example, in the hyperlow dose area the inhibitor activity was observed, and the concentration increase changed it to stimulating and then back again to the inhibitor effect.

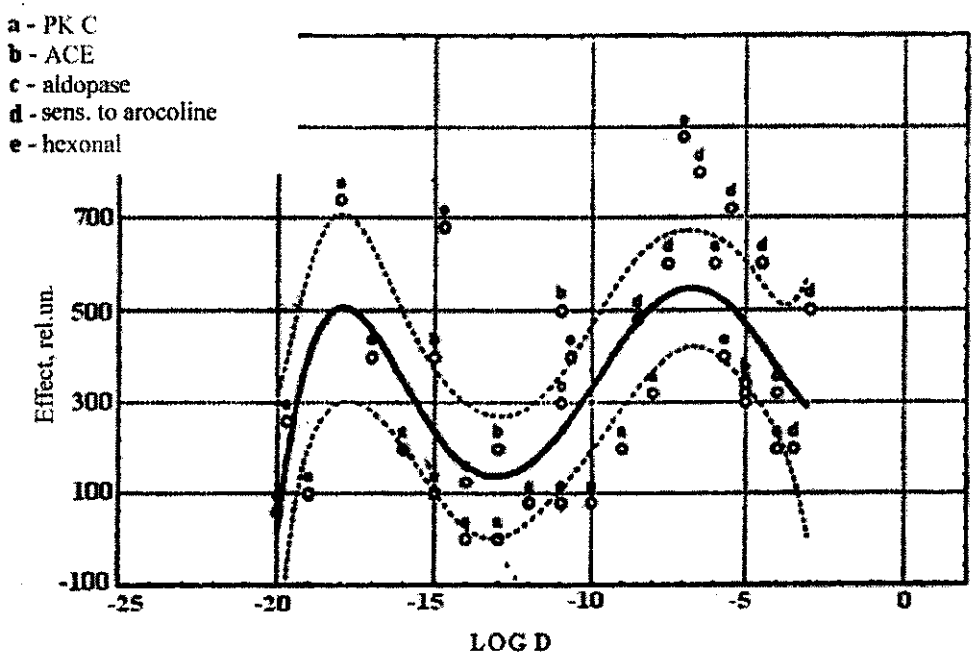

Figure 2. The dependence of biological activity of phenosan on the antioxidant concentration

Figure 2 shows the dose dependence of various effects for phenosan K. The activity was determined experimentally by phenosan influence on the enzymes: protein kinase C and acetyl cholinesterase, on physiological activity – hexenal sleep duration for rats and mice, and sensitivity to cholinomimetic arecoleine.

Clearly different types of activities manifest a general dependence of the effect on dose. It is suggested that at different AO concentrations injected to the animal organism, the leading place in discussion is devoted to the ways of antioxidant effect. As the concentration decreases, the antioxidant loses abilities to effect on metabolism. For example, at the concentration of 10^{-6} M

or higher the compound may interact with lipid radicals, whereas in $10^{-9} - 10^{-7}$ M concentration it does not practically directly interact with $RO_2^{\bullet}$.

Another feature – the change of sensitivity to the action of various factors after injection of hyperlow doses of the substance – also pretends for versatility. For instance, this feature forms the basis for such effects as synergism in action of two antitumor agents, one of which is injected in HLD [86], and increase of herbicide activity above the additive level under the same conditions [87].

So far as concerns another property – the effect sign, it can be indicated that its value (positive or negative) depends on the initial parameters of the biological object. For example, it has been found that antioxidant HLD acts differently on isolated neurons with different action potentials. Hence if the potential is high, antioxidant decreases it, and vice versa increases low potential [82]. Analogous results were obtained in the study of antioxidant effect on membranes of erythrocytes possessing different antioxidant activity of lipids [82].

To understand the mechanism of hyperlow dose effect on biological objects, we first should explain from kinetics viewpoint the possibility of reactions between so low quantity of molecules and their targets. As the concentration is 10^{-15} M or lower, the Van't Hoff law of acting masses is not obeyed anymore, and the term "concentration" becomes somewhat meaningless [88].

L.A. Blumenfeld has suggested an idea about parametric resonance as a possible mechanism of action of hyperlow quantities of biologically active substances and the cellular and subcellular levels [89, 90]. He suggested the parametric resonance to occur at coincidence of temporal parameters of intracellular processes, started-up by biologically active substances, and specific time for the substance to approach the target. As a result of active substance binding to the target, an enzyme (receptor) converts to conformation nonequilibrium state, which provides its maximal activity at a definite relaxation stage.

In the framework of these ideas, the observed decrease of enzyme activity with increasing dose of active substance may also be explained.

We have suggested another approach to explaining of kinetic paradoxes [91]. It is based on the idea about allosteric interaction of catalytic sites in the enzyme molecule.

The ideas are also considered [91] that any biological system subject to hyperlow doses of biologically active substances may response to the initial

quickest single molecules rather than their stationary concentrations (" the first approach moment").

There are several hypotheses [92], which interpret the mechanism of biological signal amplification under the effect of hyperlow doses. Ashmarin has presented a scheme of signal "multiplication" and describes the main systems required for realization of the HLD effect. They are:

a) cascade systems amplifying the signal;
b) collective, convergent systems;
c) accumulators and transporters of signal molecules;
d) superffinic receptors.

An adaptive mechanism is also suggested [93], according to which the low dose effects are explained analogously to the chemotaxis effect – the change in biological object response is determined by BAS concentration gradient in space and time rather than by BAS concentration itself.

It is our point of view that the main difficulty in composing these hypotheses is explanation of the primary act of single molecule interaction with biological targets. As observed in our investigations, every time, when a hyperlow dose of biologically active substance was injected to the animal organism, cellular structure or model system, containing suspension of membranes, structural characteristics of membranes are changed. In its turn, changes in the membrane structure may cause variation of the functional state of the cell, whereas polymodality of the response may be explained by a change in the action mechanism of the substance by the membrane structure in any concentration range. But the question is still urgent: how could the primary act of interaction between biologically active substance HLD with membrane protein or lipid be explained, if the ratio of the number of molecules of this substance to the number of protein molecules equals $1:10^6–10^9$?

Table 5 shows results of antioxidant HLD influence on the isolated enzymes and enzymes in the cell composition [84].

As follows from the investigation results on protein kinase C and acetyl cholinesterase activity, phenosan K and α-tocopherol act uniformly both in usual (10^{-5} – 10^{-3} M) and hyperlow (10^{-18} – 10^{-9} M) concentrations. If α-tocopherol acts as protein kinase C inhibitor in the concentration about 10^{-4} M, it also inhibits it in the concentration range of 10^{-17} – 10^{-15} M; if phenosan K in 10^{-6} – 10^{-5} M activates protein kinase C, it also activates it in 10^{-18} M dose. Moreover, if phenosan K inhibits acetyl cholinesterase in the dose of 10^{-5} –

10^{-4} M, it also inhibits this enzyme in 10^{-14} – 10^{-13} M concentration. Basing on these facts, one may conclude about the uniformity of the action mechanisms of ligands on the enzyme activity both in usual doses and HLD. However, the results obtained may not be explained from the viewpoint of classical biochemistry. The ligand–enzyme ratio equal, on average, one ligand molecule per 10^4 – 10^9 enzyme molecules eliminates the possibility of explaining the HLD effect by the ligand–enzyme complex formation. Biochemical mechanisms of response amplification, for instance, via systems of control by cyclic nucleotide, the phosphoinositide cycle, applicable to cell, may not be used for explaining the effects observed in model systems.

Table 5

Hyperlow BAS doses and their influence on the activity of enzymes

Enzymes	Ligand	Concentration, M	Effect	Ligand / enzyme
Protein kinase C from rat cerebrum	Phenosan	10^{-5} – 10^{-4}	inhibitor	1:2400
		10^{-14}	inhibitor	
Protein kinase C (smooth muscle cells of rat aorta)	Phenosan	10^{-7} – 10^{-5}	activator	1:10^4
		10^{-17}	activator	
Protein kinase C (human osteo-sarcoma cells)	Phenosan	10^{-5} – 10^{-4}	activator	1:10^4
		10^{-8}	activator	
Acetyl cholinesterase	Phenosan	10^{-9}	activator	1:(10^4–10^5)
		10^{-15}	activator	

Hence it is natural to suggest that biologically active substances in the concentration range of 10^{-18} – 10^{-11} M may implement other mechanisms of influence on the activity of enzymes. We have compared biochemical changes in an animal organism after intraperitoneal injection of hyperlow dose (10^{-14} M) of antioxidant phenosan and usual dose (10^{-4} M).

Table 6

Comparison of phenosan effect for injections in 10^{-14} and 10^{-4} M doses on biochemical and biophysical parameters of tissues of mouse cerebrum membranes (rel.un)

Biochemical and biophysical parameters	Antioxidant dose	
	10^{-14} M	10^{-4} M
1. Peroxide oxidation products	1.25 – 1.4	0.8
2. Microviscosity of membranes	1.3 – 1.4	2
3. Sphingomyelin	1.25 – 1.5	1.4
4. AC		
V_{max}	1.6 – 1.75	0.9
K_M	1.5	52
5. LDG		
V_{max}	1.6	1.6
K_M	0.8	0.8
6. Aldolase		
V_{max}	1.6	1.6
K_M	0.8	0.8
7. Influence on DNA adsorption by nitrocellulose filters (spleen)		
1 h after	1.25	1.17
24 h after injection	0.85	1.32
8. Changes in p53 protein content		
24 h after injection	1.25 – 1.3	1.0 – 2.0

Table 6 shows results of such investigation. Amazingly, the range of changes and their directivity were practically equal for doses different by 10^{10} times.

We would like to pay special attention to the change in gene expression, for example, p53 gene, which activity increased for 10^{-14} and 10^{-3} M doses. Despite different temporal dependence, the changes were of the same character. Thus the presence of close effects for usual and hyperlow doses cannot be explained from the viewpoint of antioxidant use of various biochemical mechanisms and targets in these concentration ranges.

Many authors tended to explain the observed regularities from the viewpoint of antioxidant HLD effect on structural characteristics of water [94 – 96].

However, the experimental data of these models are not enough and, which is most important, there are no data on the long-term existence of water clusters.

Meanwhile, it should be assumed that many HLD phenomena could be simply resolved, if we yield from the water structure change. For example, the fact that in some cases the sign and direction of the effect depend on the initial properties of the biological object can be simply explained. If the enzyme is highly active – it reduces activity, but it is low – it increases under these circumstances. The most remarkable fact is that the levels of activity changes are very close. This can be simply explained by the fact that in solution of biologically active substance water changes the protein structure similarly for both higher and lesser activity.

Also, it becomes nonparadoxical that substances effect on biotargets, when their concentration is by many orders of magnitude lower than the ligand–receptor complex dissociation constant or the protein concentration.

Despite many ambiguities, specially in the field of biological activity mechanisms of antioxidants in HLD, the above-mentioned investigations form the new field of studies and provides for broad practical prospects in it. For the same of example, let us dwell on two aspects of antioxidant HLD application. Firstly, it is application of antioxidants as environmental protectors against low-rate radiation.

It is well-known that classical radioprotectors, applied in common doses, show no protective effect in the case of low-rate chronic radiation. This is associated with limitation of their application due to toxicity of radiaprotectors, which must be injected during a long time, and that, to the great probability, the low-rate radiation possesses another mechanism compared with high doses of acute radiation.

Already in 1989, we have shown that the "critical degree" of membranes increases with dose and intensity decrease, i.e. the membrane damage contribution into total cell damage increases [97]. In some works (unfortunately, very scanty) membrane-active compounds – natural antioxidants – were used. Kudryashov, Goncharenko et al. [97] suggest the use of food additive containing antioxidants.

However, these approaches are somewhat indefinite in relation to the amount of bioantioxidants present in food additives. Moreover, it is often unknown how the components present in mixture interact with one another. In our work we have used a synthetic antioxidant from the class of shielded phenols. Indices of peroxide oxidation of lipids in cerebrum, liver, blood

corpuscles, the composition of lipids, activity of membrane-bound enzymes – ACE and cytozole enzymes (aldolase and lactate dehydrogenase), and structural characteristics of membranes were studied. It has been found that both one-time and multiple injection (prophylactic, before radiation impact, and treatment, after it), decelerate changes in organs and tissues of irradiated animals, induced by the effect of low-dose, low-intensity radiation. This makes HLD application of this antioxidant to the most variable impacts of this kind [98], including ecological consequences of radiation bombs, very attractive.

The idea to use HLD antioxidants in gerontology and tumor prophylaxis is also of great interest and importance. We have studied the influence of phenosan, dosed 10^{-4} and 10^{-14} M, on the live duration and the rate of spontaneous leukemia development in AKP strain mice. It is found that leukemia in these mice develops at lower rate and the life duration of animals treated with antioxidant is much longer than in control group [99]. Preservation of such activity at 10^{-14} M dose and the absence of secondary effects makes the application of HLD antioxidants as anticarcinogen compounds very attractive.

It would be said that this is the reason why HLD AO application is one of the most perspective directions in gerontology and cancer prophylaxis. In this case, the effectiveness of antioxidants can be forecasted by studying their kinetic parameters for both HLD and usual dosage. Four antioxidants with different constants were tested [87] for herbicide activity. In all cases, bimodal effect–dose dependence was observed. The concentration, at which the low-dose maximum is observed, increases with k_7 decrease, whereas the "dead zone" and the second (high-dose) maximum values increase with k_7.

It is my viewpoint that the future of antioxidants and enhanced interest to them are related to their important role in gene expression and the influence on genome stability. There are different mechanisms explaining AO effect on the gene expression, suggested in the literature. However, in the majority of cases, this explanation is reduced to the idea about an important role of AO in changing concentration of free radicals, which are ascribed the key role in gene activity regulation.

Two more interesting directions, recently developed in Institute of Biochemical Physics (RAS, Moscow), should be mentioned. They are:

1) antioxidant properties of odorants and their use in treatment of various pathologies;
2) relations between AO and nitric oxide regulation systems.

These relations are extremely variable, discussed in the broad-range literature on this topic. Kuropteva, Baider *et al.* (Institute of Biochemical Physics, RAS, Moscow) are carrying out experiments on the role of ascorbic acid in nitric oxide synthesis: it may effect of nitric oxide synthesis; unfortunately, t is unclear yet if this is the property of ascorbic acid only, or of other antioxidants, too.

Concluding this review, we must state that bioantioxidants are very important for living beings and have wide prospects in future, not completely studied yet.

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PEROXIDE OXIDATION OF LIPIDS IN BIOLOGICAL MEMBRANES AND FOOD ADDITIVES

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Contrary to the popular viewpoint, peroxide oxidation of lipids is a physiological process, which proceeds in normally metabolizing tissues. It obeys the same regularities as the liquid-phase oxidation of lipids *in vitro*. Precise regulation of lipid oxidation in a cell is carried out both chemically and enzymatically. Low oxidation rate of lipids is supported by several systems; each system functions in strictly determined limits, on specific stages of the process and has its own rate-limiting reaction. The low level of lipid peroxides in normal tissues is a result of well-balanced processes of peroxide formation and consumption. Disturbance of this balance leads to a change in the antioxidant status of an organism. Food additives can modify the rate of an oxidation stage, even being not antioxidants. However, the most powerful antioxidants proved to be additives, which components interact with peroxide radicals.

Recently, scientists of various specialties became more and more interested in oxidative stress. The origin and the mechanism of stress are studies; pathologies induced by stress increase are detected; substances capable of decreasing the destructive action of stress. Among substances counted on are antioxidants (AO), present in food and biologically active food additives.

In chemical kinetics of antioxidants substances, small additives of which quickly decelerate oxidation of model systems, are mentioned. The effectiveness of low concentrations of additives is the main typical feature of chain oxidation processes.

One of the features of chain oxidation reactions is formation of dozens or even hundreds of peroxide molecules from one peroxide radical, occurred by

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any possible way. Some of these peroxide molecules may decay to new radicals (chain branching), included in the chain transfer reaction.

The decelerating action of additives may be stipulated by two types of reactions: the substance either directly reacts with radicals, propagating the oxidation chain, and decreases their concentration (radical process inhibitors) or molecularly, without free radical formation, destroys peroxides, which are branching agents [1, 2].

From the point of view of the action mechanism, AO may be divided into several groups:

1) substances terminating oxidation chains by reaction with peroxide radicals;
2) substances reacting with alkyl radicals with oxidation chin termination;
3) substances degrading peroxides without free radical formation and, therefore, releasing peroxides from the reaction sphere;
4) deactivators of alternate valence metal ions, which promote oxidation by degradation of peroxides to free radicals;
5) substances reacting with free, nonperoxide radicals capable of initiating oxidation [2].

The oxidation rate *in vivo* depends on many factors, which define AO status of the organism. Analysis of the literature indicates the most clearly expressed oxidative changes in lipid bilayer of cellular membranes, because comparing with proteins and nucleic acids lipids are oxidized much easier. It may be considered that lipid bilayers of membranes are primary targets for action of free radicals and reactive oxygen species of various etiologies in the cell [3, 4].

Peroxide radicals of lipids formed are short-living particles ($10^{-2} - 10^{-1}$ s), but live longer than reactive oxygen species ($10^{-9} - 10^{-6}$ s). Compared with long-living structurally bound free radicals of proteins and nucleic acids, peroxide radicals of lipids are more active and enter multiple exchange reactions with the oxidation substrates, mostly unsaturated fatty acids (UFA), with formation of hydroperoxides.

In membranes of animal cells, over 100 various hydroperoxides are formed during POL, total amount of which may be registered by definite physicochemical methods.

POL investigations were started 50 years ago by Prof. B.N. Tarusov, Department of Biophysics, Biological Faculty M.V. Lomonosov Moscow State

University and Academician N.M. Emanuel, Institute of Chemical Physics, Academy of Sciences of the USSR [6 – 9].

For a long time, POL has been considered as the process undesirable and even unfavorable for the organism, which development should be prevented by any possible method.

This was proved by the following facts:

1) The highest frequency of peroxides was observed in animal and human organs at development of pathological states. At present, it is found that over 150 pathologies are developed at increased POL level.
2) Oxidized lipids injected to animals were found toxic for the organism.
3) Accumulation of lipid oxidation products in membranes increases hydrophilic nature of molecules, damage of membrane structures, disturbance of their permeability, and the change of functional activity of membranes.

Intensive investigations of POL processes have altered estimation of biological role of lipid peroxides, and some points were found important for justification and stipulation of the statement about a significant role of peroxide oxidation for normal metabolism [10 – 12]:

1) Modernization of methods determining lipid peroxides and the increase of their sensitivity allowed for detection of peroxides in many organs and tissues of intact animals.
2) Discovery and study of enzymatic systems regulating the rates of both formation and utilization of peroxides show that these processes are typical of normal metabolizing cells, and the quantity of peroxides in the membranes is supported at a definite stationary level.
3) Thorough studies of oxidized lipid toxicity indicated dangerous toxicity for the organism of peroxides themselves, to the lesser extent, and products of deeper oxidation of lipids (aldehydes, ketones, oxy-acids), to the greater extent.
4) Peroxides are intermediates, used in the organism for the synthesis of active substances – eicosanes: prostaglandines, prostacycline, thromboxanes, steroid hormones, etc. Therefore, peroxides may not be considered as secondary and unwilling products of oxidation. Synthesis of eicosanoids from UFA is performed in several stages via formation of short-living, labile endoperoxides under the influence of specific

enzymes. With respect to enzymatic system involved in biosynthesis, biologically active substances with different, sometimes opposite properties, are synthesized from the same precursor – arachidonic acid endoperoxide. It may be suggested that peroxides represent the universal raw material, used by the organism for various purposes.

5) Oxidation of lipids is interrelated to the process of renewal of phospholipid composition in membranes. It is shown that POL intensification accelerates the exchange of phospholipids, changes their relative composition, lipid-protein ratios and, consequently, the membrane structure. The relation between POL rate and lipid composition change is based on the acceleration of oxidized phospholipids' outcome from the membranes [12].

All these data confirm a suggestion to accept POL as a physiological process, and peroxides – exchange products of normal metabolizing cells. Low level of lipid peroxides typical of normal tissues does not mean that the peroxidation rate in them is low. The reason is the peroxide formation and consumption processes are balanced in tissues, and the lipid oxidation proceeds at a definite stationary level.

Oxidation of lipids possesses specific features, first of all, provided by participation of some enzymes in it, structuring of the lipid bilayer, low partial pressure of oxygen, and low oxidation temperatures.

Unfortunately, many investigators still consider POL as unwilling process, which must be suppressed. It is very popular now to attribute AO properties to many drugs and food additives. It is believed that the higher AO level in the organism, the better. However, it is not always true.

Contrary to oxidation *in vitro*, the rate of which may be varied in broad ranges by changing temperature, composition and ratio of the components, such changes in membranes are only possible in strictly limited range. The rate of lipid oxidation is extremely sensitive to low changes in the composition of components, specially minor admixtures, action of agents increasing or decreasing the amount of free radicals (radiation, the presence of initiators, inhibitors, etc.) that indicates the chain type of the process.

May chains reactions proceed in membranes? Yes, they may. Despite heterogeneity of the membrane composition, they have heterogeneous zones, in which lipids are present in the liquid-crystal state with hexagonal type packing, forming bilayers, in which oxidation reactions may proceed. The assumption of mosaic structure of membranes with "lipid seas" allows a suggestion that

conditions in membranes are sufficient for oxidation in the liquid phase, but it is obvious that structural features of biological membranes must seriously affect intensity of this process. In all probability, oxidation proceeding in lipids of membranes consists of the same elementary reactions and possesses the same development stages as for oxidation *in vitro*: initiation, propagation, branching and termination of chains [1, 2, 10]. The rate of elementary reactions may be controlled by two methods: enzymatically, via operation of corresponding enzymes, and chemically. In the organism, liquid-phase oxidation proceeds. It is very finely regulated owing to existence of several related ways of transformation in lipids providing specificity and selectivity of oxidation [10, 12].

Free-radical chain oxidation represents extremely labile process, which rate may change in one way or another with:

- amount of AO;
- chemical origin of the oxidation substrate;
- the initiation rate of free radical formation.

The changes may:

- be reversible, returning to the normal state;
- transfer the process to another stationary oxidation level;
- disengage autoregulation mechanism.

Apparently, disturbances of free-radical regulation mechanism accompany many diseases (up to 150 pathologies).

We suggest that systems defining AO-status of the organism and changing the rate of lipid oxidation may be considered to be regulatory. They may be conditionally divided into 4 groups [10, 11]:

I. Structural organization of lipids as a system defining the rates of oxidation chain initiation, propagation and termination.

II. The system of enzymes of AO-action [13], responsible for formation and death of reactive oxygen species and free radicals of different etiology. This group also includes the system of enzymes of antiperoxide action [13], which decompose peroxides without free radical formation.

III. The system of enzymes regulating exchange of phospholipids in membranes, outcome of oxidized species of lipids from them that changes UFA composition in phospholipids, lipid/protein, phopsholipid/cholesterol ratios, etc. [12].

IV. Lowmolecular substances, including vitamins acting as AO, initiators, synergists, $O_2^{\bullet}$ extinguishers, etc., which affect the chain branching and termination stage [10, 11].

To all probability, the action of the above-counted system is interrelated. Hence, each of them operates in strictly defined limits at definite oxidation stages and has different limiting reactions.

CHAIN INITIATION AND PROPAGATION STAGES

Lipids in membranes are oxidized at rather low temperature, at which spontaneous formation of free radicals is of low probability. To all probability, formation of free radicals of lipids in membranes may be initiated not only by light, ionizing radiation, reactions with ozone and other initiators, as it happens in usual models, but mostly by enzymes. The latter either directly react with UFA (for example, lipoxygenases) or act indirectly via formation of reactive oxygen species. Among these species are oxygen-containing radicals – superoxide anion-radical ($O_2^{\bullet}$), hydroperoxide radical ($HO_2^{\bullet}$), hydroperoxyl radical ($HO^{\bullet}$), and nitric oxide radical ($NO^{\bullet}$). These radicals may be formed in various enzymatic metabolic processes with participation of oxygen [14].

AO-action enzymes (superoxide dismutase, catalase) deactivating oxygen-containing radicals significantly suppress initiation of lipid oxidation, i.e. change AO-status of the organism.

The change in activity of enzymes participating in formation and recombination of free radicals and reactive oxygen species (system II) causes **the main** influence on the total rate of lipid oxidation. Meanwhile, activity of such enzymes also depends on the structuring of lipid bilayer in membranes (system I).

Operation of enzymes responsible for formation of peroxide radicals requires "liquid" state of lipids [15]. The increased viscosity of the medium decelerates operation of these enzymes and decreases the rate of peroxide

radical formation. Viscosity of the lipid component of membranes will cause a significant influence on degradation of peroxides (outcome of radicals from the cell), as well as on the diffusion rate of oxygen and its reactive species to the most labile UFA bonds.

Since oxidation in tissues proceeds at low partial pressures of oxygen, physical state of the lipid bilayer affects the rate of oxygen diffusion and the ratio of alkyl and alkyl peroxide radical concentrations, showing different activities [2]. The denser packing of phospholipid UFA in membranes, the lower access of O_2 and $O_2^{\bullet}$ to them and the lower the rate of free radical initiation. Any factors breaking UFA packing, chaotropic agents, for example, accelerate oxidation of lipids.

Total rate of oxidation of lipids will be defined by the chain propagation reaction and depend on the number of double bonds in UFA. Therefore, the change in composition of membrane phospholipids (system III) may affect total rate of the oxidation of lipids. Substances able to deactivate $O_2^{\bullet}$, e.g. singlet oxygen extinguishers, will also decrease the rate of free radical initiation and total rate f the oxidation of lipids (system IV).

To-date, it is found that there are several types of free radicals in the cell, differing by the way of synthesis and activity. Independently of the origin of the radicals formed, in accordance with the principle of free valence indestructibility, they are substituted by peroxide radicals of lipids, and the rate of such exchange is rather high. Meanwhile, total rate of the oxidation of lipids, proportional to square concentration of lipid radicals, increases. The change in concentration of lipid radicals, their additional synthesis and recombination, induced by variation of the operation rate of one enzyme or another, consecutively changes the stationary state of the process. Thus, the factors affecting conditions of active radical formation in lipids of membranes change total rate of oxidation of lipids; one of such factors is structuring degree of the lipid bilayer (system I).

The structure of membranes is interrelated to its physicochemical properties, such as microviscosity, permeability, charge, etc., and defines activity of membrane-bound enzymes.

The ability to stabilize lipids in membranes by increasing the structuring degree of the lipid bilayer is demonstrated by some vitamins, which have long side chains (vitamins E, K and ubiquinones). Recently, a hypothesis about structural interaction between tocopherols and components of biological membranes has been widely spread (system IV) [16 – 21].

Vitamins of different structure (A, C, D, F), which produce radical species, in high concentrations may act as initiators and accelerate oxidation of lipids, increasing the rate of free radical synthesis. Alternate valence metal ions, specially Fe^{2+} and Cu^{1+} (system IV), may also be initiators [22 – 26].

Thus total rate of oxidation of lipids at the stage of chain initiation and propagation depends on operation of all four systems, namely on:

1) the change of the structuring degree of lipids in membranes (systems I and IV);
2) activity of antioxidant enzymes participating in formation and recombination of free radicals and reactive oxygen species (system II);
3) composition of phospholipid UFA (system III);
4) the presence of substances, acting as oxidation initiators, in lipids, and singlet oxygen extinguishers (system IV).

To all probability, the main factors are structuring degree of the lipid bilayer and activity of antioxidant enzymes.

Food additives, which are not antioxidants, but interacting with reactive oxygen species or breaking the structuring level of the lipid layer in membranes, will affect the AO-status of the organism.

CHAIN BRANCHING STAGE

Lipids are characterized by high concentration of highly unsaturated fatty acids and very unstable peroxides, formed from them. Phospholipid exchange enzymes, which change their LC-composition (III), will affect concentration and activity of lipid radicals, oxidability of lipids, and stability of hydroperoxides formed. Stationary quantity of hydroperoxides in the lipid bilayer depends on both the rate of their formation and the rate of their release from the membrane.

Enzymes, which utilize hydroperoxides (for example, glutathione peroxidase or catalase) reduce their quantity and, consequently, change the rate of chain branching reaction and total rate of oxidation (II). Total rate of oxidation is also affected by some lowmolecular admixtures, which induce degradation of peroxides in lipids without free radical synthesis (IV).

At rather low temperatures, when oxidation of lipids in biomembranes is initiated, the rate of spontaneous dissociation of hydroperoxides in lipids to free radicals is low. On the other hand, concentration of hydroperoxides in the membrane in norm is supported by the system of enzymes and the presence of alternate valence metals, sulfur- and phosphorus-containing substances. Therefore, oxidation of lipids may be considered as a stationary process, initiated by radicals approaching the membrane from the environment. Stationary mode of the oxidation of lipids in membranes imposes definite limits on the rate of degenerate branching reaction. This reaction must proceed at extremely low rate and not proceed at all. If by any reason the control of peroxide concentration stationary mode is broken, increase of the concentration will intensify the chain branching reaction and induce system transition to another stationary level.

Total oxidation rate at the stage chain branching directly depends on the action of three factors:

1) activity of enzymes regulating exchange of phospholipids in membranes (system III);
2) activity of enzymes utilizing hydroperoxides without free radical synthesis (system II);
3) concentration of alternate valence metal ions and the presence in lipids of sulfur- and phosphorus-containing substances, which induce degradation of peroxides without free radical formation (system IV).

Thus, to all probability, for the chain branching stage, the main factor defining the rate of oxidation of lipids is activity of enzymes utilizing peroxides without free radical formation.

Food additives able to release peroxides from the oxidation medium will actively affect the AO-status of the organism.

CHAIN TERMINATION STAGE

This stage includes quadratic and linear reactions of the chain termination. Particular mechanism of quadratic chain termination depends on the oxygen concentration. In case of physiological concentrations of oxygen, preferable proceeding of the reaction

$$RO_2^{\bullet} + RO_2^{\bullet},$$

should be expected, which in case hypoxia and ischemia will be added by the reaction

$$R^{\bullet} + R^{\bullet}.$$

The termination reaction involving two peroxide radicals proceeds by disproportionation mechanism with formation of polar monomeric products of oxidation (ketones and alcohols), usually well soluble in water, which caused their release from the lipid bilayer. Recombination proceeding with participation of alkyl radicals produces crosslinks of two phospholipid molecules that induced long-term changes in membranes. Changes in the composition of peroxide radicals induce variations of the total rate of oxidation of lipids in membranes and in structure of the latter.

Besides oxygen diffusion, the concentration and activity of lipid radicals are determined by UFA composition, CH-bond strength in them (III) and the presence of substances promoting breaks of these bonds (IV).

Total rate of oxidation of lipids strictly depends on changes in concentrations of the substances, on which molecules linear chain termination happens. *The ability to interact directly with peroxide radicals under natural conditions is demonstrated by natural AO exclusively (mainly, phenols). None of the first three protection systems interacts with peroxide radicals of lipids. None of antioxidant enzymes (II) terminates the oxidation chain.* If any impact on lipids in membranes produced an additional amount of free radicals, which was not prevented by structural or enzymatic protection system, the excess can be eliminated by natural AO only, which are inhibitors of radical processes. Meanwhile, their influence on the total oxidation rate is much higher than effectiveness of other systems. This defines the unique role of natural AO in the control of intensity of oxidation of lipids [10 – 12].

The exchange reaction between natural AO and Peroxide radicals of lipids produces inhibitor radicals, which demonstrate high activity. To our point of view, this is the reason for the dual effect of natural AO on the oxidation process – the ability of high dose to intensify oxidation of lipids [28]. The effectiveness of prooxidant action increases with decreasing temperature and increasing nonsaturation degree of unsaturated fatty acids.

Substances regenerating natural AO radicals, tocopherols, in particular, or releasing them from the reaction will possess the synergic action. Among such substances, ascorbic acid, glutathione and some sulfur-containing substances may be mentioned. Interestingly, oxidized forms of synergists are reduced enzymatically [29].

Recently, it has been shown that synergists for tocopherol may be reduced forms of ubiquinone [29 – 32], which affinity to peroxide radicals is by an order of magnitude lower compared with tocopherol. Phospholipids may also be synergists to tocopherol, though they are not antioxidants themselves.

We have indicated that tocopherol radicals show high effectiveness in reactions with UFA. The lower UFA nonsaturation, the higher rate constant of hydrogen atom detachment by tocopherol radical, e.g. its regeneration [33].

The specific feature of oxidation of natural lipids is that effective deceleration of thee processes by elimination of the excess of lipid radicals is provided only by compounds with increased affinity to them. They are mostly natural AO [34, 35]. To all probability, this is the reason for the unique role of natural AO in POL intensity regulation.

The rate of oxidation of lipids at the chain termination stage depends on:

1) the composition of peroxide and alkyl radicals (III);
2) concentration of natural AO possessing high affinity to the radicals (IV).

Food additives reacting with peroxide radicals, to the greater extent change the AO-status of the organism. However, they should be used in definite concentration limits.

CONCLUSION

Concluding this article, the following points should be outlined:

1) POL is the usual physiological process, the integral part of metabolism. It proceeds by the chain mechanism, at a definite stationary level, which is supported by physicochemical regulatory system *with negative feedback.*

2) In the lipid layer of membranes, there are several systems, which regulate POL intensity. Each of these systems is active at its specific stage of oxidation only.

3) *Natural AO – the inhibitors of radical processes possessing high affinity to them – are the only substances*, capable of direct interaction with peroxide radicals of lipids.

4) An abrupt change in the quantity of peroxide radicals is unwilling for the organism. *The chain oxidation mechanism is the process, responsible for high changes in the oxidation rate of lipids at the occurrence of even low excess of peroxide radicals.*

5) Food additives are capable of changing the rate of POL due to variations in:

- structuring degree of the lipid bilayer;
- activity of antioxidant and antiperoxide action enzymes;
- fatty acid composition of the lipid bilayer;
- natural AO activity due to direct interaction with peroxide radicals of lipids or synergic effect.

The highest effect of food additives is observed, if they are inhibitors of radical processes.

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THE INFLUENCE OF ANTIOXIDANTS ON THE SIGNALING SYSTEM OF SPHINGOMYELINIC CYCLE AND APOPTOSIS

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For long years Nikolai M. Emanuel was interested in the aging mechanisms. He was one of the first among scientists all over the world who suggested a hypothesis of free radical participation in this process. On this basis suggestions about geroprotective action of antioxidants capable of slowing down aging processes and prolonging human life were made. At present, the outstanding anticipation is proven on the molecular level. Aging of the whole organism cannot be understood apart from the processes providing death of a separate cell. Recently, molecular mechanisms of the cell death are of close attention, the understanding of which will allow for controlling lifetime of both separate cell and the whole organism. Among various types of the cell death of special importance is genetically programmed cell death or apoptosis [1, 2]. The term "*apoptosis*" is a word of modern coinage, combined from two Greek words: *apo* – detachment and *ptosis* – falling down e.g. a process similar to the leaf fall in autumn. This romantic image was associated with the end of the living cell life.

Apoptosis has specific morphological and biochemical features, which distinguish it from aggressive and catastrophic cell death – necrosis. Apoptosis is the instrument, which removes damaged, naturally dead or "*undesirable*" cells from multicellular organism without destroying cellular microenvironment, whereas at necrosis the damaged cell content effuses into the environment and induces inflammatory processes and necrotic damages of the surrounding tissues. The key event of necrosis is cellular membrane damage, whereas for apoptosis it is activation of an enzyme (endonuclease), which provides for intramicrosomal DNA degradation [3]. The earliest apoptosis demonstration is clearly observed indurations of nuclear chromatin, localized at the internal nuclear membrane, and some cytoplasm condensation. This demonstration is detected by electronic microscopy methods. The intensification of cytoplasm condensation proceeds with the nucleus division

and cell content fractionation into separate fragments, encased in membranes, which form "*apoptotic bodies*" of various sizes.

In adult organism cells apoptosis is the process proceeding at any time and everywhere. At the same time, the apoptosis mechanism forms the basis for pathogenesis of many diseases, which may be divided into two main groups: diseases associated with inhibition of apoptosis and those induced by intensification of apoptosis. Among diseases associated with apoptosis inhibition, the following are most frequently observed:

1) tumor diseases (mammary gland, ovary and prostate tumors);
2) autoimmune diseases (exanthematous lupus erythematosus);
3) viral infections (herpes, adenoviral);
4) spasmodic asthma;
5) schizophrenia.

Diseases proceeding with apoptosis intensification are the following:

1) AIDS;
2) neurodegenerative diseases (Alzheimer's and Parkinson's diseases, pigmented retinitis, back muscular dystrophy);
3) blood diseases (aplastic anemia);
4) ischemic damages (acute myocardial infarction, apoplectic attack, reperfusion damages);
5) toxic damages of liver (hepatitis and cirrhosis);
6) kidney diseases (cystic disease);
7) radiation disease.

So broad list of the most widespread diseases outlines the importance of investigations of apoptotic mechanism and the search for inhibitors and activators of this process, which may be used as curative preparations in cases of these and many other diseases. In the case of apoptosis proceeding, the highest therapeutic efficiency may be reached by early and complex application of pharmacological preparations [2]. Searching for effective compounds preventing or activating apoptosis is based on the knowledge of apoptotic biochemical mechanisms.

Apoptosis activates many signaling cascades, which id different combinations may be activated at both mitosis and differentiation of cells. Besides DNA degradation, the key event in apoptosis is proteolytic irreversible protein cleavage, when primary protein molecule loses its structural and

functional properties and forms a set of peptides, which conduct apoptotic signals [1, 3, 4]. In apoptosis, of special importance are calcium ions, which serve as activators of many enzymes participating in the cell death program [4, 5]. For apoptosis development, phospholipid degradation is of great importance. They are structural elements of cellular membranes. They either activate or inhibit protein kinases – the enzymes, which phosphorylate proteins changing their properties or affect the change of Ca ion concentration in cells [1, 6].

Also, plasmatic membranes are of the highest importance for cellular death realization. Many receptor systems mediating the action of apoptosis inductors are localized on them. Therefore, one of possible ways for therapeutic intervention may be variation of external cellular membrane properties. Among such preparations affecting cellular membranes emulsions of perofluorocarbons (perfluorane, for example) should be mentioned. These compounds were widely and successfully studied in Russian laboratories and clinics [2].

Numerous data in the literature indicate a tight connection between the level of oxidative processes in the call and its physiological state [1, 2]. For instance, it is shown that the oxidative stress may induce programmed cell death – the apoptosis. The apoptosis development is accompanied by increasing concentration of active oxygen forms in the cell and decreasing activity of enzyme antioxidant protection [4].

An attention should be paid to the properties of antioxidants to inhibit cell death due to apoptosis. For example, N-acetylcystein, ionol and co-enzyme Q inhibit apoptosis [4]. This means that natural and synthetic antioxidants, which are widely investigated in many Russian laboratories (the most famous among them are laboratories of the Institutes of Chemical and Biochemical Physics) can be successfully included in the complex therapy of the diseases associated with apoptosis intensification. Actually, in the complex therapy of AIDS, Alzheimer's disease and ischemic damages of organs antioxidants significantly decrease negative displays of these diseases [7].

Among signaling systems participating in apoptosis induction, the key role is played by the sphingomyelin cycle system [4 – 6, 8]. The attention to sphingomyelin cycle, which primary component is sphingomyelin, was paid rather recently (about 20 years ago). It was made on the background of intensively studied phosphatidilinozitide cycle and secondary messengers of the lipid origin – diacylglycerol (DAG) and phosphoinozitides, arachidonic and phosphatide acids. The investigations of the sphingomyelin cycle as the source

of regulatory components of the signal pathways began in 1986, when the inhibiting effect of sphingomyelin enzymatic degradation product - sphingosine and their derivatives - on protein kinase C activity (PK C) was determined [9]. However, in 1976 the authors [10] were the first who noticed a decrease of sphingomyelin content in nuclei and cells of regenerating liver at the cell transition from rest to active proliferation. Within the cellular cycle, sphingomyelin content regularly changes at different stages of the process [10].

Many authors consider ceramide as a critical component of apoptosis [6]. TNF-α, Fas-ligand, radiation or chemotherapeutical agents cause ceramide accumulation in cells which, in turn, induces apoptosis features [11 – 14].

Experiments carried out on synthetic analogue of ceramide having shorten fatty acid chain (ceramide C-2 or C-6) show that ceramide itself is capable of cell death induction according to apoptotic character then induces internucleosome DNA degradation and typical morphological changes in the cell structure [6, 9]. The authors of these investigations have suggested that ceramide can be one of the main components of programmed cell death. Ceramide is able to activate some enzymes or participate in cascade enzymatic reactions typical of apoptosis.

It is known that a special role in apoptosis is played by cysteine proteases, belonged to ICE protease family which split interleukine-1 (IL-1) precursor. Among potential substrates of ICE-like proteases, poly(ADP-ribose)polymerase (PARP) is the most well studied one. It participates in DNA regeneration and activity modification of some nuclear proteins. The specific PARP degradation to two fragments of 85 and 24 kDa is the early event at apoptosis. PARP degrading enzyme is CPP32. It is shown that ceramide is capable of activating CPP32/Yama protease with consequent PARP degradation into apoptosis specific fragment of 85 kDa [1]. Cysteine protease activation by ceramide was also confirmed in experiments with purified PARP, which hydrolysis had been discovered at PARP incubation with cytosol of cells preliminarily treated with ceramide during 6 h. A potential role of ceramide-activated protein phosphatase during PARP hydrolysis induced by ceramide, was detected [1, 6].

In some works, it is shown that at neutrophils and cardiomyocytes treatment with OTNF-α and HL-60 cells with phorbol ester, both ceramide and SPH are accumulated [8]. The authors have also found that a single injection of TNF-α in mice leads to ceramide and SPH accumulation in liver cells and their nuclei [11, 15].

SPH and N,N-dimethylsphingosine exogeneously added to tumor cells induce apoptosis in 90% of cells 6 h after injection. The apoptosis was detected by internucleosome DNA degradation and morphological changes typical of apoptosis (Nucleus condensation, apoptotic bodies formation, etc.) similar to those, induced by protein kinase C inhibitors in these cells (staurosporin and H7) [5, 8]. Ceramide added to HL-60 cells at the differentiation stage easily transforms to SPH indicating ceramidase activation. The observations testifying that sublethal concentrations of SPH and sphinganine synergically increase proapoptotic activity of ceramide (10 μM) [2]. Therefore, to our point of view, it is assignable that the action of apoptosis inducers causes accumulation of both ceramide and sphingosine in cells. Similar to SPH, as combined with ceramide, the synergic effect is also caused by other PK C inhibitors, i.e PK C inhibition by sphingosine bases and other inhibitors sharply increases the proapoptotic action of ceramide. Moreover, as known, ceramide is the phosphatase activator. Cells resistant to apoptosis are unable to accumulate ceramide and SPH simultaneously. For example, radiation of radiation-resistant malignant cells A431 causes no increase of ceramide concentration in them, however, SPH level was increased [8]. Compared with SPH, higher concentrations of ceramide induce apoptosis. Apparently, some part of it must be consumed by a cell for sphingosine generation. It may be suggested that the combined action of ceramide and sphingosine is the most effective for apoptosis proceeding under various toxic effects on the cell.

Besides the main target of SPH–PK C, an important role in apoptosis is played by Rb (retinoblastoma) gene product dephosphorylation independently of PK C [8] and DNA-primase inhibition [7]. Specific activation of retinoblastoma protein correlates with cell growth inhibition and their retarding in the G_0/G_1 stage of the cellular cycle. Recently, it has been found that SPH may affect *bcl*-2 gene expression, which product inhibits apoptosis development in cells. It is also found that SPH directly affects the level of *c-myc* transcripts accumulation. SPH also influences on RNA-polymerase activity and promotes free enzyme conversion to a matrix-linked state [16]. It inhibits phosphorylation of nuclear proteins, induced by retinoic acid which is known to activate PK C nuclear form.

For the purpose of confirming possible SPH participation in TNF-α toxicity and its role in apoptosis signal transduction, the authors injected exogenic SPH to mice (20 μg per mouse) [1, 11, 12]. It is found that single injection of SPH induces reversible DNA degradation into highmolecular fragments in liver and spleen. In thymus, DNA fragmentation was more

intensive. Combined injection of SPH and TNF to mice (20 μg per mouse) produced sharp increase of apoptotic DNA degradation in spleen and thymus [13]. It is typical of SPH that toxic effect on cell of various antitumor agents is intensified. Apparently, in the present case, SPH also intensifies toxic effect of cytokine which is of great interest for pharmacology and clinical use of TNF-α combined with SPH.

Among possible mechanisms of SPH participation in apoptosis the ability of it to interact with DNA [17] and affect activity of replication and transcription enzymes are mentioned [16]. SPH may affect DNA-binding properties of some regulatory proteins, including transcription factors and topoisomerases [18].

The ability of SPH to affect the set of enzymatic systems in a cell is displayed in extremely variable biological activity of this molecule [8]. It is found that SPH affects cell growth and differentiation, aggregation of thrombocytes, inhibition of blood coagulation, mitogenesis stimulation, and possesses antitumor [2] and antimicrobial activity [8]. SPH obtains special meaning due to its ability to induce apoptosis [2, 12].

Summing up SPH properties one may imagine a mechanism of its participation in apoptosis signal transduction at the level of cytoplasm and cellular nucleus. In cytoplasm, it may control enzymes participating in apoptosis signal transduction, among which phosphokinases and proteases are mentioned, whereas in the nucleus it may influence on the DNA degradation via direct interaction with it.

The connection between sphingomyelin cycle and oxidative system of the organism is confirmed by data on ceramide and sphingosine ability to induce the peroxide process and, therefore, accumulate POL products [19, 20]. Apparently, this is one of the reasons leading cell to apoptosis under the effect of these products of sphingomyelin degradation. The mechanism of POL intensification under sphingosine effect is not quite clear, because the structure of its molecule does not indicate its oxidation properties. However, there are data on toxic effect of sphingosine on isolated mitochondria. Meanwhile, an abrupt increase of hydrogen peroxide synthesis was observed that may intensify peroxide oxidation of lipids.

The observed effect of POL components on the signal system intensity of the sphingomyelin cycle will allow for decoding and then more successfully correcting many processes in cell, which are controlled by these systems in various pathologies developing at the apoptosis background [21 – 28].

However, in contrast with sphingosine, its phosphorylated analogue (SPH-1-P) inhibits apoptosis which occurs at the background of increase ceramide concentration, induced by TNF-α, Fas antibodies, sphingomyelinase or ceramide analogues penetrating into the cell [6]. Moreover, PK C inhibition similar to sphingosine kinase inhibition by dimethylsphingosine induces apoptosis which may be prevented by adding SPH-1-P [6]. These results allow for a suggestion that sphingosine kinase activation increasing SPH-1-P concentration in a cell is the important target for PK C in apoptosis inhibition process. Therefore, sphingomyelinase, ceramidase and sphingosine kinase regulation may define is the signal is transmitted via ceramide and sphingosine or via SPH-1-P, changing the balance between apoptotic and mitogenic signals, and thus define the cell fortune: to die or to live.

Because sphingomyelin cycle is very important for the cell life, the investigation of key enzyme activity control in this signal system becomes of very high interest [29]. Despite the long-term investigations of sphingomyelinase properties and the influence of various factors on their activity, in the recent five years only it has been found that activity of some sphingomyelinase isoforms are controlled by the oxidative system of cell [29, 30].

The oxidative stress directly induced by prooxidant agents (UV radiation, hydrogen peroxide or mediated by different influences (cytokines, lipopolysaccharide, ischemic reperfusion) [31] activates the sphingomyelin cycle. The activation mechanism is not completely studied yet; however, to-date, there are data in the literature about a dependence of the main sphingomyelin cycle enzyme (sphingomyelinase) activity on oxidative process intensity in the cell.

Scanty investigations carried out at the cellular level or in isolated fragments show that neutral sphingomyelinase activity depends on glutathione present in the cell in both reduced and oxidized forms [29, 31].

Glutathione concentration in the cell (the physiological content equals 1 – 20 mM) is so important for the organism functioning that the ratio of reduced (GSH) and oxidized (GSSG) forms of it is considered as a controller of the cell development [30]. GSH concentration in various types of cells exceeds GSSG concentration by 100 – 500 times [30]. The state of GSH–GSSG system in cells may change reversibly or irreversibly. Sustaining of glutathione – glutathione disulfide system state at a definite level is necessary for normal functioning of the cell and the whole organism. The effect of compounds inhibiting glutathione synthesis on cells activates POL.

To-date, it is found that sphingomyelinases effect on glutathione concentration in cell. Addition of exogenic neutral sphingomyelinase from *Bacillus cereus* to cultivated hepatocytes increases GSH concentration in cell, whereas acidic sphingomyelinase from human placenta hydralyzing sphingomyelin equally to neutral sphingomyelinase reduces GSH depot [31]. Interestingly, despite different effects caused by these isoforms on GSH concentration, both sphingomyelinases increased mRNA level of γ-gluthamyl cysteinase heavy chain. Moreover, it is found that acidic sphingomyelinase depolarizes mitochondria and activates caspase 3 that may lead to oxidative stress and apoptosis development [20].

There is a definite relation between glutathione concentration and sphingomyelinase activity. Neutral sphingomyelinase extracted from plasmatic membrane is inhibited by reduced glutathione [31]. However, its isoform connected to endoplasmic reticulum membranes is inhibited by oxidized glutathione and free-radical oxidation products [31].

On a cell culture, the effect of glutathione, its analogues and some fragments on the intensity of various sphingomyelinase isoforms action was studied. As a result, it has been found that GSH inhibits neutral Mg^{2+}–dependent sphingomyelinase. Despite an unambiguous influence of reduced glutathione on other antioxidant concentrations and POL intensity in cell, the inhibition of neutral sphingomyelinase by glutathione has specific character, not associated with its antioxidant properties. This is testified about by both direct glutathione action on purified enzyme and the fact that γ–glutamyl–component instead of SH–component is active in glutathione [31]. The majority of experimental data indicate that enzyme inhibition is a reversible process and does not result GSH metabolism or any irreversible changes in sphingomyelinase. The data on effects of other antioxidants on enzyme activity are rather conflicting. For example, the studies of apoptosis induction in cells having no serum showed simultaneous inhibition of POL, neutral sphingomyelinase and apoptosis caused by ascorbate, tocopherol and co-enzyme Q injections [32]. It is shown that synthetic antioxidant ionol and superoxide dismutase prevent apoptosis, induced by sphingomyelinase activation by oxidized low density lipoproteids [33]. SH-containing antioxidants (N-acetylcysteine and pyrollydine dithiocarbamate) also have ability to inhibit apoptosis and suppress neutral sphingomyelinase [34]. All cited works were carried out on cellular cultures.

In our experiments, it is shown for the first time that GSH affects activity of neutral sphingomyelinase in animal organism [30]. Similar to the

cellular level, GSH inhibits enzyme activity and induces sphingomyeline accumulation. The content of diene conjugates and diene ketones decreases synchronously during the whole time of experiment that confirms GSH ability to act as antioxidant e.g. inhibit oxidative processes in cell. It should be mentioned that the type of POL product content variation duplicates the type of neutral sphingomyelinase activity change under GSH effect.

Among effective antioxidants, the protective role of which is demonstrated for various pathologies – ischemic reperfusion, atherosclerosis, haemorrhagic insult, anaphylactic reactions, and chemical mutagenesis – cholochrome bilirubin should be mentioned [36]. This pigment is of recent great interest, because the main enzyme responsible for its formation – hemoxygenase – is highly inducible compound. It is shown that the cell response to oxidative stimuli represents manifold increase of hemoxygenase activity. The system bilirubin–hemoxygenase is currently considered as one of the most important components of antiperoxide protection together with superoxide dismutase, catalase, glutathione peroxidase, and water and fat soluble antioxidants [36]. It may be suggested that bilirubin will also cause a definite effect associated with its antioxidant properties on apoptosis.

Recently, we have shown [12] that at momentary sphingosine injection reversible DNA degradation to lowmolecular fragments in liver, spleen and thymus of animals are observed. As sphingosine is injected, temporary and concentration dependencies of POL intensification in liver of mice coincide with analogous dependencies observed at apoptosis induction by this compound. At present, the function of bilirubin as one of the most important antioxidants of extracellular fluids, such as blood plasma, in unambiguous for every investigator. At the same time, its role and place in the intracellular membrane lipid protection system against oxidative damage are disputable in many points. However, recently, indirect proofs that bilirubin might play an important role in protection from POL as the intracellular antioxidant have been obtained [36].

In our experiments, we show that bilirubin affects POL activity in animal organs as the compound is directly injected to it. Similar to blood serum [35], bilirubin inhibits POL in cells of heart and kidneys of test animals. This effect is rather short-term which is associated with quick elimination of excessive bilirubin by liver cells.

Since bilirubin has antioxidant properties and oxidative reactions play an important role both in induction of hemoxygenase (the enzyme responsible for bilirubin synthesis) and in apoptosis induction, a relation between bilirubin

metabolism and systems responsible for programmable cell death may be suggested.

This suggestion was confirmed in the experiments, in which apoptosis was induced by sphingosine and UV radiation of thymus cells in rats. Apoptosis induced by momentary injection of sphingosine to animals is accompanied by expressed intensification of POL reactions in mouse liver. There are multiple data in the literature confirming protective role of antioxidants in apoptotic processes, whereas no data about bilirubin ability to protect cells from programmed cell death was observed. Bilirubin inhibition of apoptotic DNA degradation induced by UV radiation or sphingosine, shown by the authors [36], may be quite useful for developing tactics of curing various diseases accompanied with biliousness. In particular, it may be suggested that hyperbilirubinemia seriously affects the efficiency of anticancer therapy in patients with tumors in hepatopancreaduodenal zone. Treatment of these patients (by radio, chemo- and photodynamic therapy methods) is aimed at induction of oxidative stress, which leads to tumor cell death due to apoptosis. Antiapoptotic and antioxidant properties of bilirubin may reduce therapeutic effect. Despite interest to bilirubin, greatly increased in the recent 15 years and multiple investigations performed, the action mechanism remains not enough clear yet. Our data indicate that, apparently, besides direct antioxidant action in free-radical oxidation reaction and membrane lipid protection, bilirubin is also important for sphingomyelinase inhibition and correspondingly affects apoptotic cell death.

It is found that bilirubin binds to sphingomyelin and hinders sphingomyelinase access to its substrate and, therefore, inhibits the enzyme activity [37].

Shown up regularities indicating sphingomyelinase dependence on POL activity and bilirubin effect on both POL and the signal system of sphingomyelin cycle and apoptosis may be found suitable for better understanding of very important processes as proliferation and cell death.

Analysis of authors' data and the literature, shown in the present review, indicates that among antioxidants able to display antiapoptotic action the highest activity is shown by those able to inhibit both the signal system of sphingomyelin cycle due to interaction with enzymes or substrates and peroxide oxidation of lipids in cellular membranes. Since induction of the oxidative system of cells and sphingomyelin cycle is the main reason for initiating the cell death process, their simultaneous inhibition is the most effective protection of cells against apoptosis. Antioxidants displaying this dual function are the most perspective compounds as geroprotectors.

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THE FEATURES OF BIOLOGICAL MEMBRANE LIPID PEROXIDE OXIDATION UNDER THE EFFECT OF ULTRA-LOW CONCENTRATIONS OF ANTIOXIDANTS

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The comparison study of kinetic regularities of primary (dienic conjugates – DC) and secondary (malonic dialdehyde – MDA) accumulation, unsaturated lipid substrate (double bonds – DB) consumption during spontaneous and Fe^{2+}-ascorbate initiated peroxide oxidation of lipids (POL) in endoplasmic reticulum membranes of mouse liver cells *in vitro* is carried out. On these models, the action of natural (α-tocopherol – α-TP) and synthetic (phenosan potassium salt – PPS) antioxidants in concentrations between 10^{-18} and 10^{-3} M was also studied. It is found that α-TP and PPS inhibit both spontaneous and initiated POL, estimated by DC and DB criteria, within the concentration ranges of 10^{-5} – 10^{-6} and 10^{-8} – 10^{-18} M. For MDA used as the criterion, an effect sign change is observed for PPS: POL inhibition in the range of 10^{-4} – 10^{-10} M is converted to POL promotion in the range of 10^{-12} – 10^{-15} M. In the case of use of all three criteria, the dose–effect dependencies for α-TP and PPS show typical maxima at high and ultra-low concentrations and the "*silent*" zone between these maxima, though the extension of this zone is different for α-TP and PPS. Such regularities are typical of biologically active substances demonstrating the current effect at ultra-low concentrations. The correlation analysis, carried out for the purpose of estimating the effect of antioxidants on two oxidation models by three above-mentioned criteria, allows for a conclusion about advantages of the use of spontaneous POL model and DC and DB change criteria for the estimation of POL inhibition. The studies carried out with the spin probe method, allow for a supposition that a definite role in the mechanism of antioxidant action play the structural changes happening in membranes.

Keywords: biological membranes, peroxide oxidation of lipids, antioxidants, α-tocopherol, potassium phenosan, ultra-low concentrations

INTRODUCTION

In 1950ies, N.M. Emanuel *et al.* have developed the fundamental positions of kinetics and the oxidation mechanisms of hydrocarbons, discovered regularities of the behavior of intermediate peroxide compounds, determined the elementary action mechanisms of catalysts and inhibitors of these processes [1 – 5]. In these years, investigations of free-radical oxidation of lipids in biological processes in norm and pathological states were also initiated [6, 7]. In 1961, for the purpose of teaching experts in this new branch of science a new specialty called "*physicochemical biology*" was established at the Chemical Department of Lomonosov Moscow State University. The first course included 6 students, who defended their diplomas in Department of Kinetics of Chemical and Biological Processes, in the Institute of Chemical Physics, Academy of Sciences of the USSR. Meanwhile, N.M. Emanuel was one of the scientific supervisors of each of these students, three of whom (N.M. Dzyuba, E.M. Molochkova and N.P. Pal'mina) were directly supervised by Prof. E.B. Burlakova. The main task put forward by our supervisors was to clear out the way of operation of natural antioxidants (if they do really exist) in lipids of animal organs and tissues, what happen to them and how oxidative processes develop in lipids during the most important physiological reactions in norm and at pathologies, such as radiation disease and malignant tumors.

It happened so that these investigations have formed the basis for creation and development of a new scientific direction in biology and medicine and, largely, provided for an avalanche of works in this branch in the former USSR, and years later in other countries. In 1984, the group of scientists headed by N.M. Emanuel and B.N. Tarusov, carried out a series of works on the "Physicochemical mechanisms of free-radical peroxide oxidation of lipids in biological membranes", were honored the State Prize of the USSR. To-date, the peroxide oxidation of lipids (POL) in biological membranes is one of the main directions in the modern biochemistry, and natural and synthetic antioxidants are widely used in therapy of many diseases. However, besides the expansion of our knowledge, more and more questions are brought up concerning the role of POL in regulation of various metabolic processes in the living systems and more and more new properties of antioxidants requiring comprehensive study and explanation are discovered.

As an example, one of the outstanding discoveries of the recent decade is the discovery of biologically active substance (BAS) action, including antioxidants in a broad range of ultra-low concentrations (10^{-22} – 10^{-15} M), on

the living systems of different structure complexity [8 – 12]. Essentially, it can be suggested that the antioxidant effect in ultra-low doses (ULD) is definitely stipulated by their influence on POL. Ascertaining of this question is the basic target of the current investigation. Obtaining of an adequate answer to this question demanded solution of the following task: firstly, designing of a suitable quantitative POL model for biomembranes, which allows estimation of the ultra-low dose BAS effect; secondly, the study of the natural (α-tocopherol) and synthetic (potassium phenosan) antioxidants in a broad range of concentrations (including ULC) on POL. For this model, we selected nonenzymatic POL proceeding in membranes cof endoplasmic reticulum of liver cells *in vitro*.

MATERIALS AND METHODS

Endoplasmic reticulum membranes we isolated from mouse liver cells according to the method described in [13].

Spontaneous POL was carried out at 37°C at continuous air blowing through the suspension of membranes (1 mg protein/ml) in the incubation medium containing 100mM NaCl + 50 mM tris-HCl without adding of any initiators.

POL was initiated in a similar manner, except for addition of 10 μM Fe^{2+} and 0.5 mM L-ascorbic acid prior to the oxidation phase [14]. Oxidation was stopped after definite periods of time, and lipids were extracted according to the method [15].

Primary oxidation products – dienic conjugates (DC) were determined after dissolution of lipids in the methanol : hexane (7:1) mixture in 0.5 – 0.7 mg/ml concentration and absorption spectrum registration in the range of 200 – 300 nm on spectrophotometer DU-50 Beckman. The level of POL development was characterized by the relation of optical density peaks A_2 (at 225 – 232 nm) presenting the absorption maximum of dienic conjugates to optical density A_1 (at 205 – 210 nm) showing the absorption maximum of nonoxidized lipids [16].

The content of secondary POL products (malonic dialdehyde – MDA) was determined on the basis of calorimetric reaction of MDA with 2-thiobarbituric acid. MDA content was estimated by the quantity of colored reaction products formed, fixing the optical density at 532 nm [17, 18].

Total unsaturation of the lipid substrate (the content of double bonds – DB) was determined by the ozonation method with the help of automated analyzer of double bonds (manufacturer – AMMO Company) [19].

Various BAS concentrations were prepared by consecutive 100-fold dilution of initial 10^{-3} and 10^{-4} M solutions. Every studied substance was added to a suspension of membranes in 1 – 2 μl of corresponding solution prior to oxidation at determination of the effect on POL. Average values of measurable parameters, fixed in 5 – 11 independent experiments.

Parameters characterizing the structural state of membranes were calculated from spectra, measured on Brucker-200D ESR-spectrometer (Germany) applying the spin probe method [20]. The probe used for the study of surface area (~ 8 Å) rigidity of the lipid bilayer represented a stable 5-doxylstearic acid (C_5) nitroxyl radical, whereas for studies of microviscosity, thermally induced structural transitions and their effective activation energy in deeply submerged areas (~ 20 Å) of the lipid bilayer it was 16-doxylstearic acid (C_{16}). The membrane rigidity in the localization area of the probe C_5 was characterized by parameter $2\Delta H_{II}$, which is the distance between low-polar and high-polar components of the spectrum, the value proportional to the regularity parameter S. The latter characterizes mobility of fatty acid chains of lipids in the radical localization area. Microviscosity of the membrane in C_{16} probe localization area was characterized by rotary correlation times τ_{C_1} and τ_{C_2}, determined by formulae for quick anisotropic rotation of radicals

$$\tau_{C_1} = 6.65\Delta H_0\left(\left(\frac{I_0}{I_{-1}}\right)^{1/2} - 1\right)\cdot 10^{-10}, \text{ s}$$

$$\tau_{C_2} = 6.65\Delta H_{+1}\left(\left(\frac{I_{+1}}{I_{-1}}\right)^{1/2} - 1\right)\cdot 10^{-10}, \text{ s [20].}$$

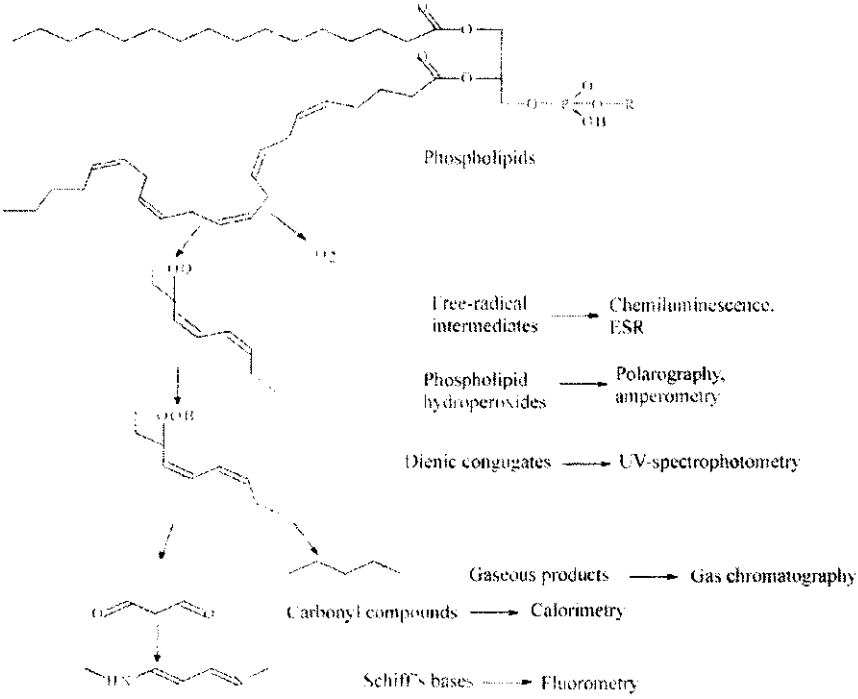
Statistical processing of the results was carried out using STATISTICA software.

RESULTS AND DISCUSSION

1. Characteristics of spontaneous and initiated POL in membranes of endoplasmic reticulum in vitro

As mentioned above, the prime object of the current investigation was the study of natural and synthetic antioxidant influence in a broad spectrum of concentrations (10^{-18} – 10^{-4} M) on nonenzymatic POL in membranes of endoplasmic reticulum. Estimation of the effect of one or another substance on POL is usually carried out with the help of induced (Fe^{2+}-ascorbate) POL model [21, 22]. However, since we suggested estimation of α-TP and PPS ultra-low dose effect and strived to the maximal approach of the performance of experiments to *in vitro* conditions, where Fe^{2+} is present but in extremely lower concentrations, we have carried out a comparative study of the antioxidant effect using both "common" model of initiated POL and much less frequently used model of spontaneous (without initiator injection) POL [23]. It should be noted that biomembrane lipids represent a mix of various substances, which oxidation produces such a broad spectrum of products that they are not completely identified yet. Nevertheless, the main classes of substances formed during POL and may be determined by one method or another can be presented schematically (see the Scheme below) [21].

Despite the fact that the studies of POL in biological membranes last for more than 50 years and many books and reviews are devoted to the mechanisms of this phenomenon [21, 22, 24 – 26], kinetic studies of the current process were rather scanty. The criteria for estimating this process are, usually, changes in accumulation of one POL product or another, most often, hydroperoxides or final oxidation products – malonic dialdehyde (MDA) or Schiff's bases. Recently, the method of POL level estimation by dienic conjugate formation was established. We have set a task to carry out the comparison study of nonenzymatic spontaneous and induced POL proceeding in membranes of liver cell endoplasmic reticulum in mice using three criteria: accumulation of primary oxidation products, dienic conjugates (DC), secondary oxidation products, MDA, as well as consumption of unsaturated lipid oxidation substrate and total content of double bonds (DB). Figure 1 shows kinetic curves of DC (a) and MDA (c) accumulation, and DB (b) consumption at initiated (curves 1) and spontaneous (curves 2) POL.


Phospholipids
Free-radical intermediates → Chemiluminescence, ESR
Phospholipid hydroperoxides → Polarography, amperometry
Dienic congugates → UV-spectrophotometry
Gaseous products → Gas chromatography
Carbonyl compounds → Calorimetry
Schiff's bases → Fluorometry

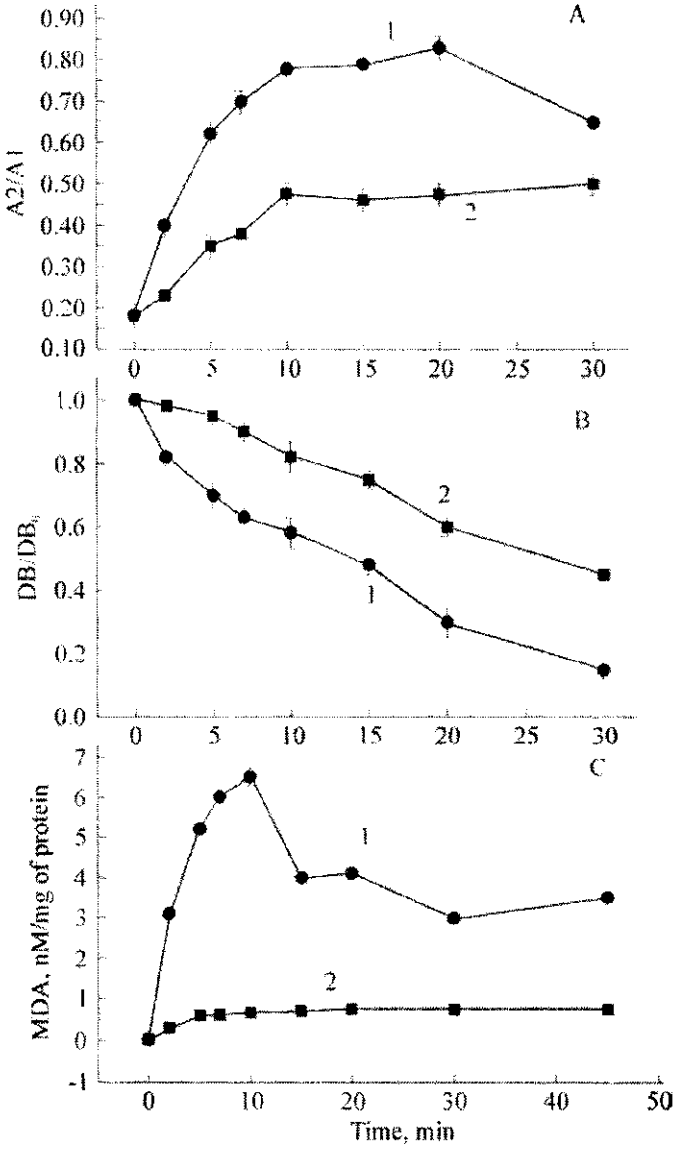


Figure 1. The change of dienic conjugate (DC) content (a), unsaturated lipid substrate (relative units) – DB/DB_0 (b), malonic dialdehyde – MDA (c), at initiated (curve 1) and spontaneous (curve 2) POL in endoplasmic reticulum membranes with respect to oxidation time. Protein concentration is 1 mg/ml, $T = 37°C$

Table 1

Average rates and accumulation constants for primary (DC) and secondary (MDA) POL products and consumption of unsaturated substrate (DB) at spontaneous and initiated POL

Criterion	POL		Measurement units
	spontaneous	initiated	
Average rate, V			
DC	$2.4 \cdot 10^{-2}$	$7.4 \cdot 10^{-2}$	$(\text{min} \cdot \text{mg} \cdot \text{lipid})^{-1}$
MDA	$7.5 \cdot 10^{-2}$	$104.7 \cdot 10^{-2}$	$(\text{min} \cdot \text{mg} \cdot \text{lipid})^{-1}$
DB	$0.4 \cdot 10^{-2}$	$6.4 \cdot 10^{-2}$	$(\text{min} \cdot \text{mg} \cdot \text{lipid})^{-1}$
Constants			
DC	$6.1 \cdot 10^{-2}$	$15.7 \cdot 10^{-2}$	$(\text{min})^{-1}$
DB	$1.76 \cdot 10^{-2}$	$5.5 \cdot 10^{-2}$	$(\text{min})^{-1}$

The Figures indicate that, expectedly, accumulation of both primary (DC) and secondary (MDA) POL products and, correspondingly, consumption of the lipid substrate (DB) proceed at much higher rate in case of initiated rather than spontaneous POL. Meanwhile, the dependence of the product accumulation has a maximum, because both DC and MDA represent the intermediate products of lipid oxidation. For spontaneous POL, this maximum is not observed due to low rate of the process. Lipids of biological membranes represent a complex mixture and, therefore, POL product accumulation and oxidation substrate consumption curves can hardly be described by any simple kinetic equation. For the sake of comparing spontaneous and induced POL processes, we approximated the initial parts of kinetic curves for DC and DB by the first order reaction as follows:

$$Dt = Dt_0 \cdot e^{kt},$$

and substituting them in semi-logarithmic coordinates (Figure 2), calculated the rate constants of DC accumulation and DB consumption. The data are shown in Table 1. For MDA accumulation, the average rate of the process development can only be estimated. Basing on the present data, one may conclude the following:

- the initiated oxidation process proceeds with 16-fold higher lipid consumption rate compared to the spontaneous process;
- kinetic constants characterizing this process are 3 times different;

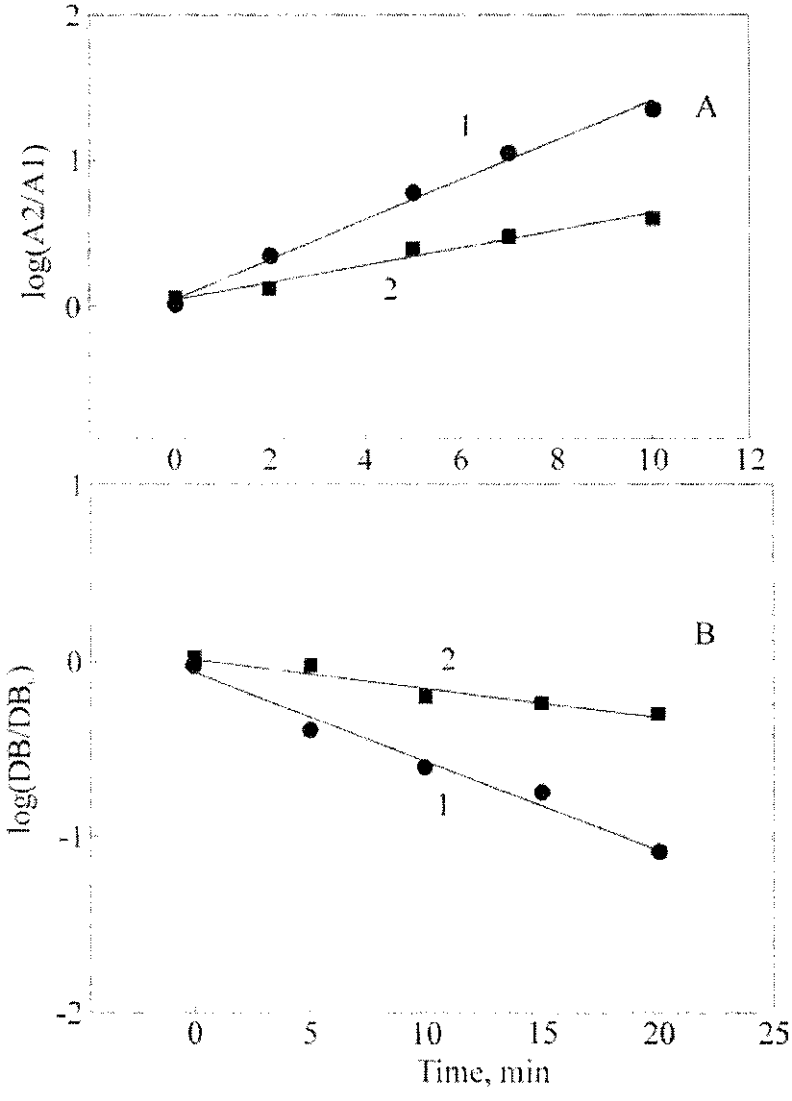


Figure 2. Semi-logarithmic anamorphoses of the initial areas of kinetic curves describing accumulation of primary oxidation products (a) and consumption of unsaturated lipid substrate (b) at initiated (1) and spontaneous (2) POL in endoplasmic reticulum membranes with respect to oxidation time. Protein concentration is 1 mg/ml, $T = 37°C$

- the average rate of primary oxidation product (DC) accumulation exceeds is 2.5 – 3 times higher for initiated rather than spontaneous POL;
- the rate constant of initiated POL is two times higher;
- the average rate of secondary POL product (MDA) accumulation is 10 – 15 times higher for initiated rather than spontaneous process.

Kinetic characteristics are significantly different with respect to oxidation time and selection of estimation parameter for POL rate.

Analysis and comparison of DB, DC and MDA molar ratios give for following results:

1) for spontaneous oxidation – 500 M DB at the oxidation maximum produce 90 M DC and 1 M MDA
2) for initiated oxidation – 50 M DB at the oxidation minimum produce 17 M DC and 1 M MDA.

As follows from the above results, the initiation affects, to a greater extent, accumulation of secondary products (MDA) rather than the increase of primary product formation (DC). Apparently, this may be associated with the fact that Fe^{2+}-ascorbate also increases the reaction rate of DC conversion to MDA, which is confirmed by data on the rates of these reactions.

Since different criteria for estimation of POL development and the influence of antioxidants on it are used in the literature, the authors have compared time dependencies of POL product accumulation and the lipid substrate consumption for both spontaneous and initiated POL. It has been found that spontaneous POL has statistically reliable between DC and DB changes ($r = -0.81$; $p < 0.003$); DC and MDA ($r = 0.93$; $p < 0.05$); DB and MDA ($r = -0.92$; $p < 0.05$). For the initiated oxidation, the reliable correlation is observed only for DC and DB ($r = -0.95$; $p < 0.02$), whereas for DC and MDA ($r = 0.8$; $p < 0.22$), DB and MDA ($r = -0.65$; $p = 0.4$) the interrelation between studied parameters is untrue. Therefore, basing on these results one may conclude about advantages of spontaneous POL model application to the estimation of BAS influence, because in this case, the control displays synchronous changes by all three criteria for estimation of the POL development level.

In the case of POL initiation, it should be taken into account which criterion is used, because deviations formed already at the criterion selection stage may occur. We have suggested that the spontaneous POL model will

provide an opportunity for estimating the influence of ultra-low doses of antioxidants. However, to check this supposition, we have implemented a series of experiments estimating the antioxidant effect in a broad range of concentrations using two models – spontaneous and initiated POL.

2. The influence of α–TP on spontaneous and initiated POL in a broad range of concentrations in membranes of endoplasmic reticulum isolated from mouse liver in vitro

We have studied the influence of α–TP on spontaneous and initiated POL in a broad range of concentrations (10^{-18} – 10^{-4} M) [23]. The study was performed kinetically e.g. concentrations of POL products and DB level were determined in the control and at injection of each α–TP concentration species after 2, 5, 7, 10, 15 and 20 min of oxidation in relation to oxidized control not added with an antioxidant. The inhibition degree was estimated 10 – 15 min after. Figure 3 shows data on the influence of various α–TP concentrations on accumulation of DC (a), MDA (b), and consumption of DB (c) at spontaneous POL. Clearly all three criteria displayed significant POL inhibition and, moreover, the effect of low doses of the preparation was quite comparable with the action of physiological concentrations of α–TP: full inhibition is observed for extremely different α–TP concentrations of 10^{-6}, 10^{-11}, and 10^{-16} M. Hence, no effect on the process at 10^{-7} M α–TP concentration is observed, and the minimal effect is observed for 10^{-13} – 10^{-14} M concentrations of the antioxidant. The general shape of the dose dependence is typical of the substances able to display any properties, as applied in ultra-low doses: the maximum is observed in the area of high concentrations, followed by a decrease of elimination of the effect (the dead zone), and then the second maximum occurs in the area of rather low concentrations [8]. Comparing dose dependencies of spontaneous POL inhibition change by DC and DB criteria, one can clearly observed their identity, which is also confirmed by statistically evident correlation between these parameters (r = 0.78; p < 0.05). The estimation of the inhibition degree by the criterion MDA gives a different dose dependence than may be associated with complications induced by the low absolute level of this oxidation product in the case of spontaneous POL proceeding. The correlations between the inhibition percentage by DC and MDA as well as by DB and MDA are incorrect.

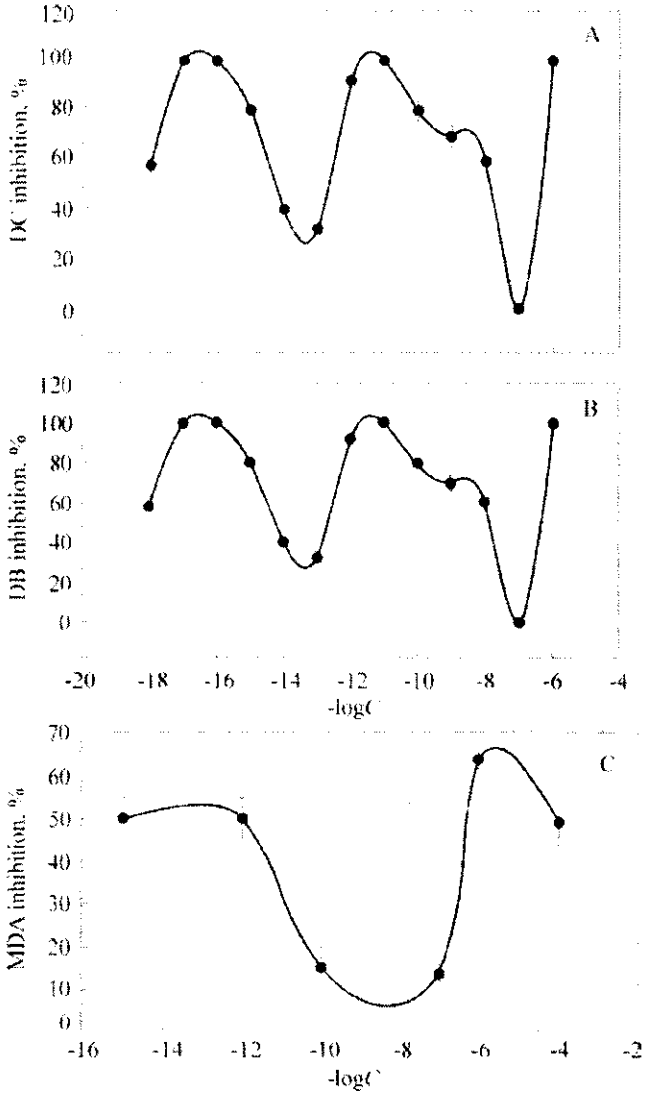


Figure 3. Inhibition of dienic conjugate (DC) accumulation (a), and consumption of unsaturated lipid substrate (DC) (b) and malonic dialdehyde (MDA) (c) in percents under the effect of α–tocopherol in various concentrations on spontaneous POL in endoplasmic reticulum membranes. Protein concentration is 1 mg/ml, $T = 37°C$

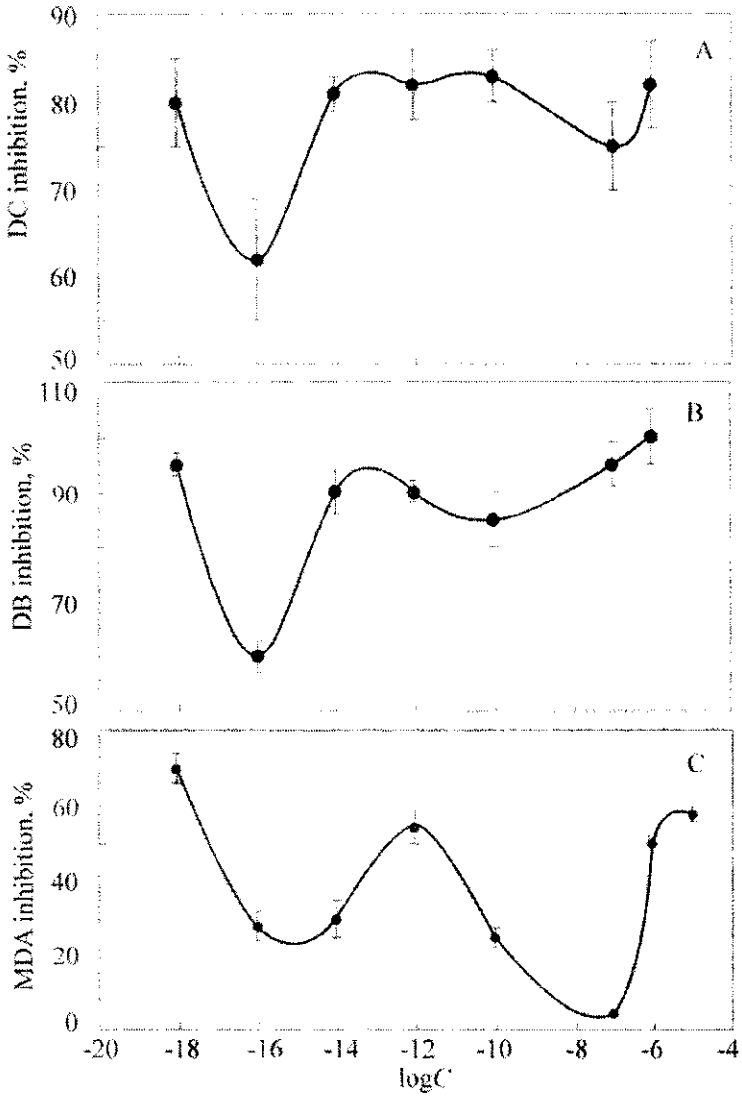


Figure 4. Inhibition of dienic conjugate (DC) accumulation (a) and consumption of unsaturated lipid substrate (DC) (b) and malonic dialdehyde (MDA) (c) in percents under the effect of α–tocopherol in various concentrations on initiated POL in endoplasmic reticulum membranes. Protein concentration is 1 mg/ml, $T = 37°C$

Figure 4 shows analogous dose dependencies for initiated POL inhibition by α–TP. As indicated by the curves, the changes in POL inhibition by criteria DC and DB are similar: almost all studied concentrations, except for 10^{-16} M, extremely high POL inhibition (85 – 100%) is observed. This can be explained by α–TP regeneration from its radical shape due to the interaction with ascorbic acid present in the reaction mixture and initiating POL, and which is also hydrogen donor [27]. Hence, the reliable correlation between these indices is also observed (r = 0.83; p < 0.005). The concentration dependence of the initiated POL inhibition by α–tocopherol according to MDA criterion is of somewhat different character: the effects are lower by the absolute value; a reliable correlation by criteria DC and MDA, DB and MDA between them are absent. The estimation of general character of the dependencies shows lower expression of bimodality, typical of the substances displaying ULD effect, for DC and DB rather than for MDA.

Comparing the results obtained on the study of antioxidant action of α–TP on spontaneous and initiated POL models, one may conclude that, firstly, unitypical dose dependencies typical of ULC acting substances were principally obtained for both models; however, for the initiated POL model the absolute values of inhibition were found overestimated due to α–TP regeneration by ascorbic acid [27]. The results different by the type of changes (Figures 3c and 4c) were obtained for MDA criterion. This circumstance is of importance, because in the majority of biological and medical investigations this very criterion forms the basis for comparison of POL development in the samples obtained from tissues and membranes, initially possessing different lipid composition and, consequently, accumulation kinetics, and the MDA level by the moment of it measurement. Despite the exhaustive analysis carried out in [21], which stipulates inadequacy of application of the current method for POL characterization under such conditions, unfortunately, it is applied still. Our results once again show that even at the substrate oxidation with the same lipid composition different determinations of antioxidant activity of BAS with respect to the POL model used (spontaneous or initiated) and criterion of its development.

3. The effect of PPS on POL in a broad range of concentrations in endoplasmic reticulum membranes isolated from mouse liver in vitro

Similar experiments were carried out with synthetic antioxidant PPS [23]. For spontaneous POL, the results of these experiments are shown in Figure 5 (a, b, c) indicating that PPS displays its antioxidant properties by all three criteria used in the concentration range of 10^{-6} M and 10^{-10} – 10^{-18} M; according to each parameter, oxidation is suppressed by 30 – 100% with respect to concentration. However, it should be mentioned that the action of high PPS concentration (10^{-6} M) is comparable with the ULC effect (10^{-18} M). Concentrations of 10^{-5} M intensify POL significantly, whereas 10^{-7} – 10^{-8} M – insignificantly by criteria DC and DB (Figure 5a,b). The dose–effect curve is of the polymodal character typical of compounds active in ULC, moreover, contrary to α–TP, possessing zones of both POL inhibition and activation. The correlation analysis discovered high, statistically reliable POL inhibition correlation for DC and DB ($r = 0.83$; $p < 0.005$); for DB and MDA ($r = 0.79$; $p < 0.005$); and lower expressed correlation for DC and MDA ($r = 0.68$; $p < 0.015$).

At the initiated oxidation (Figure 6a,b,c) PPS is a quite strong inhibitor of POL in the concentration ranges within 10^{-8} – 10^{-5} M and 10^{-16} – 10^{-11} M by criteria DC and DB that corresponded to a polymodal dependence also typical of spontaneous oxidation. According to changes in MDA criterion, there is a significant difference in the effect dependence on concentration: the effect sign changes, when inhibition in the dose range of 10^{-10} – 10^{-4} M is converted to accelerating effect for 10^{-15} – 10^{-12} M. Such type of curves is also typical of various low-intensive effects, but is observed less frequently than bi- or polymodal type of curves [8]. The initiated POL simulation gave no statistically reliable correlation between POL inhibition level according to criteria DC and DB, DC and MDA, DB and MDA.

Comparing antioxidant properties of α–TP and PPS on models of spontaneous and initiated POL, it may be concluded that, despite some differences and features, both preparations possess general ability to modulate significantly POL in a broad range of concentrations; hence, the dose–effect dependence is of poly- or bimodal character typical of BAS in ultra-low concentration. It is very probable that these features form the basis for many physiological and biochemical effects of these substances. α–TP possesses only POL inhibiting properties in both models and for all three criteria used, whereas in spontaneous POL model PPS may represent a pro-oxidant.

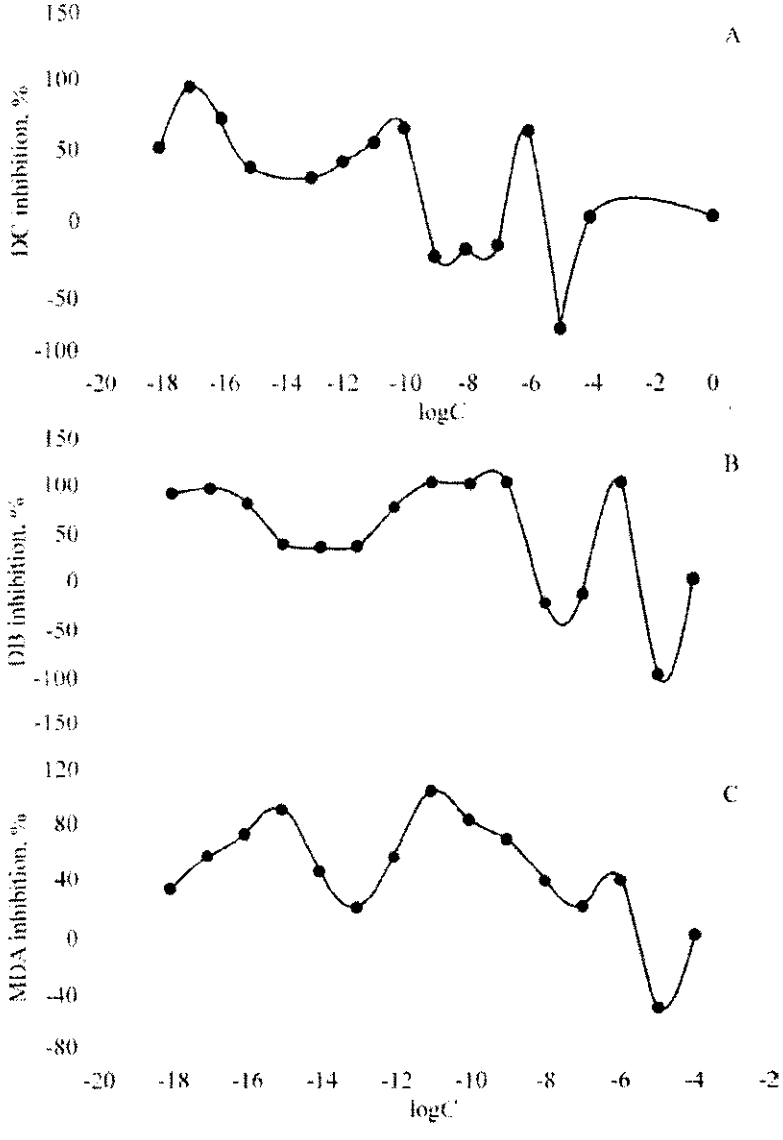


Figure 5. The influence of various concentrations of potassium phenosan (PPS) on dienic conjugate (DC) accumulation (a) and consumption of unsaturated lipid substrate (DC) (b) and malonic dialdehyde (MDA) (c) in percents on spontaneous POL in endoplasmic reticulum membranes. Protein concentration is 1 mg/ml, $T = 37°C$

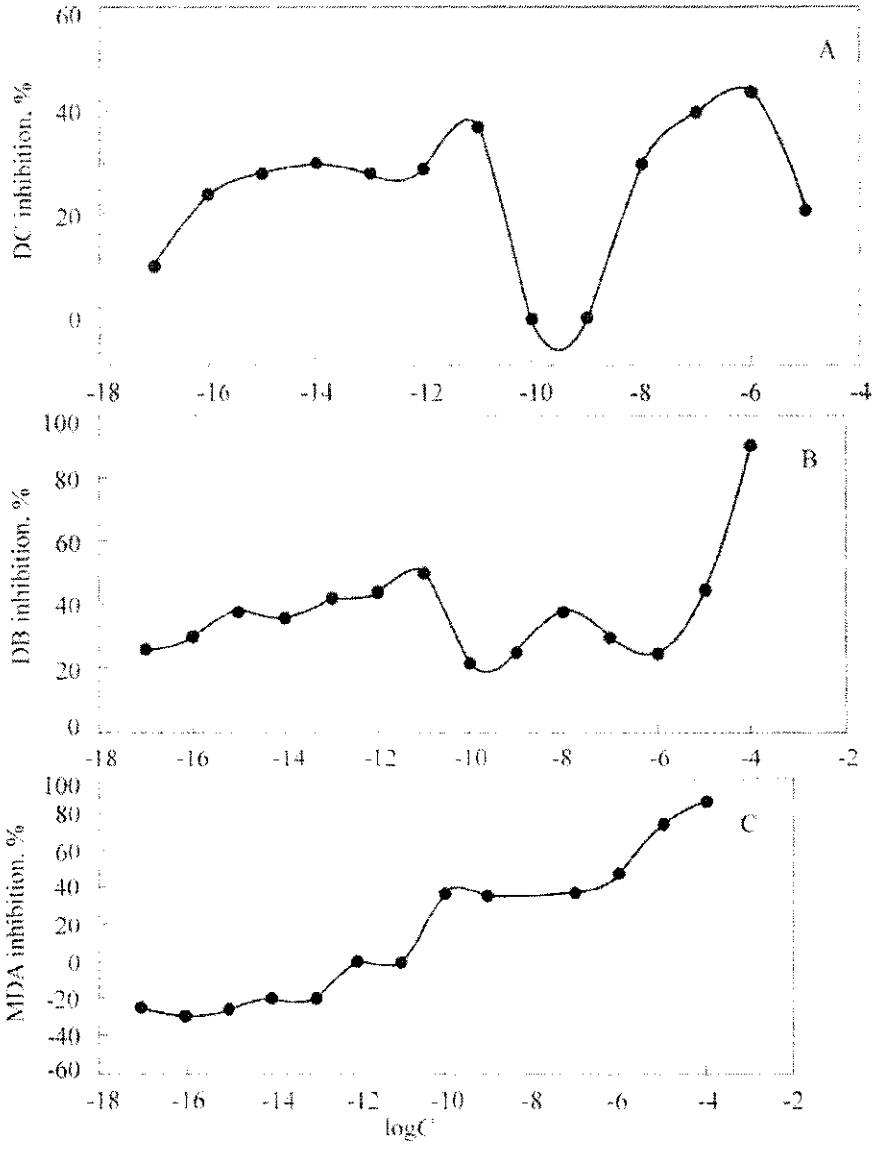


Figure 6. The influence of various concentrations of potassium phenosan (PPS) on dienic conjugate (DC) accumulation (a) and consumption of unsaturated lipid substrate (DC) (b) and malonic dialdehyde (MDA) (c) in percents on initiated POL in endoplasmic reticulum membranes. Protein concentration is 1 mg/ml, $T = 37°C$

Table 2

Possible mechanisms of ULC antioxidant action

Concentration (in the environment)	Concentration (calculated per lipids)	Possible mechanism
Below 10^{-9} M	10^{-6} M	Antioxidant action
$10^{-13} - 10^{-10}$ M	$10^{-10} - 10^{-7}$ M	Structural factor
$10^{-18} - 10^{-14}$ M	$10^{-15} - 10^{-11}$ M	Ligand–receptor theory
$10^{-22} - 10^{-15}$ M	$10^{-19} - 10^{-12}$ M	Water structure

All data obtained produce a suggestion that the spontaneous POL model (without injection of initiators) is more adequate for the estimation of antioxidant action of BAS.

Concluding the discussion, let us consider the ideas currently existing on possible ultra-low concentration BAS mechanism, including antioxidants. Since the greater part of POL inhibitors represent phenol origin substances, well soluble in lipids (occurring in an organism or membranes, water-soluble PPS transform to phenosan – the acid, and also accumulates in lipids), then the concentration of studied substances, calculated for lipids, will decrease by three orders of magnitude. Table 2 shows several possible mechanisms, suggested by different authors for several concentration ranges of these compounds. By 10^{-9} M concentration (10^{-6} M per lipids), the classical antioxidant mechanism is absolutely rightful [26]; in the particular case, the ligand–receptor theory may be excluded from the consideration, because in our experiments the membranes of endoplasmic reticulum are used, having no receptors to antioxidants. To estimate the role of the structural factor in the antioxidant action of α–TP, the authors have implemented special investigations, namely, studied α–TP effect on microviscosity of various areas of biological membranes *in vitro* in a broad concentration range.

The investigation was carried out by the spin probe method, for which stable radicals with different depth of nitroxyl radical submerging into the lipid bilayer (16-doxylstearic acid – 22 Å, and 5-doxylstearic acid – 8 Å) were used [20]. As a result, polymodal concentration dependencies of the effects expressed in percent relation to the control, typical of ULC substance effect, were presented in Figures 7 and 8. It is of importance that all α–TP concentrations (except for 10^{-22} M area) induce parameter τ_{C1} and τ_{C2} increase, i.e. microviscosity in the area of probe C_{16} localization. Moreover, the values of effects caused by α–TP ULC are comparable with the changes induced by

the action of its "*physiological*" concentrations (10^{-9} – 10^{-6} M; Figure 7, curve 1). Besides, in deep areas of lipids, an increase of amount and occurrence of higher cooperative thermally induced structural transitions, as well as the increase of their effective activation energy with temperature [28].

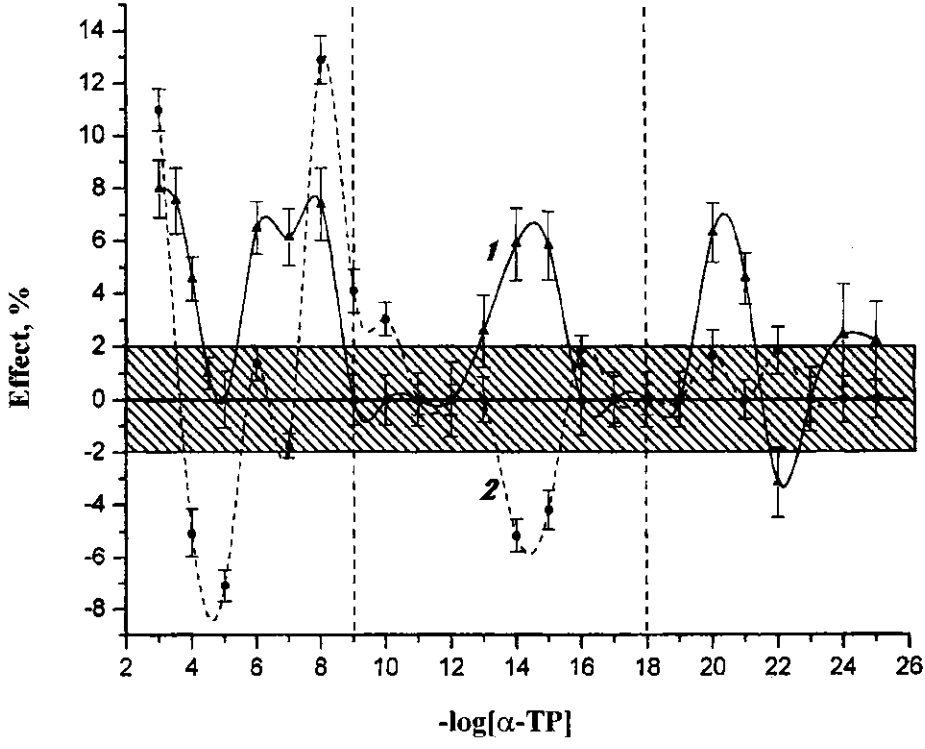


Figure 7. The effect of α–tocopherol in polar (1) and nonpolar (2) solvents on microviscosity of deeply occurring areas of microsomal membrane lipids at 293 K and protein concentration equal 4 mg/ml in the membrane suspension

Figure 8 shows change in rigidity (ΔH_{II}) of the surface areas (~8 Å) of the lipid bilayer in microsomal membranes induced by α–TP in the concentration range of 10^{-18} – 10^{-3} M at T = 293 K. The concentration dependence of α–TP effects is of bimodal shape typical of ULC actions with the maxima at 10^{-13} and 10^{-5} M, separated by a broad "*dead zone*" including

five orders of magnitude by concentration. The effect of all α–TP concentrations, except for $5 \cdot 10^{-4}$ M, increases parameter ΔH_{II} value, which indicates increasing membrane rigidity. Moreover, in ULC range the effect is twice higher than at usual "*physiological*" concentrations of α–TP.

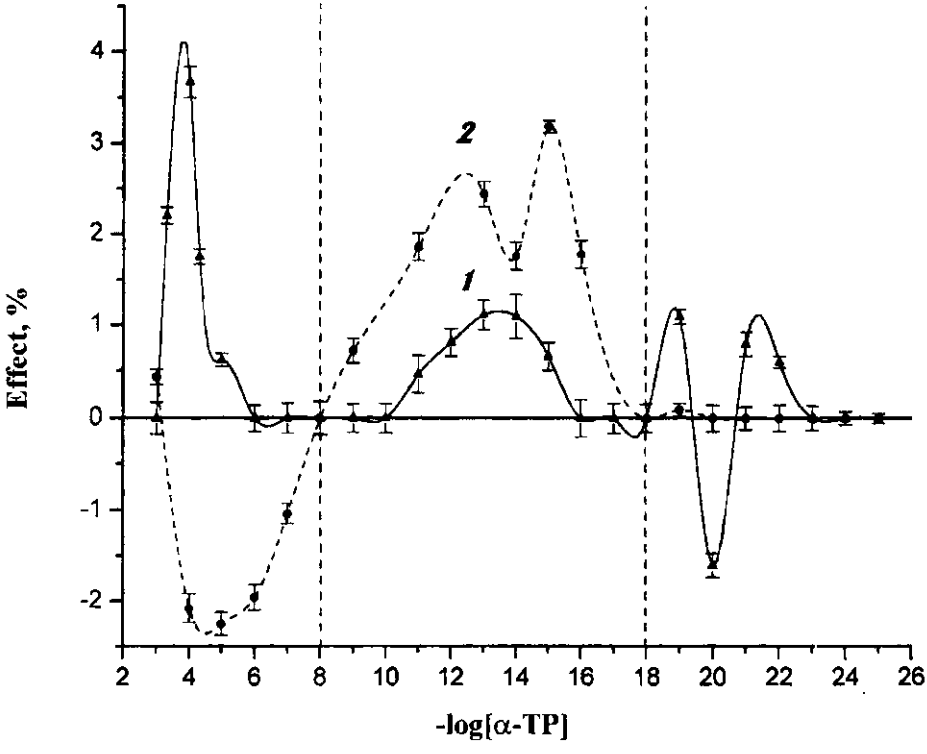


Figure 8. The effect of α–tocopherol in polar (1) and nonpolar (2) solvents on rigidity of surface areas of microsomal membrane lipids at 293 K and protein concentration equal 4 mg/ml in the membrane suspension

Thus the experiments *in vitro* carried out on endoplasmic reticulum membranes of liver cells reliably confirm α–TP ULC effect on both surface and deeper areas of lipids, which is strictly related to its antioxidant action. Nevertheless, there are questions still about possible mechanisms of chemical reagent action on biological objects in the area of so-called "*imaginary*" concentrations (10^{-18} M or lower), when the probability of occurrence of even

a single BAS molecule in the membrane suspension approaches zero. Many authors tend to explain the observable regularities from positions of the idea about structure-forming properties of polar, in particular, aqueous solvents [8, 30]. Therefore, for the purpose of determining the role of the solvent polarity in the mechanism of BAS ULC action, we have carried out a comparative study of the influence of natural antioxidant α–TP prepared in on viscosity properties of the lipid bilayer in microsomal membranes of liver cells in mice *in vitro*. The results are shown in Figures 7 and 8, curves 1 for polar solvents (alcohol – aqueous solutions with predominant water content as diluted) and curves 2 for nonpolar solvents (based on petrolatum oil) in a broad range of concentrations (10^{-25} – 10^{-3} M), including "*imaginary*" concentrations (10^{-18} M or lower).

For nonpolar (petrolatum oil) solvent used polymodal concentration dependencies of the effects typical of substance ULC action were obtained (Figure 7, curve 2). Maxima and minima of the effects relate to increasing and decreasing microviscosity of deeper areas, respectively; moreover, the values of effects in ULC and "*physiological*" concentration areas are similar for both types of solvents. It is very important that in the area of so-called "*imaginary*" concentrations (10^{-18} M or lower), where the probability of even a molecule of α–TP occurrence approaches zero, only polar solvents (currently, aqueous α–TP solutions; Figure 7, curve 1) demonstrate the reliable effect. This indicates a significant role of the solvent polarity in the transfer of "*information*" about the substance during dilution, when the solvents are being prepared.

Concentration curves also possess the so-called "*dead zones*" characterized by zero values of effects. Hence, with the solvent change to nonpolar (petrolatum oil) type the "*dead zone*" width decreases by two orders of magnitude (Figure 7, curve 2).

Both concentration curves shown in Figure 7 can be divided into three areas:

- 10^{-25} – 10^{-18} M – the "*imaginary*" concentration area, where the effect is displayed by α–TP polar solutions only;
- 10^{-18} – 10^{-9} M – the ULC area, where the dose–effect curves for α–TP in polar and nonpolar solvents are counter-phased and comparable by the amplitude;
- 10^{-9} – 10^{-3} M – the area of "*physiological*" concentrations, in which α–TP is present in the organism. In this area dose dependencies are

synchronized and for α–TP solution in petrolatum possess high amplitude.

Figure 8 shows concentration dependencies of polar and nonpolar α–TP solution effects on rigidity of the surface areas (~8 Å) of the lipid bilayer in microsomal membranes at T = 293 K. The dose–effect curves are both of polymodal type, possessing maxima of the effects corresponded to lipid bilayer rigidity increase in the probe C_5 localization zone, and minima – to decrease. Similar to effects in deeper lipid zones, in this case, the same concentration zone may be separated on dose dependencies for both types of solvents. Particularly, in α–TP "*physiological*" concentration area (10^{-8} – 10^{-3} M) the dose curves are counter-phased for both types of solvents, possessing much higher amplitude for polar α–TP solutions. The ULC zone (10^{-18} – 10^{-8} M) shows synphase curves with high amplitude for petrolatum-based nonpolar solvent. However, in the area of "*imaginary*" concentrations (10^{-25} – 10^{-18} M) reliable and statistically valuable effect is demonstrated by α–TP aqueous polar solvents only, which strictly confirms the hypothesis about the significant role of the solvent polarity in the substance "*information*" transfer at dilution. The "*dead*" zones showing the absence of the reliable effect are also observed for the polar solvent only.

Thus the experiments performed indicate the counter-phase type of the "dose–effect" curves in the surface and deeper zones of the lipid bilayer in microsomal membranes in the areas of α–TP "*physiological*" concentrations and ULC in both polar and nonpolar solvents. Hence, at transition to nonpolar solvent, the "*dead*" zone either disappears (probe C_5), or reduces in width (probe C_{16}). Meanwhile, in the area of α–TP "*imaginary*" concentrations (10^{-18} M of lower) only polar aqueous solutions of it possess the reliable effect on the structure of microsomal membranes. Totally, these results indirectly support the suggestions, present in the literature, about the possible role of water in the substance "*information*" transfer of any kind at dilution during the solvent preparation.

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STRUCTURE, PHARMACOLOGICAL PROPERTIES AND MEDICAL APPLICATION OF HETEROAROMATIC ANTIOXIDANTS

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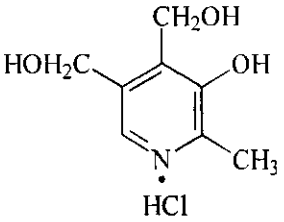
Compounds of the antioxidant type compose a new pharmacological group of medical preparations possessing an original spectrum of biological action. High treatment effectiveness of these compounds in medical practice is determined. The theoretical premise for development of this direction was detection of the important role of free radicals of oxygen, the so-called reactive oxygen species (ROS) in biological systems [1].

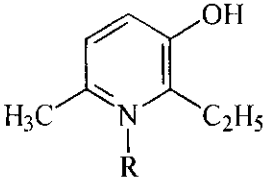
Disturbances in the systems controlling free-radical process intensity activate peroxide oxidation of lipids, damage lipids in biological membranes and, consequently, cause development of various pathological states [2].

The fundamental studies, initiated by N.M. Emanuel, have justified the necessity of searching for effective antioxidants, which limit or interlock free radical synthesis in the human organism or their reactions initiating development of pathological processes.

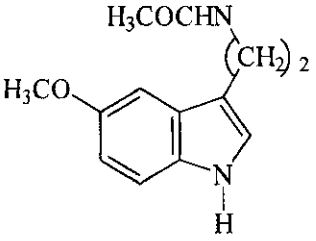
Heteroaromatic antioxidants used in medicine



Pyridoxol (I)
(Vitamin B_6)



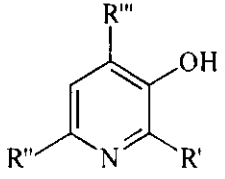
Emoxypine (II)
(R = HCl)
Mexidol
(R = HOOC–$(CH_2)_2$–COOH)



Melatonin (III)

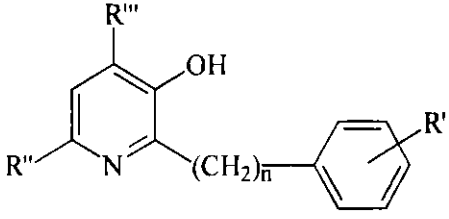
Among various synthetic antioxidants, aromatic and heteroaromatic phenols are of special attention, particularly, 3-oxypyridine (3–OP) derivatives, which are structural analogues of vitamin B_6 and possess a broad spectrum of pharmacological properties, stipulated by their antioxidant and membrane-stabilizing action [3 – 5].

Among the apparent advantages of this group of antioxidants is water solubility of their salts in organic and mineral acids that allows for creation of injectable forms of drugs. Accessible methods of 2-alkyl-(IVa), 2-alkaryl (V) and 2-phenylethyl-(VI), 2-aryl-(VII), 2.6-dialkyl-(4b), 2,4,6-trialkyl-3-oxypyridines (VIc), and many derivatives have been developed [6 – 9].



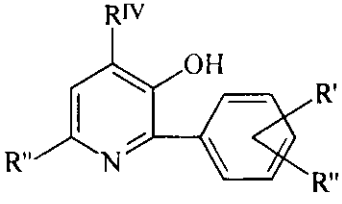
a) R′ = Alk; R″, R‴ = H
b) R′ = R″ = Alk; R‴ = H
c) R′ = R″ = R‴ = Alk

IV



R′ = R″ = R‴ = H; n = 1, 2
R′ = H; R″, R‴ = CH_2NR_2
R′ = OH; R″ = R‴ =H

V (n =1); VI (n = 2)



a) R′ = R″ = R‴ = R^{IV} = H
b) R′ = R″ = H; R‴ = R^{IV} = CH_2NR_2
c) R′ = Alk; R″ = R‴ = R^{IV} = H

VII

It is found that alkyl- and aryl-substituted 3–OP possess high antiradical activity [10 – 11] and high reactivity in relation to radicals of biological molecules – polypeptides and proteins, oxidative and reduction

radicals [12 – 13]. The effective concentration of antioxidants and their relative activity were determined by kinetic methods of studied drug additions to the model reaction of initiated oxidation of cumene (at 40 or 50°C) with the chemoluminescence (CL) intensity control. The results obtained were related to the data of the known inhibitors: ionol, α-tocopherol and its analogue chromane C_1 [14, 15].

The dependence of antiradical activity of 3-oxypyridine derivatives on their structure was tracked. Table 1 shows that unsubstituted oxypyridine is practically unable to react with free radicals. The introduction of electron-donor substituents into pyridine circle increases electron density at OH-bond and promotes homolytical detachment of hydrogen from hydroxyl group. It also leads to a significant (by 10 – 100 times) increase of 3–OP antioxidant activity.

The ARA increase for 3–oxypyridine is defined by the electron-positive substituent effect and the place of its injection to the pyridine ring. The greatest increase of k_7 was observed at introduction of alkyl substituents into the *ortho*-position at OH-group.

In the sequence of 2-alkyl-3-OP, the highest k_7 value is observed for 2-methyl-3-oxypyridine and 2-phenyl-3-oxypyridine. For *ortho*-isopropyl substituents, the increase of OH-group shielding slightly reduced k_7 value. The introduction of additional methyl group into position 6 increased the antiradical activity (ARA) of 3-oxypyridine.

A greater increase of ARA was observed at introduction of three alkyl groups into 3-oxypyridine structure. For example, k_7 value for 2,4,6-trimethyl-3-oxypyridine equaled 10^5 $(Ms)^{-1}$.

For phenol derivatives, the analogous tendency was observed. For instance, introduction of alkyl groups into phenol ring increases its antiradical activity: 2,4,6-trimethylphenol possesses higher constant k_7 rather than other Me-substituted phenols.

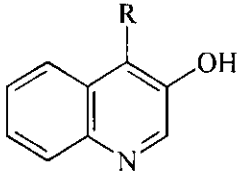
The introduction of phenol group, possessing negative inductive and positive mesomeric effects, into position 2 of the pyridine cycle increased k_7 value by the same value, induced by introduction of CH_3 group.

The transition from 2-methyl- to 2-benzyl-3-oxypyridine was accompanied by k_7 value decrease, which is associated with demonstration of only negative inductive effect by methyl group; hence, this effect decreases electron-donor properties of CH_3-group.

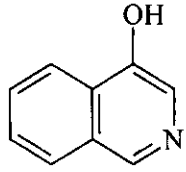
However, introduction of methoxy-group into 2- and 5-benzyl-3-OP structure or one or two oxy-groups into 3-OP molecule causes a significant increase of antiradical activity (by 10^3 times, approximately).

The introduction of electron acceptor NO_2-nitrogroups into β-pyridol ring abruptly decreased ARA of 3-oxypyridine derivatives. A substitution of functional OH-group by amino group in 3-oxypyridine also induced a noticeable decrease of k_7 value that testifies about higher proton mobility in OH-group compared with NH_2-group.

By analogy, introduction of benzene ring into 3-OP (VIII, IX) molecule also increases their antiradical activity [11].



VIII



IX

Therefore, analysis of the data obtained (see Table 1) on 3-OP ARA allows for determining the low for various substituent influence on the constant of interaction with 3-OP derivative peroxy-radicals. This provides for a targeted synthesis of the most effective inhibitors in the studied class of compounds.

The antioxidants mentioned show high effectiveness acting as membrane protectors. The membrane-stabilizing action of alkyl- and aryl-substituted 3-OP was detected due to their ability to increase blood resistance to mechanical trauma and hypotonic hemolysis [16, 17]. The highest protection level was provided by alkyl-substituted 3-OP and theirs oxymethyl derivatives. Substitution of phenol hydroxide by amino group or nonsubstitution led to full loss of membrane-stabilizing properties. It is found that 3-OP derivatives may effectively inhibit PDE of cyclic nucleotides from rabbit heart [18, 19].

It is found that 3-OP derivatives demonstrate an original spectrum of neurotropic action (anxiolytic, anti-stress, anti-amnesic, and anti-alcoholic properties), combining properties typical of tranquilizers and nootropics [5, 20 – 22].

Table 1

Antiradical activity of 3-OP derivatives in relation to ethyl benzene peroxy-radicals (T = 50°C, RH = EB; $W_i = (2 \div 5)\cdot 10^{-8}$ (Ms)$^{-1}$, activator – DBA)

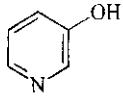
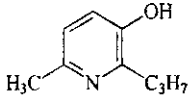
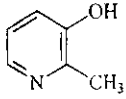
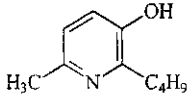
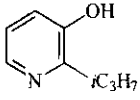
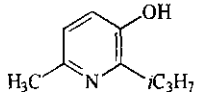
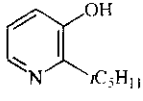
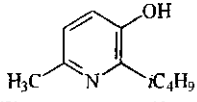
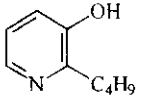
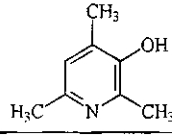
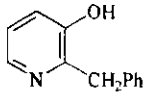
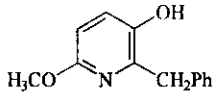
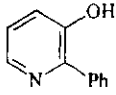
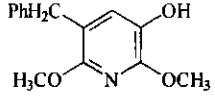
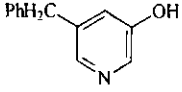
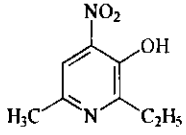
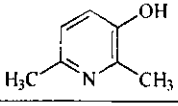
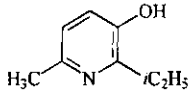
No.	Compound	$k_7\cdot 10^{-4}$ (Ms)$^{-1}$	No.	Compound	$k_7\cdot 10^{-4}$ (Ms)$^{-1}$
1		0.70	10		3.40
2		1.40	11		4.70
3		1.20	12		2.30
4		1.10	13		2.70
5		1.94	14		9.50

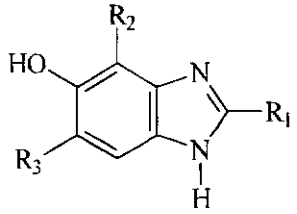
Table 1 (continued)

6		0.60	15		70.0
7		1.50	16		30.0
8		0.10	17		0.05
9		4.40	18		3.30

Among 3-OP derivatives, the compounds with actoprotector properties have also been found. For example, 2-phenylethyl, 5-methylbenzyl and adamantyloxy-3-OP demonstrated similar action in relation to the known actoprotector bimethyl. They increased physical workability of experimental animals at prophylactic injection under usual and complicated conditions (hyperthermia and hypoxia) [23, 24].

Besides the neurotropic activity, 3-OPs demonstrate anti-ischemic and lipid-regulating properties, as well as ability to normalize carbohydrate and energy exchange [25, 26].

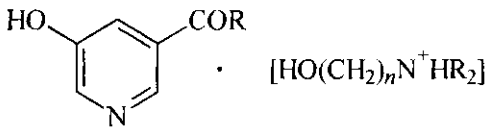
Among heteroaromatic antioxidants, 5-hydroxybenzimidazole (5-HBI) derivatives in the composition of vitamin B_{12} fragment should be mentioned [15]. The study of 5-HBI influence on liver microsome and cerebrum synaptosome membranes at POL activation shows their inhibiting effect at both ascorbate- and NADPH-dependent induction of POL [27]. It is found that 5-HBI derivatives are more effective POL inhibitors at the nonenzymatic induction method.



X (R_1 = H, CH_3, SH; R_2 = R_3 = H, CH_3)

The inhibiting effect of 5-HBI in liver microsomes may be compared to the action of α-tocopherol, but second to it in effectiveness at POL activation in cerebrum synaptosomes. The study of psychotropic activity of 5-HBI derivatives indicated high actoprotector properties of 2-mercapto-substituted 5-HBI [28].

Another class of membrane-active compounds of interest, possessing a broad spectrum of pharmacological effects, was found 5-oxynicotinic acid (5-ONA) derivatives. For example, 5-ONA derivatives containing aliphatic and cyclic amine fragments are perspective cardiovascular drugs.



R =C_2Cl_2OH; CH_3; n = 2, 3

XI

XII, R = $NH(CH_2)_3COOH$

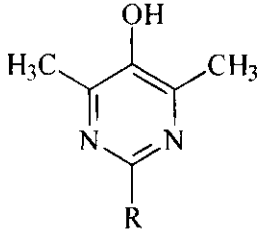
XIII, R = $NHCH(CH_2)_2COOH$
|
COOH

The most perspective 5-ONA derivative is protecor (aminium salt), characterized by high antianginal activity and ability to prevent formation of necrotic areas at short-term transient ischemia of myocardium [29, 30].

It is found that protecor toxicity (LD_{50} is above 5,000 mg/kg) is much lower than for compounds from the group of β-adrenoceptor blocking agent and calcium antagonists. Protecor dosed within 0.2 – 1.0 mg/kg possesses lipid-regulating properties and exceeds drug probucol by hypolipidemic activity. Applied in the same doses, protecor also demonstrated anti-aggregation properties [29, 30].

High anti-hypoxic and anti-amnesic properties are also demonstrated by amino acid 5-oxynicotinic acid derivatives (XII and XIII). Compared with the known nootropic drugs (piracetam etc.), one of the compounds, N-(5-hydroxynicotinoyl)-L-glutamic acid – nooglutyl (XIII) – possesses higher specific activity [31, 32].

The perspective class of membrane-active compounds also includes 3-OP aza-analogues, representing 5-hydroxypyrimidine (XIV) derivatives. The advantage of this group of antioxidants is their low toxicity [10].



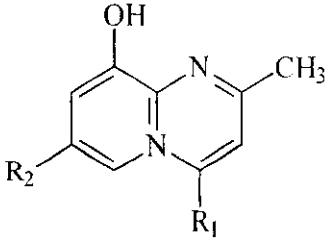
(R = H, CH_3, Ph)

XIV

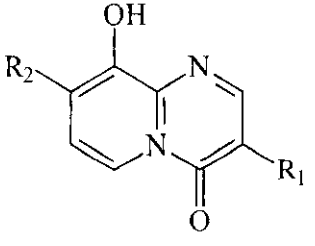
5-Hydroxypyrimidine derivatives cause the inhibiting influence on the cell-mediated immune reactions without suppression of the humoral immunity

system [10, 33, 34]. They reduce cytotoxic activity of immune lymphocytes affecting cells-target, have no effect on the functional activity of T-suppressors, and produce the dose-dependent stimulation or suppression of lymphocyte transformation, induced by phytohemagglutinin. These derivatives do not affect mature antibody producers and do not inhibit synthesis of hemagglutinins at the primary and secondary immune response. At the same time, these compounds decelerate development of the reaction of hypersensitivity of decelerated type for dinitrochlorobenzene. Among 5-hydroxypyrimidine derivatives, immunosuppressors, mostly affecting T-lymphocytes and perspective for application at transplantation of tissues and organs, and treatment of autoimmune diseases, were detected [33].

9-Hydroxypyrido[1,2a]pyrimidines possessing tertiary (group A) or quaternary bridge atom of nitrogen (group B) and representing 3-OP heteroanalogues, containing condensed pyridine cycle, show a complicated spectrum of psychotropic activity [35, 36].



XV (A)



XVI (B)

According to the psychotropic action, the studied compounds manifest themselves as serotonin antagonists and are distinguished by high tropic property in relation to the catecholamine system. Hence, the preferable activating action is typical of pyridopyrimidines from the group A. This action was determined in tests on the interaction with apomorphine and phenamine. These compounds manifest themselves as potential antidepressants both in behavioral screening-test detecting antidepressant activity and by the type of influence on monoamine systems (antiserotonin action and action synergism with apomorphine and phenamine). Pyridopyrimidines of the group B possess more complex spectrum of psychopharmacological activity and with the change of the type of substituents in position 8 of bicycle converts its action from antidepressant (activation) to sedative type. Thus, 9-

hydroxypyridopyrimidines may be related to a new group of psychotropic compounds combining properties of antidepressants and tranquillizers [35].

As a result of pharmacological screening carried out in various institutes (N.M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences; Research Institute of Pharmacology, Russian Academy of Medical Sciences; All-Russian Scientific Centre of Biologically Active Substances (Staraya Kupavna); S. Ordgonikidze All-Russian Research Chemicophysical Institute), the most effective drugs for introduction into medical practice – emoxypine and mexydol (2-ethyl-6-methyl-3-oxypyridine hydrochloride and succinate, respectively) and nooglutyl (N-(5-oxynicotinoyl)-L-glutamic acid) have been selected.

The comprehensive studies of biological properties of emoxypine and mexydol indicated that these antioxidants may act as potentially protective agents against various factors damaging the organism and demonstrate high effectiveness as radio-, photo-, hepato-, and geroprotectors. Therefore, they can be successful as universal drugs of antioxidant pharmacotherapy [10, 37].

The mentioned compounds show high effectiveness in various branches of medicine: neurology, psychiatry, cardiology, ophthalmology, surgery, dentistry, endocrinology, etc.

Emoxypine has found a broad clinical application in ophthalmology for treating subconjuctival and intra-eye hemorrhages, including in retina and vitreous body, at post-traumatic hemorrhages, hyphemas, angioretinopathies of various causation, including diabetes, as well as for cornea and retina protection from high intensity light impacts (sunlight, laser radiation at laser coagulation) [37, 38]. This drug is recorded to the Register of the most vitally important medical compounds.

Mexydol is related to the group of antihypoxants with nootropic and anxiolytic properties and antioxidant action [20 – 22]. It possesses a broad spectrum of pharmacological activity: it is antihypoxic, stress protective, nootropic, anticonvulsive and anxiolytic compound, which inhibits free-radical processes of oxidation of lipids [39, 40]. This compound increases the organism resistance to the influence of various damaging factors, oxygen-dependent pathological states (stress, hypoxia and ischemia, cerebral blood circulation disturbances, alcohol and anti-psychotic drug intoxication [41 – 43].

This compound improves cerebral metabolism and blood circulation in cerebrum, increases microcirculation and rheological properties of blood, and reduces aggregation of thrombocytes [42]. It also stabilizes membrane structures of blood corpuscles (erythrocytes and thrombocytes) preventing their

hemolysis. It demonstrates hypolipidemic action and, therefore, reduces concentration of general cholesterol and low-density lipoproteids [25]. Mexydol also decreases enzymatic toxemia and endogenic intoxication at acute inflammation.

The mexydol action mechanism is defined by its antihypoxic, antioxidant and membrane-modulating action.

Mexydol affects the main pathogenesis stages of various diseases, which are associated with free-radical oxidation processes and oxygen-dependent pathological states. Moreover, this mechanism explains very low secondary effects caused by mexydol and its ability to initiate other central-acting substances [37, 43].

The pharmacological properties of mexydol are realized at least at three organizational levels – neuronal, vascular and metabolic. It stimulates direct glucose oxidation by pentosophosphate shunt, increases the level of reduced nucleotide pool (NADPH), therefore, intensifying antioxidant protection of the cell and stabilizing the level of endogenic antioxidants. Therapeutic effects of mexydol are observed for the concentration range of 10 – 300 mg/kg.

Mexydol increases the organism resistance to the influence of various extreme damaging factors, such as sleep disturbance, conflict situations, stress, cerebrum traumas, electrical shock, physical loads, hypoxia, ischemia, and various intoxications [44, 45].

Mexydol has high tranquilizing and antistress action, the ability to eliminate anxiety, fear, tension, and agitation [39, 46]. At parenteral injection, it demonstrates the effect similar to diazepam (seduxen) and alprazolam (xanax). The antistress action of mexydol is manifested in normalization of the after-stress behavior, somato-vegetative indices, reduction of sleep-wakefulness cycles and disturbed learning and memory processes, decrease of the stomach ulcer, dystrophic and morphological changes, formed due to stress in various cerebrum and myocardium structures.

Mexydol demonstrates expressed antispasmodic action affecting both primarily generalized spasms, mostly induced by HAMC-ergic substances and epileptoid cerebral activity with a chronic epileptogenic focus [39, 47].

Nootropic properties of mexydol are manifested by the ability to improve the learning process and memory, promote preservation of the memory trace, and preventing a decrease of learnt and trained skills and reflexes. Mexydol has exressed antiamnesic effect, reducing memory disturbance, induced by various imparts (electrical shock, cerebrum traumas, sleep deprivation, injection of scopolamine, ethanol, benzodiazepine, etc.) [39, 48].

This compound also has demonstrates high antihypoxic and anti-ischemic action; moreover, its antihypoxic activity is much higher than for pyritinol and pyracetam. The compound also displays antihypoxic effect on myocardium. According to the mechanism of realization of these effects, mexydol is antihypoxia compound of direct energizing action, which effect is related the influence on endogenic mitochondrion respiration and activation of energy-synthesizing functions of mitochondria [49]. Antihypoxic action of mexydol is also stipulated by the presence of succinate fragment in its structure, which under hypoxia conditions is capable of oxidizing by the respiratory chain, when entering inside the cell.

Mexydol effectively suppress manifestations of acute cerebral ischemia: it increases cerebrum stability to the lack of oxygen, preserves autoregulatory reactions, reduces total power and amplitude of the cerebrum potential, supports activity of the buffer blood systems, limits permeability of vascular walls for protein and water, increases oxygen and glucose consumption by the cerebrum, and reduces lactic acid accumulation in it. It also suppresses development of the main stages of pathogenesis at post-ischemic disturbances in the cerebrum [42].

Another property of this compound is geroprotector action, indicated by clear correcting influence on the learning and memory processes, disturbed by aging, improvement of information recording, storage and playback, promotes restoration of the emotional and vegetative status, decreases manifestations of neurological deficiency and the level of aging markers – lipofuscin, malonic aldehyde, and cholesterol [50, 51]. The geroprotector mechanism of mexydol is related to its antioxidant properties, the ability to inhibit POL, direct membranotropic action, the ability to reduce ultra-structural disturbances in granular endoplasmic network and mitochondria, and modulate operation of receptor complexes.

Mexydol possesses antiaterogenic action. The compound decelerates humoral manifestations of atheroarteriosclerosis: it reduces hyperlipidemia, prevents activation of peroxide oxidation of lipids, increases activity of the antioxidant system and protects local vascular mechanisms of atherogenesis, preventing development of pathological changes in the vascular wall, and reduces aorta damage degree [52, 53].

Mexydol decreases concentration of atherogenic lipoproteids and triglycerides, increases the level of high-density lipoproteids in blood serum, and prevents deficiency of highly saturated phospholipids [25].

Mexydol induces regression of atherosclerotic changes in main arteries and reduces lipid homeostasis; it also corrects disturbances in the regulatory

and microcirculation systems, which is indicated by the absence of arteriole and precapillary constriction, and their diameters is similar to the control; in venules only local aggregates are observed, and spasms of afferent microvessels are completely removed [53].

Mexydol possesses high antialcoholic and hepatoprotective effect at alcoholic damage of liver, demonstrated by reliable decrease (by 53%, on average) in the hepatocyte population of "regressive" cellular form concentration, characterized by necrobiotic changes of nuclei in the chromatin lysis form. Simultaneously with the decrease of hepatocyte quantity at nucleus lysis, mexydol reduces the level of chromatin lysis in cells subject to this pathological process [54]. At the application of mexydol, lower level of chromatin lysis in hepatocyte nuclei at the application of mexydol promotes quicker reduction of this important structural component of the cell. On the third day of the experiment, this of observed by reliably higher positive changes in morphometric indices (the increase of granule square and decrease of light zones of chromatin) compared with ethanol and tocopherol. On the seventh day, the analysis of these indices indicates that mexydol application also intensifies reduction of total genome of hepatocytes, which was decreased in the initial period of the experiment due to death of a part of population of these cells, induced by toxic effect of ethanol.

Mexydol also normalizes the disturbance of energy balance of hepatocytes, induced by ethanol, which was expressed by preservation of higher compared with single ethanol effect, concentration of the main reserved energy carrier (glycogen) in liver tissues. On the third day of the experiment, the intensity of this protective effect of mexydol was reliably higher compared with analogous action of tocopherol [54, 55].

For liver damages with carbon tetrachloride, mexydol reduces necrosis zones and the volume of fatty degeneration of hepatocytes, normalizes the energy balance of hepatocytes, and protects nuclear and cytoplasmic pool of nucleic acids [54].

The model of liver damage in rats by chronic injection of alcohol and desulfiram treatment at the estimation of the morphofunctional state of the liver have shown clear hepatoprotective action of mexydol against toxic doses of alcohol and decrease of hepatotoxic effect of desulfiram [54, 56]. The hepatoprotector effect of mexydol is accompanied by decreasing level of lipofuscin in cerebrum of rats and elimination of neurological and neurotoxic manifestations of acute alcoholic intoxication.

For two-week injection of toxic doses of tuberculostats into rats, the changes in biochemical indices indicating the increase of hepatocyte cytolysis

and intensification of POL in the liver. Mexydol application increases catalase and glutathione reductase activity, decreases MDA level and free iron concentration in the blood serum. It also promotes limitation of cholestasis, induced by tuberculostats [55, 57].

It is shown that mexydol application prevents high POL activation, reduces the yield of Transaminases ALT and AST to blood plasma, and suppresses the damaging effect of relanium. At this background mexydol and other antioxidants normalize catalase activity in blood plasma, liver and kidneys [55].

It is noted that mexydol effectively corrects activation of peroxide oxidation of lipids, causes hepatoprotector effect, corrects lipid and carbohydrate exchange disturbances, developed at combined influence of ethanol and experimental diabetes [58].

For the first time, it has been found in the experiment that mexydol possesses high hepatoprotector properties in relation to pancreatogenic hepatic failure (HF) and is specially observed at heavy acute toxic HF, induced by carbon tetrachloride (CCl_4). At intraperitoneal injection mexydol decreases lethality, POL activity and intensity of hepatodepressive syndrome to much greater extent than comparison compounds [59].

Data obtained in experiments on animals became the foundation for mexydol application in clinics at liver damages of various geneses. Inclusion of mexydol into the basic therapy of patients with pancreatogenic HF increases effectiveness of the standard treatment scheme, allows for a significant decrease of the treatment time, the number of complications and lethality. Moreover, mexydol has higher hepatoprotector effect than essentiale [60].

Possession of hepatoprotector effect by mexydol, which is manifested by limitation of cytolysis syndrome, a decrease of break frequency in protein- and cholesterol-synthesizing function of liver provides for wide possibilities of their use in oncology, during chemotherapy course at breast cancer, in particular [55]. Mexydol application decreases the frequency of development and severity of endogenic intoxication syndrome, corrects disorders of electrolytic and microelemental composition of blood plasma in patients with breast cancer and, therefore, allows for reduction of complication risk after chemotherapeutic treatment by cytostatics [55].

Other fields of clinical application of mexydol are neurology, psychiatry and cardiology.

NEUROLOGY

Vascular ischemic damages of cerebrum, first of all, acute disturbances of cerebral blood circulation, are now among the main reasons for lethality and disabling of patients in neurological clinics and neuroreanimation.

Despite the broad spectrum of indications for mexydol use, this compound is an effective neuroprotector, because the frequency of cerebral insults testify about insufficient attention and improper organization of prophylaxis of acute disturbances of cerebral blood circulation. In genesis of ischemic insult the key role is devoted to oxygen exchange disorder, which may cause cerebrum hypoxia, cerebral blood circulation and metabolism disturbance, and POL activation. Among drugs applied to the treatment of ischemic disorders in cerebral blood circulation, antioxidants are of special importance. Therefore, modern approaches to pharmacological correction of acute disorders of cerebral blood circulation are based on development of medical compounds inhibiting POL processes.

It is shown that prophylactic and curative effect of mexydol on various manifestations of acute (insults) and chronic disorder of cerebral blood circulation, discirculatory encephalopathy, vegetovascular dystonia, aterosclerotic disorders of cerebrum functions [61 – 63].

The effectiveness of mexydol in treatment of patients in acute period of ischemic insult is confirmed. It is expressed by an increase of life duration for patients with the most severe damages of cerebrum and significant decrease of lethality among them (10 – 15%) [61].

Mexydol has a high effect for primary and secondary prophylaxis of ischemic insults [62]. At the background of already performed treatment, positive dynamics was observed for 80.1% patients, for 19.9% the state remained unchanged. The amount of symptoms detected for one patient varied in the range between 10 and 25 points. Prior to the treatment, neurological status was estimated as (12.6 ± 0.5) points, after the end of treatment – as (4.8 ± 0.5) points ($p < 0.05$). The regression of neurological symptoms equaled 61.9% of the initial level.

Analyzing dynamics of blood lipid spectrum index change in patients with insult, treated by mexydol, one may conclude about the following. The lipid spectrum of blood was changed, which may be characterized as antiatherogenic process. At the background of treatment, the level of general cholesterol decreased by 8.9% ($p < 0.05$), the atherogeneity index – by 10.95%

($p < 0.05$), B–XC – by 6.8%, whereas α-YC level increase by 15.7% ($p < 0.05$) and apoprotein A1/apoprotein B ratio – by 2.3%. The thrombocyte aggregation decreased by 15.9% ($p < 0.05$), whereas thrombocyte deaggregation increased by 14.2%. Positive change of hemostasis plasma unit was not so high. Deceleration of phases I and II of blood coagulation according to TEG data was observed: R increased by 7.5%, R + K by 3.8%, and Ma decreased by 5.2%. The amount decrease of coagulation plasma factors was testified by VCTV increase by 4.9% ($p < 0.05$). Positive dynamics was also observed in fibrinolytic system of blood, FAC increased by 8.7% ($p < 0.05$), whereas fibrinogen level decreased by 9.2%. Paracoagulation processes also demonstrate positive tendencies: optical density PST decreased by 7.5% ($p < 0.05$), and positive ET was observed for 11.9% cases. At the background of treatment aggregation of erythrocytes reliably decreased by 28.8% ($p < 0.05$).

Positive changes were also observed in POL system: levels of primary (diene conjugates) and secondary (malonic dialdehyde) POL products decreased by 21.6% ($p < 0.05$) and 30.1%, respectively. Superoxide dismutase activity (the main ACC enzyme) increased by 18.9% ($p < 0.05$) [62].

Positive dynamics in the hemostasis system was observed for both vascular-thrombocyte and coagulation chains.

Mexydol demonstrates high therapeutic effectiveness for chronic cerebrovascular diseases, when the treatment effect is observed at both vascular and neural levels. According to USD results, mexydol causes an increase of voluminous cerebral blood circulation, vasodilation, reduction of cerebral vascular resistance, and increase of venous outflow in patients with dyscirculation encephalopathy and vegetovascular dystonia. A significant advantage of mexydol is its low toxicity accompanied by therapeutic broadness, as well as the absence of secondary effects typical of traditional compounds, applied in the treatment of cerebrovascular dysfunctions. Also, mexydol improves cognitive functions, mood, sleep, removes anxiety and fear.

It is found that mexydol has a modulating effect in relation to antithrombogenic properties of vascular wall endothelium at disturbances in the hemorheology system; regional cortical perfusion increases in the cerebrum zones with the highest reduction of cerebral blood flow and in frontal and temporal lobes. This property of mexydol makes possible elimination of the negative effects of common compounds used in combination for insult treatment (cavinton, stugeron, sermion) [63].

In this connection, mexydol can be applied to all types of vascular disease of the cerebrum at both chronic and acute cerebrovascular diseases that makes it irreplaceable for the first aid conditions.

PSYCHIATRY

Mexydol possesses high ability to potentiate the effects of other neuropsychotropic drugs. It causes a synergic effect as combined with tranquilizers, neuroleptics, antidepressants, hypnotic and anticonvulsive drugs, and analgetics. This gives an opportunity to reduce their doses and, therefore, decrease the secondary effects. In particular, a combination of mexydol and carbamazepine allows for the latter concentration decrease by two times without reduction of the therapeutic effect. It also allows for carrying out adequate pathogenetic therapy of partial epilepsy, decrease the secondary effect of carbamazepine in case of long-term application without a decrease of therapeutic effectiveness and, therefore, optimize the treatment of patients with epilepsy [47, 64].

The clinical and biochemical study of injecting forms of mexydol applied as curative and hepatoprotector drug to patients with alcoholism and drug abuse indicated its high efficiency [65 – 67]. At the stage of alcoholic abstinent syndrome, a single intravenous injection of mexydol (200 mg) reduced vegetative and somatic disturbances in the majority of patients (68%). During initial 3 – 5 days patients from the main group, treated with mexydol, showed almost complete elimination of vegetovascular disorders, improved sleeping, increased mental and physical workability, normalized and generally stabilized behavior (patients from the control groups experienced disturbances during 7 – 8 days). It should be noted that mexydol application improved tolerance to neuroleptics and antidepressants for patients with alcoholism, which reduced the frequency and severity of extrapyramidal disorders by almost 1.5 times [66].

Moreover, since the second day of mexydol treatment, a tendency to significant decrease of dystrophic damages and symptoms of neurological pathology was observed.

In the postabstinent period, clinical observations indicated that mexydol improved general psychic conditions, increased mental effectiveness and memory of the patients with alcoholism, which, inevitably, testifies about its nootropic action.

Biochemical studies indicated the hepatoprotector action of mexydol in relation to patients with alcoholism, which is manifested in shortening the period of liver and myocardium cell regeneration. The high antioxidant effect

of mexydol promotes a quick decrease of lipid peroxide concentration in blood plasma and ascorbic acid plasma pool recovery [67].

Mexydol also produces high immune correcting effect, thus, promoting complete recovery of T-lymphocyte helper population, decrease of the concentration of apoptosis receptor carrier cells, normalization of NK-cell quantity and 5′-nucleotidazo-dependent signaling transduction of μ-opioid receptors. Meanwhile, the quantities of neurophils, eosinophils and monocytes reach their upper limits [67].

As a consequence, clinical, experimental psychological, and biochemical studies implemented indicate the accelerated recovery and readaptive effect of mexydol. Therefore, this drug is recommended for application as the intensifying agent able to shorten the periods of psychic, physical and immunological rehabilitation of patients with alcoholism and drug abuse possessing signs of liver and myocardium damage, exhaustion of antioxidant protection system factors and the immune status disorders [66].

CARDIOLOGY

The question of modernizing pharmacotherapy of ischemic heart disease (IHD) is still one of the urgent tasks in modern cardiopharmacology. The questions of therapy optimization of myocardium infarction complications, such as cardiac rate disturbance, acute cardiac insufficiency, and limitation of myocardium necrotic damage size are of special urgency. At present, for this purpose various groups of drugs are used: antiarrhythmics, cardiotonics, compounds affecting tissue exchange and regeneration processes; unfortunately, their effectiveness is insufficient.

The reliable reason for development of atherosclerotic changes in vessels is lipid profile disturbance affected by POL activation. In this case, LDLP CS is the most aggressive fraction determining development of pathological changes in vessels. It is found that endothelium, damaged by oxidized LDLP CS, becomes unable to produce endogenic vasodilatory substance. As a consequence, the damaged vascular zone obtains an affinity to spasm occurrence, in coronary arteries, in particular. This circumstance confirms the urgency of searching for anti-ischemic agents, in the mechanism of which inhibition of POL is of high importance [68, 69].

The perspective approach to treatment of IHD is combined application of antioxidants and other antianginal compounds, with nitrates, in particular.

During formation of compensatory coronary-dilatory responses of the myocardium, in case of stenocardia, nitric nitrite (NO) formation intensity and action duration as one of the strongest vasodilating drugs are of importance. Recently determined fact that the lifetime of NO is much defined by activity of POL processes justifies the use of antioxidants for pharmacotherapy of stenocardia as the drugs potentiating the action of nitrates [25. 69].

All the above-mentioned was the premise for the study emoxypine and mexycor (encapsulated mexydol) effectiveness on hyperlipidemic model in experiment and in patients with atherosclerosis combined with various IHD forms.

The important component of antiatherogenic action of mexycor is the ability to increase HDLP CS, whereas the comparison drug probucol induces potentially unfavorable effect of this fraction of lipoproteids, which implements the reverse transport of cholesterol. Moreover, in contrast with probucol, mexycor possessed high hypotriglyceridemic action. As injected during three-month course at the background of atherogenic diets, mexycor decreased the zones of lipoid damages in aorta, demonstrating protective effect on local vascular mechanisms of atherogenesis. Clinical observations of 96 patients with IHD demonstrating stable exertional angina, in randomized placebo-controlled study, confirmed the favorable effect of the drug on the lipid profile [25].

For example, the initial level of total cholesterol before treatment equaled 7.8 mmol/l, a month after its content was reduced by 14%. Further on, 2 months after treatment (100 mg of mexycor 3 times a day), this index was reduced by 25% ($p < 0.05$) and reached its normal value. The level of LDLP CS was decreased by 32%. Analogous changes were observed for triglycerides: after 2 months of treatment the content of this lipid fraction decreased by 14%. Positive effect of mexycor on HDLP CS level was also observed.

The initial state of POL processes in patients with IHD and stable exertional stenocardia was characterized by sharp increase (by 1.8 – 2.1 times) of primary (diene conjugates) and secondary (malonic dialdehyde) POL products.

At the background of treatment by mexycor a noticeable decrease of POL product content in the patient blood was observed, reaching 30 – 37% decrease by the end of the second month of the treatment. The integral index of treatment effectiveness of patients with IHD and stable exertional stenocardia is increased tolerance to physical loads. For example, according to veloergometric sampling of patients with stable stenocardia 2 FC its average value equals 115 W. After one month of treatment this index increased by 20%,

to 139 W ($p < 0.05$), and after 2 months – by 27.4%. The number of stenocardia attack and the amount of nitroglycerine intake decreased [70].

The ability of mexycor to intensify the action of nitrates is the important effect significantly increasing its clinical effectiveness for patients with IHD. Particularly, experimental models studying tonus of isolated coronary arteries and portal vein, as well as collateral blood supply at coronary occlusion show that combined injection of mexycor and nitroglycerine significantly increases the main parameters of coronary blood flow and, therefore, possesses the serious advantage before separate use of these drugs [69].

The randomized placebo-controlled study of 68 patients with IHD and unstable stenocardia (basic therapy: isosorbide dinitrate – 60 mg/day, nifedipine – 40 mg/day, anapriline – 120 mg/day; for the main group the treatment was added by peroral intake of mexycor – 300 mg/day during 10 days) indicates that the mexycor action intensifies stenocardia stabilization (5.9 days in the main group and 11.3 days in the control), reduces daily frequency (by 46%) and duration (by 67%) of myocardium ischemia periods compared with the control group, significantly decreases the frequency of rhythm break episodes and improves clinical proceeding of the disease [70, 71].

According to everyday Holter monitoring, during initial 5 days of treatment by mexycor, the patients with unstable stenocardia demonstrated a relative increase of the painless ischemia periods.

The mentioned clinical effects were accompanied by faster, compared with the control, decrease of POL product content in blood – diene conjugate and malonic dialdehyde [70, 71].

Therefore, the unique combination of pharmacological properties of mexycor makes it very perspective drug for the treatment of acute and chronic IHD forms, possessing antiatherosclerotic, anti-ischemic and antianginal activity, moreover, it has no Russian and foreign analogues. Summing up the above-discussed data, one may conclude the following: antioxidants of heteroaromatic sequence, related to 3-oxypyridine derivatives, are perspective drugs for prophylaxis and treatment of free-radical pathologies.

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THE MECHANISMS OF PHOTODAMAGING OF VISUAL RETINAL AND PIGMENT EPITHELIUM CELLS OF THE EYE

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INTRODUCTION

In the vision, light may not only be the carrier of visual information, but also the risk factor. In both cases, the key molecule absorbing light and starting up normal photoreceptor process and photodamaging is retinal. It should be outlined at once that besides retinal, photodamaging in photoreceptor and pigment epithelium cells may also be initiated by other effective photosensibilizers, first of all, retinal phototransformation products (A2E and its derivatives), lipofuscin granules, flavins and flavoproteins, and under definite conditions, melanin and melanopsin.

If photochemical isomerization of chromophore group in the molecule of the visual pigment – 11-*cys* retinal, is the trigger of the photoreceptor process, the photodamaging is based on photosensibilized reactions of free-radical oxidation.

Owing to the features of 11-*cys* retinal as the chromophore and its nearest protin surrounding, rhodopsin molecule possesses the unique photochemical activity: high quantum isomerization yield (0.65), which is almost 3-fold higher than the quantum yield of retinal isomerization in solution (0.22 – 0.24), and fantastically high rate of photoisomerization, which is 146 femtoseconds, whereas in solution this reaction takes 1 – 2 picoseconds [1]. At present, the molecular organization of the chromophore site of rhodopsin and the basis of its high luminous sensitivity is studied comprehensively, including the methods of molecular simulation or modeling [2]. In completely *trans*-shape, retinal itself isolated at the latest stage of rhodopsin photolysis and, moreover, products of its transformation in photoreceptor and pigment epithelium cells (A2E and A2E epoxy-forms) are phototoxic and, therefore, dangerous as photosensibilizers of oxidation processes (Figures 1 and 2). Meanwhile, as being the amphiphilic molecule, A2E is bound to a membrane,

then hydrophilic epoxy-A2E is released to cytoplasm due to A2E photooxidation. Hence, it is extremely toxic and, therefore, extremely dangerous for the cell, because it may damage DNA, for example [3].

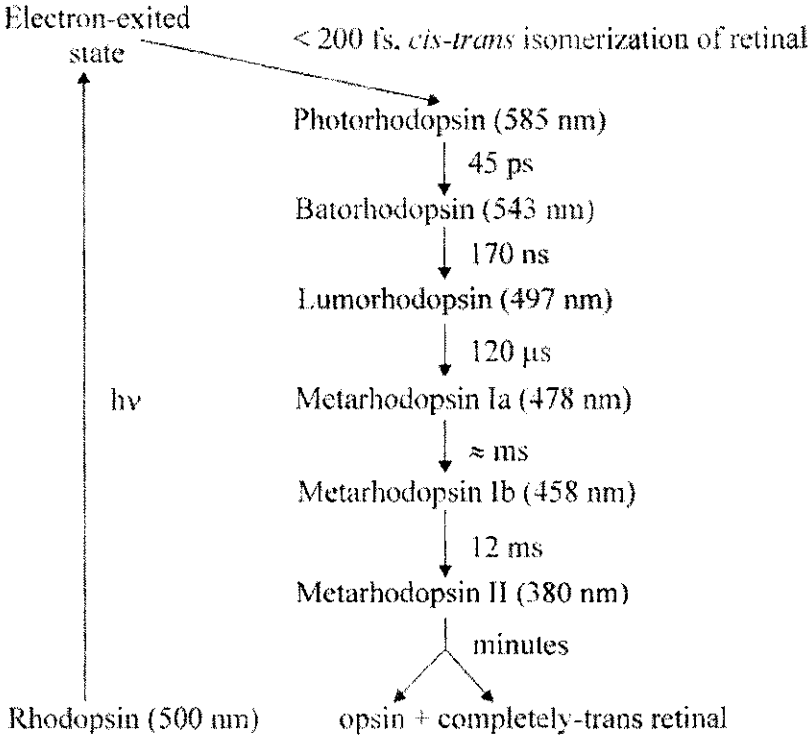

Figure 1. The scheme of phototransformation (photolysis) of rhodopsin (see text for details)

Compound A2E is the main but not unique fluorophore of lipofuscin granules. As mentioned above, illuminated lipofuscin granules effectively generate reactive oxygen species, including singlet oxygen, superoxide radicals, and hydroperoxides. Being extremely toxic, they initiate (directly or indirectly) oxidation of lipids and proteins, damage of lysosomes and, finally, the cell death.

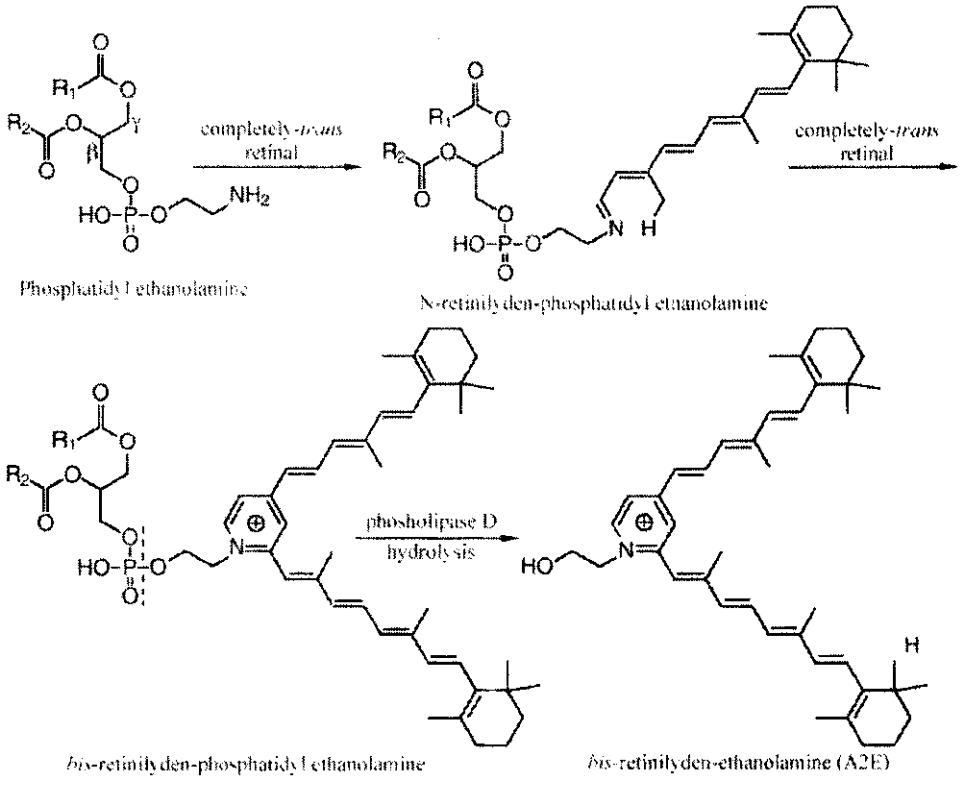

Figure 2. Synthesis of the main fluorophore of lipofuscin granule– *bis*-retinilyden-ethanolamine (A2E) from two molecules of completely *trans*-retinal and phosphatidyl ethanolamine

For the mechanisms of retinal and pigment epithelium photodamage, of special importance is the knowledge of the action spectrum of photosensibilized reactions, which induce such damage. The totality of currently obtained data indicates occurrence of the maximum of the reaction spectrum in the blue region. The most impressive is almost complete coincidence of photodamage spectrum for pigmantary epithelium of the primate eyes in the Ham tests (the so-called "blue light damage") [4] and oxygen consuption by lipofuscin granules at their illumination [5]. In both cases, we consider the blue region of the spectrum. Therefore, the probability that phototoxicity of the lipofuscin granules is generally responsible for the damage of pigment epithelium cells, and the damage of these cells, as is

commonly known, induced consecutive degeneration of photoreceptor retinal cells.

Lipofuscin granules, or the "age pigment", are implacably accumulated in pigment epithelium cells with the age and do not disappear anywhere from these cells till the end of the life. Many experimental data on the relation between accumulation of these phototoxic granules and intensification of retina degeneration, including one of the most widespread shapes of it – senile macular retina degeneration – were obtained. It is shown that accumulation of lipofuscin granules is chronologically coincident with development of this disease, and the area of retina with the highest concentration of accumulated brightly fluorescing granules is the most sensitive to degeneration [6]. The danger of retina photodamage with the age correlates with accumulation in pigment epithelium of lipofuscin granules and development of senile macular degeneration.

There are many epidemiological data on the aggravating effect of light in pathogenesis of this disease [7 – 11]. All the materials: experimental representing the studies photodamage spectrum of action, clinical – the use of intraocular lenses (IOL) without UV-absorber [12], and epidemiological, indicate that not only ultraviolet, but also, to the greater extent, blue area of the spectrum is responsible for aggravating action of light in pathogenesis of retina degeneration. Hence it follows that that use of light filters, which completely eliminate ultraviolet and partly blue or blue-green area of the spectrum from the retina, may cause a significant protective and prophylactic effect. Already in the middle 1980ies, basing on this fact, we suggested yellow colored intraocular lenses (IOL), designed for protecting retina from damages by ultraviolet and violet-blue light. In fact, transmission spectra of these lenses simulated transmission spectrum of natural lens in human eye about 45 – 50 years old [13 – 15]. Moreover, due to decreasing chromatic aberration such IOL increased contrast sensitivity of scotopic (rod) and photopic (cone) vision.

Analysis of the monitoring results obtained during 17 years of investigations (1986 – 2003, GU Interbranch Scientific-Technical Complex "Microsurgery of the Eye", Ministry of Health of the Russian Federation) of distant clinical consequences of implantation of our intraocular lenses "Spectrum" showed that postoperation complications as, for example, retina edema in the macular area (the Irwit-Hass syndrome), which usually caused vision loss by the operated eye, were observed: in 0.59% cases in the early period compares with previous 3% and in 0.06% cases in the late period compared with 1.5% [16, 17].

Our efforts, undertaken during many years, to design an intraocular lens (implant) possessing the most favorable and physiologically stipulated transmission spectrum have gained the international acceptance. Particularly, this is confirmed by recent active introduction of new yellow IOL (AcrylSof® Natural IOL) by Alcon Company into the world ophthalmologic practice. The transmission spectrum of these lenses is identical to that of our "Spectrum IOL.

Searching for optimal spectral characteristics of IOL, spectral glasses and contact lenses, which must minimize risks of retinal photodamage and, simultaneously, preserve or even improve quality of visual perception is in progress [18, 19]. Recently, three independent articles discussing in all details the prospects of light filter application were published. First of all, they discussed the advantages of application of colored IOL eliminating blue and blue-green area of the visible spectrum from retina and, therefore, suppressing developments of senile macular degeneration of retina [20 – 22]. This the apparent perspective of the nearest future, when many ophthalmologic centres will initiate wide clinical investigations of the application effectiveness of such light filters – IOL and spectral glasses for the purpose of decelerating development of various forms of retina degeneration, first of all, macular degeneration of retina.

MOLECULAR MECHANISMS OF VISUAL CELL PHOTODAMAGING IN RETINA AND PIGMENT EPITHELIUM CELLS

Basically, two classes of photodamages can be outlined. Firstly, we consider comparatively low light intensities and, which is most important, its prolonged action (from several days to several weeks); secondly, we are dealing with much higher light intensities at the impact duration varying from seconds to several hours. In the first case, mostly photoreceptors are damaged, whereas in the second case – pigment epithelium cells and then retina cells. For the first class of photodamages, the maximum in the action spectrum corresponds to the absorption spectrum of rhodopsin, i.e. blue-green area of the visible spectrum is the most effective. For the second case, the action spectrum is within ultraviolet and blue areas of the spectrum [23, 24]. For a man and diurnal animals, the greatest danger is presented by too intensive radiation in ultraviolet and blue areas of the spectrum. In this Section, we will discuss such

damages, which are based on photochemical reaction of sensibilized free-radical oxidation. The effectiveness of these reactions is determined by three factors: the presence of photosensibilizers absorbing light, oxidation substrates and oxygen. As mentioned above, all three factors in full measure are present in retina and pigment epithelium.

a) Photosensibilizers

Photosensibilizers may serve as both endogenic and exogenic dyed compounds. Among endogenic photosensibilizers of retina and pigment epithelium, first of all, retinal and corresponding transformation products should be mentioned.

Let us start our discussion with lipofuscin granules and their main fluorophore – *bis*-retinyliden-ethanolamine (A2E).

Phototoxicity of lipofuscin granules

As was believed before, lipofuscin granules represented harmless ballast, accumulated in postmitotic cells with aging of the organism. However, it has been found then that they are phototoxic, because are able to form reactive oxygen species under the effect of light – singlet oxygen and superoxide radicals [25 – 29].

The action spectra e.g. dependence of generation intensity of these reactive oxygen species on the radiation wavelength indicate that UV and blue light are much more effective (dangerous) rather than green of red light. Figure 3 shows dependence of oxygen superoxide anion-radical accumulation on the radiation wavelength impacting the suspension of lipofuscin granules, isolated from pigment epithelium of human donor eyes [27]. Most likely, the danger of blue light damaging impact on retina and pigment epithelium, described long ago [4], is related to phototoxicity of lipofuscin granules.

Acting as photooxidation sensibilizers, lipofuscin granules are able to simulate lipid and protein oxidation (inactivation of their enzymatic activity) and damage biological membranes [28, 30]. According to our preliminary data, when radiated by blue light lipofuscin granules are able to induce apoptosis of pigment epithelium cells, i.e. its programmable death. It is also shown that radiation in the tissue culture of pigment epithelium cells loaded with lipofuscin fluorophore – A2E, implacably induces their apoptosis [31]. It should be noted that apoptosis is now considered as the total final way of the

cell death at many diseases of retina, including various degeneration and dystrophy forms.

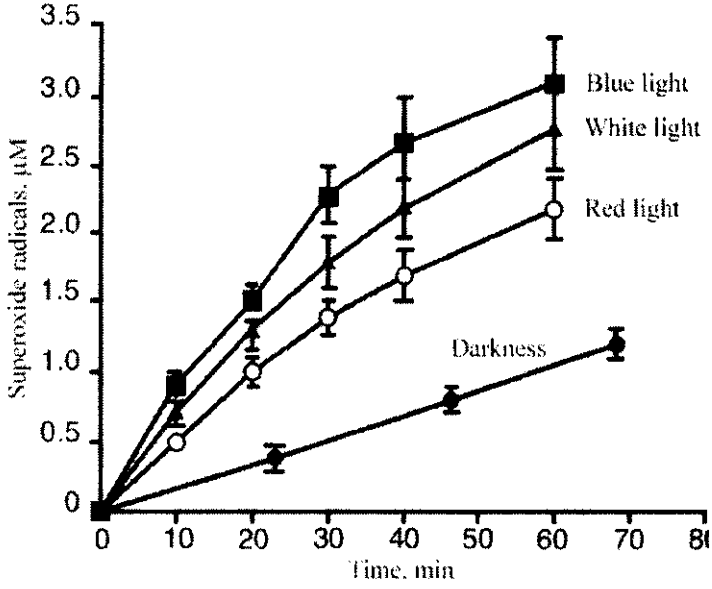

Figure 3. The dependence of superoxide oxygen radical generation by lipofuscin granules on the wavelength of radiation [27]

The mechanism of free-radical photooxidation includes both direct interaction of activated sensibilizer with the cell components and formation of reactive oxygen species, which consequently interact with and damage nucleic acids, proteins and lipids of the cell. Recently, it has been shown that highly toxic singlet oxygen, formed at blue light impact on A2E, which transform them to much more toxic epoxide form [31 – 33].

The studies of phototoxicity of lipofuscin granules and their fluorophores, specially A2E, are still continued. The aim of this work is detailed determination of their role in pathogenesis of several degenerative diseases of retina.

Retinal phototoxicity

Retinal is the chromophore in the visual pigment molecule and precursor of A2E – the fluorophore of lipofuscin granules. It is one of the most effective natural sensibilizers. As shown in Table 1, its photosensibilizating

activity is only two times lower than for riboflavin – the classical sensibilizer [34].

Table 1

Relative yield of glycyltriptophane oxidation products in the presence of various endogenic photosensibilizers (radiation by UV-light, $\lambda = 313$ nm)*

Photosensibilizer	Maximum of the absorption spectrum, nm	Relative photooxidation yield
Nicotinic acid	261.5	9.7
Flavin mononucleotide.	450	1.1
Riboflavin	372.5	1.0*
Pyridoxine	325	0.74
Retinal	380	0.51
$NADH_2$	339	0.05
Folic acid	365	0.05
Cobalamin	361	>0.05
Rutin	362	>0.05
Bilirubin	453	>0.05

Note: * For the unit the value of yield for riboflavin is taken. Glycyl tryptophane concentration is 10^{-3} mol/l, optical density of all solution at 313 nm is 0.05 and 0.01 mol/l; phosphate buffer, pH 7.0; the illumination time is 2 min.

As mentioned above, the final product of rhodopsin decolorizing (λ_{max} = 500 nm) is metarhodoprin II (λ_{max} = 380 nm), or already completely isolated *trans*-retinal (λ_{max} = 380 nm). In due course, we have shown that the light impact on decolorized photoreceptor membranes containing metarhodopsin II or *trans*-retinal induces photooxidation of both protein part of rhodopsin (SH-groups of opsin) and photoeceptor membrane lipids [35]. Here the action spectrum e.g. the dependence of protein and lipid oxidation in this membrane on the wavelength of impacting light is determined. It has been found that their photooxidation is most highly manifested near 380 nm, i.e. in the range identical to the absorption spectrum range of free retinal (or metarhodpsin II). As a consequence, rhodopsin molecules irreversibly aggregate in the photoreceptor membrane, and rhodopsin ability to regenerate at 11-*cys* retinal injection is decreased, e.g. its native properties are disturbed (see below).

Phototoxicity of drugs

Drugs as exogenic photosensibilizers, as well as dyed metabolism products such as endogenic photosensibilizers, may be dangerous, if enter eye structures accessible for light – retina amd pigment epithelium, first of all [36]. Exogenic photosensibilizers are specifically dangerous for aphakic e.g. lens-deprived eye, as well as for normal child's eye, which lens transmits some part of ultraviolet radiation together with the visible light [37].

b) Photooxidation substrates

Molecular components of the photoreceptor membrane – proteins (rhodopsin) and lipids – represent the ideal substrates for photosensibilized oxidation reaction. As any biological membrane, the particular membrane consists of the lipid bilayer and membrane proteins piercing it. The main protein of this membrane is rhodopsin: it gives up to 95 – 98% of all integral membrane proteins (Figure 4).

The three main phospholipids of photoreceptor membrane are phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl serine. The composition of these phospholipids is unique: over 60% is composed by polyunsaturated fatty acids, where about 75% is given by the long-chain, the so-called docosahexaenic acid containing 22 carbon atoms and 6 double bonds (C22:6) [38]. Thanks to this, the photoreceptor membrane possesses extremely low viscosity equal, approximately, two poises (equal to olive oil viscosity). Such low viscosity allows rhodopsin and other proteins in this membrane being subject to quick rotary and slower lateral diffusion that yields in their ability to frequent collision and interaction with one another.

Photooxidation of proteins and lipids of the photoreceptor membrane represent two independent processes, proceeding in parallel. Actually, addition of the known inhibitor of peroxide oxidation of lipids – 2,6-di-*tert*-butyloxytoluene (dibunol), completely transforms photooxidation of lipids in this membrane, and just partly decreases the rate of toluene group oxidation in the protein [35].

Proteins and lipids show different response to the change in the oxygen content: proteins are less sensitive to oxidation than lipids [39].

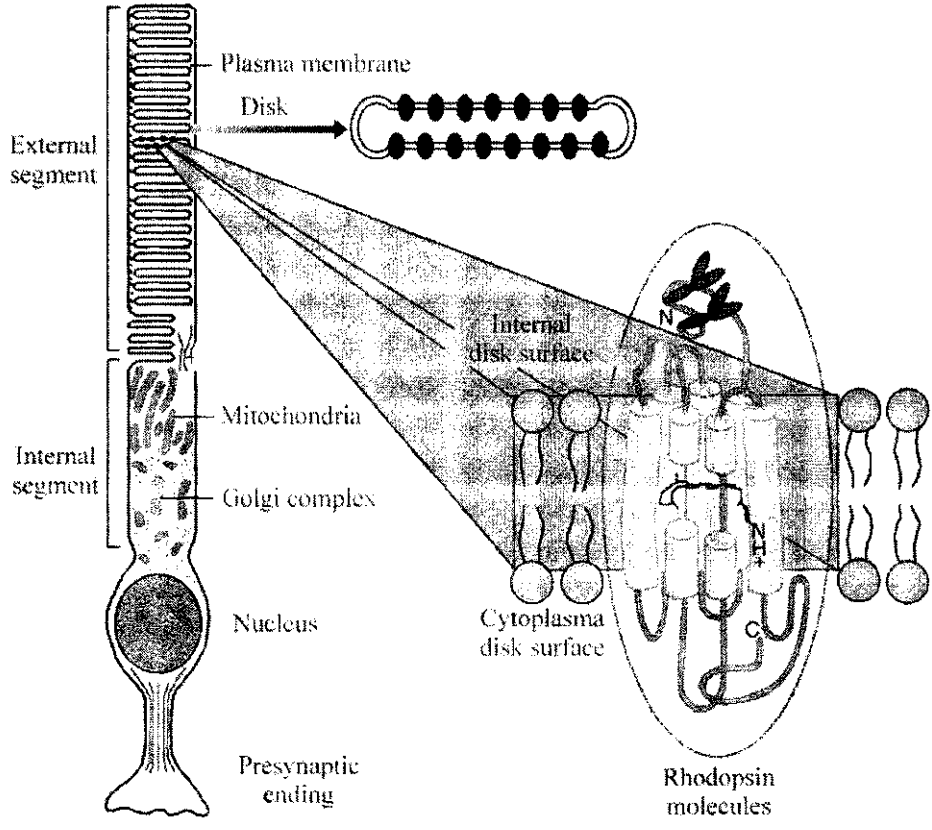

Figure 4. Schematic presentation of the rod, photoreceptor disk of the external segment, photoreceptor membrane of the disk, and rhodopsin molecule, in the center of which its chromophore group (11-*cys* retinal) is located, covalently bound to the protein part (opsin)

In this relation, it is interesting to compare quantum outputs of rhodopsin photooxidation and decoloration (photolysis) processes. The quantum output of rhodopsin photooxidation is low (about 0.04), whereas the level ofphotodecoloration is by an order of magnitude higher (about 0.7) [38]. This explains why physiological intensities of light are non-damaging for the retina.

The photopathological consequences of rhodopsin and lipid photooxidation in the photoreceptor membrane are:

- formation of intra- and intermolecular disulfide bonds (crosslinks);
- formation of irreversible protein aggregates showing extremely low mobility in the membrane;
- the loss of ability by oxidized and aggregated rhodopsin to regenerate bot *in vitro* and *in vivo* [40 – 44].

Irreversible aggregation of rhodopsin in membrane, induced by damaging light doses, causes loss of its native properties and normal functional activity. Sensitive test for the native property of visual pigment is its ability to regenerate at adding exogenic 11-*cys* retinal. In tests *in vitro* in suspension of photoreceptor membranes [42] and in tests *in vivo* at white rat eye radiation [45], we have shown that the ability of rhodopsin to regenerate is decreased, and electroretinogram is suppressed or disappears (Figure 7).

Retinal can be photosensibilizer of oxidation and damage of protein rhodopsin and other retinal-binding and retinal-transporting proteins. As is shown, it sensibilizes photooxidation and damaging of two key proteins of the visual cycle – ABCR and water-soluble interphotoreceptor retinal/retinol-transporting protein (IRBP). Recently, we has observed that protein IRBP is damages under the effect of light in the optical absorption band of retinal (in the near ultraviolet range, λ = 380 nm): a part of its cysteine and aromatic amino acid residues is oxidized and, which is most important, hence its functional properties are deteriorated, namely, the ability to bind retinol decreases [46].

Protein ABCR was found extremely sensitive to photodamage, induced by retinal as the photosensibilizer [47] (see above). Similar to rhodopsin, the molecular mechanism of ABCR damage is its aggregation (however, not owing to disulfide bond formation). Photooxidation of protein ABCR, which is able to bid and transport retinal and possesses ATPase activity, leads to the loss of *trans*-retinal ability, bound to ABCR, to stimulate its ATPase activity. As a result, retinal is not "*released*" from the external segment to the intracellular space. To put it differently, the light-induced ABCR defect (and its hereditary defect as well) causes accumulation of *trans*-retinal in the photoreceptor membrane and formation of A2E and excessive amount of lipofuscin granules from it [47] (Figure 4b). Protein ABCR was found more sensitive to photooxidation and damaging than other proteins of photoreceptor membrane, for example, rhodopsin, transducin α-subunit, protein of cHMP-reulated ionic channel, as well as peripheral proteins of this membrane – rhodopsin kinase and arestin.

c) Oxygen

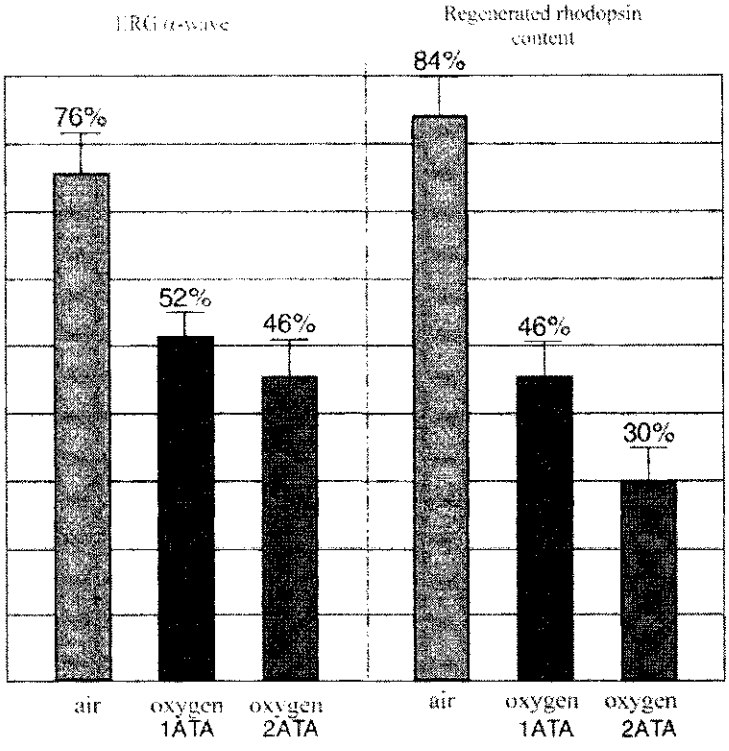

Figure 5. The decrease of α-wave in electroretinogram of white rate retina (at the left) and deterioration of decolorized rhodopsin ability to regenerate at external injection of 11-cys retinal (at the right) resulting the damaging effect of light and at different oxygen concentration in the inspired mixture [45]

Oxygen is the third necessary participant of photooxidation reaction. It should be noted that partial oxygen pressure is different along the photoreceptor cell of retina. In the distal part of the external segment, closer to pigment epithelium and choroid, the oxygen concentration is higher as compared with proximal (basal) part. Actually, at the top of the external segment oxygen pressure is $P_{O_2} \sim 100$ mmHg, which is equivalent to oxygen

concentration in arterial blood. In the proximal area (at the border with the internal segment) P_{O_2} ~ 10 mmHg in darkness and ~30 mmHg in the light. This difference can be explained by high concentration of mitochondria in the internal segment of the photoreceptor cell, which, as is known, are the main oxygen consumers.

To all probability, different partial pressure of oxygen along the photoreceptor cell is the reason for occurrence of a gradient of the external segment photodamage. Its distal part contains "old" disks, which accumulated molecular defects and are higher sensitive to damage. This is confirmed by a gradient of SH-group concentration decrease, capable of titration. This gradient is observed from the top of the external segment ("old" disks) towards its base ("new" basal disks) [48].

In the tests on rats, we have indicated that oxygen excess (hyperoxia) and especially hyperbaric oxygenation intensify the damaging effect of light on retina. Hence rhodopsin regeneration is seriously disturbed and electric activity of retina significantly decreases [45] (Figure 5).

THE PROTECTION SYSTEM OF VISUAL CELLS OF RETINA AND PIGMENT EPITHELIUM CELLS FROM PHOTODAMAGE DANGER

Thus, a combination of light, oxygen, pigments-photosensibilizers and easily oxidizing substrates (lipids and proteins) represents the real danger of photodegradation process initiation and development in retina and pigment epithelium. This paradox of vision, when light is the information carrier and, simultaneously, dangerous damaging factor, was resolved during the evolution by creation of a reliable multilevel protection system against photodamage danger. This system includes:

- renewal of photoreceptor membranes;
- a complex of endogenic antioxidants;
- a mechanism of the quickest elimination of free retinal from the visual cell;
- a system of optical filters of the eye, in which the key role is belonged to the lens, yellowing with age in primates and people;

Actually, breaks in this system cause increased risk of retina and pigment epithelium light damage.

Renewal of photoreceptor membranes

The first and the most drastic protection method is permanent renewal of photoreceptor membranes and the whole external segment of the visual cell (both rods and cones). Renewal allows avoidance of molecular defect accumulation in the external segment, owing to which molecular machinery of the photoreceptor mechanism preserves effectiveness during the whole life of the organism. The process of permanent renewal of the external segment proceeds as follows: new photoreceptor membranes (photoreceptor disks) are formed in its basal part, whereas in the apical part the stocks of "old" disks (the top of the external segment) are truncated [38, 49, 50]. These "fragments" are then effectively phagocyted by pigment epithelium cells. This process is extremely intensive: about 100 disks are renewed and truncated in each rod during the day. Since multiple ligaments of pigment epithelium cell surround more than a dozen of rods, each cell "digests" about 30,000 wasted disks per day. The pigment epithelium cell is one of the most active phagocyting cells in the organism.

However, the process of drastic renewal of the external segment in photoreceptor cell has the age-dependent drawbacks. In particular, the case at hand is the excessive accumulation of the "aging pigment" in pigment epithelium – lipofuscin granules, which essentially are fragments of external segments incompletely digested during phagocyting and possessing phototoxicity (see above). Lipofuscin granules may occupy up to 20% of the cell volume.

Antioxidants

The next protection level against photodamaging is the antioxidant protection. The case at hand is deceleration of free-radical photosensibilized oxidation. This system includes vitamins E (α-tocopherol), C (ascorbic acid), taurine, and a set of antioxidant enzymes (superoxide dismutase, peroxidase, catalase). In addition, in due course we have shown that the antioxidant protection system of the cell is strengthened by shielding pigments of the eye – melanosomes (in vertebrates and man) and ommochromes (in invertebrates) [51 – 55]. This gives a significant explanation why albinos are so sensitive to photodamaging. Interestingly, the deficiency of melanin in albinos is partly compensated by increased content of α-tocopherol in their retinas [55].

In the mechanism of the quickest elimination of free, completely *trans*-retinal from the visual cell, the key role is played by ATPase-dependent cassette transferring protein ABCR (Figure 6) [47, 56].

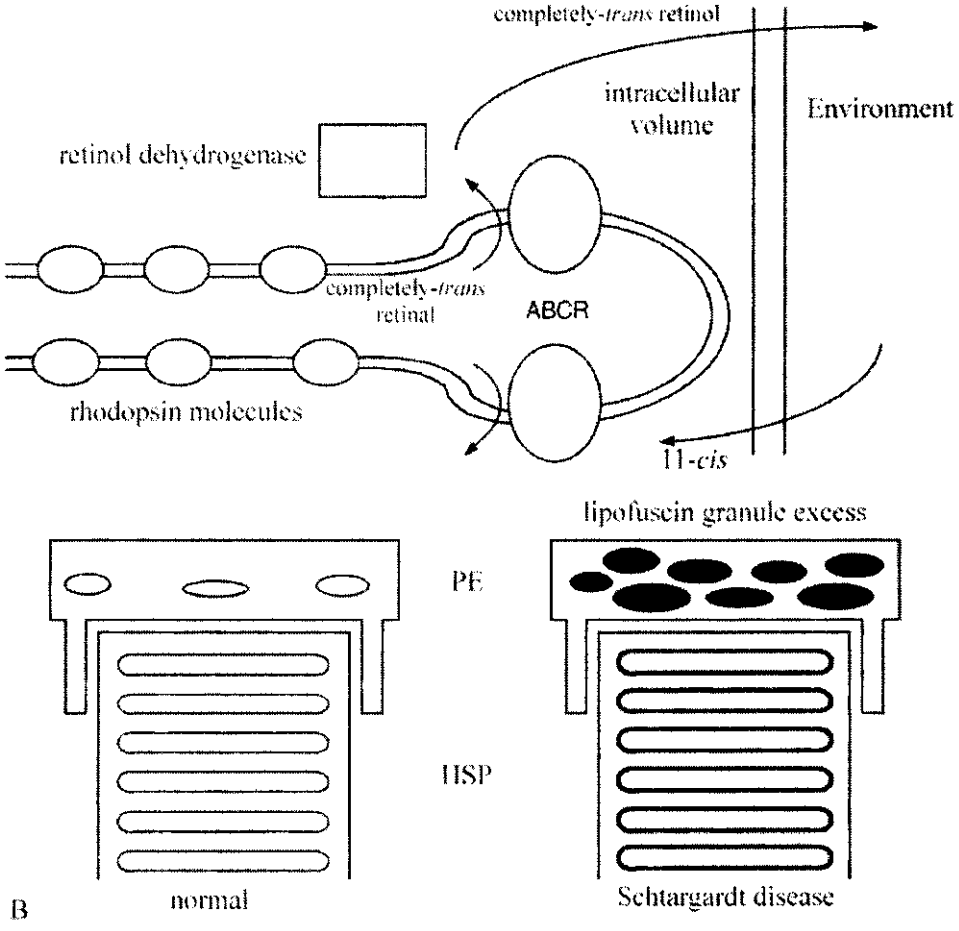

Figure 6. a) The mechanism of active elimination of *trans*-retinal from photoreceptor membrane of the disk after rhodopsin photolysis (see the text for details)

b) Accumulation of lipofuscin granules in pigment epithelium cells induced by hereditary defect of ABCR protein or its retinal-photosensibilized oxidation (damage)

Optical protection system

The final important protective system of retina and pigment epithelium from photodamages is the optical system. It consists of a sequence of light filters – cornea, lens, and shielding pigments (melanosomes and ommochromes) [54]. These light filters cut off short-wave radiation (ultraviolet and partly blue) from retina and pigment epithelium. Moreover, they decrease chromatic aberration and, therefore, improve contrast sensitivity and sharpness of vision.

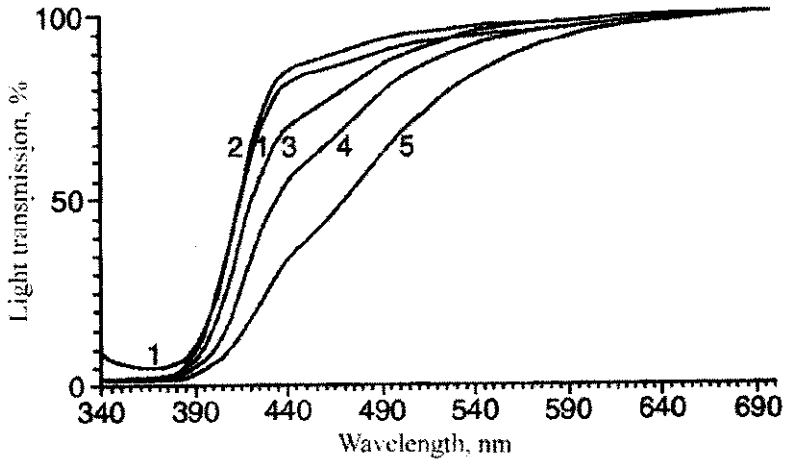

Figure 7. Natural age-related yellowing of human lens [3]:
1 – newborn children; 2 – 8 – 29 years old (10 lenses); 3 – 31 – 49 years old (25 lenses); 4 – 52 – 65 years old (14 lenses); 5 – above 70 (13 lenses)

The lens is the key light filter. For many vertebrates inhabiting in the bright-light environment, the lens is bright yellow since the birth, i.e. it intensively filters UV and blue light off the retina and pigment epithelium. Human lens becomes yellow with aging, i.e. in senile (non-cataract!) lens something like an additional yellow light filter is introduced before the retina (Figure 7) [57].

This is extremely important for the retina and pigment epithelium, because the efficiency of the optical protection increases. Hence in children and younger individuals the lens is usually colorless, effectively absorbing ultraviolet radiation only. It should be noted that in infants the lens has an

"ultraviolet window", i.e. penetrates a definite part of UV-light heating the eye to the retina. It is still unclear, if this "window" is just a consequence of the postnatal lens imperfection (its protein crystals), or it is required postnatal development of retina and pigment epithelium. Approximately, by the age of 15 the UV-window disappears and the lens becomes yellow gradually, reaching its maximum in the old age.

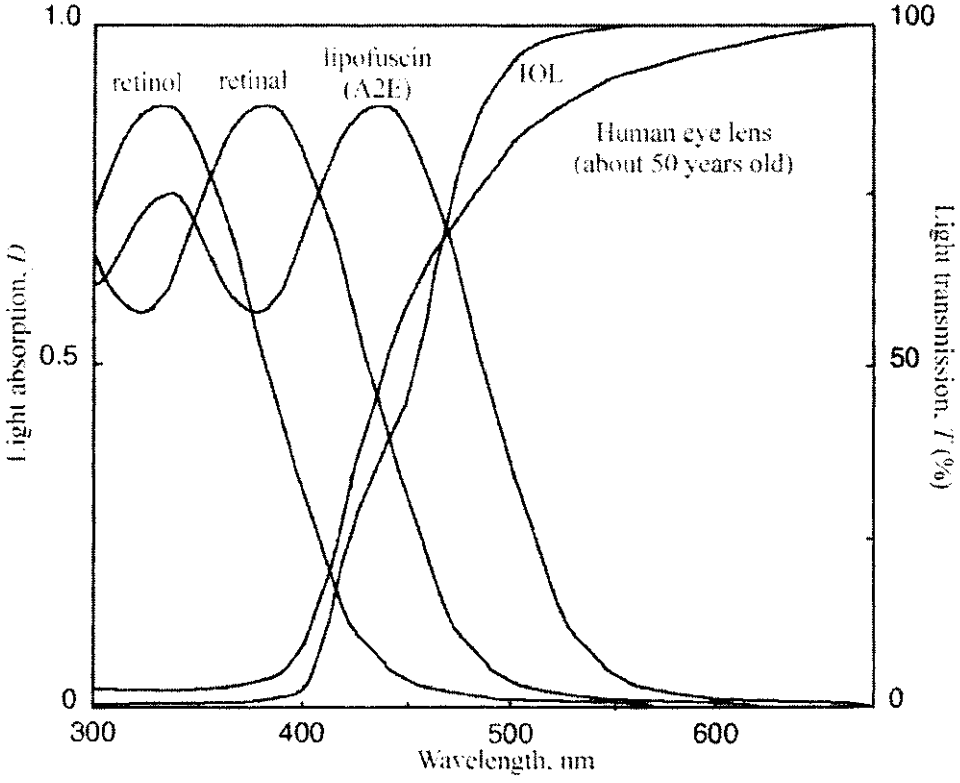

Figure 8. Absorption spectra (*D*) of endogenic photosensibilizers – retinol, retinal and lipofuscin, and transmittance spectra (*T*, %) of human lens aged about 50, and yellow intraocular lens (IOL "Spectrum")

At the early stage of opacity, even cataract-carrying lens may serve as the light filter [58, 59]. Making the image on retina not so proper, such yellow lens effectively detains the short-wave and is able, to some extent, decrease

chromatic aberration and prevent aggravation of some light-induced forms of retina degeneration.

The unfavorable consequence of the lens extirpation in case of cataract is multiple increase of the risk of retina damage by UV and blue light. Already in the middle 1980ies, basing on these ideas, we have designed and introduced to clinical practice the "*artificial lens*", representing a new colored intraocular lens "Spectrum" [13, 14, 60]. Similar to natural lens of an old man, this lens filters off ultraviolet and, to a greater extent, short-wave (violet-blue) part of the visible spectrum. It should be noted that normal color perception of a patient with implanted yellowy lens is not disturbed.

Figure 8 unites the absorption spectra of the main endogenic photosensibilizers – retinol, retinal, lipofuscin, and transmittance spectra of the aged man lens and "Spectrum" lens. Clearly the aged man lens and the yellowy lens filter off the most part of the short-wave visible spectrum, where photosensibilizers dangerous for retina are absorbed.

Unfortunately, the effective and safe drugs (antioxidant) able to prevent photosensibilized damage of retina and pigment epithelium are not found yet.

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THE ANTIOXIDANT ROLE OF SHIELDING EYE PIGMENTS – MELANINS AND OMMOCHROMES, AND PHYSICOCHEMICAL MECHANISM OF THEIR ACTION

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INTRODUCTION

Shielding pigments of animal eyes are melanins for vertebrates and ommochromes for invertebrates. Melanins (eumelanins) represent a product of tyrosine or dioxyphenylalanine oxidative polymerizationon a protein matrix. In human eyes and eyes of vertebrates melanin is present as intracellular organelles – melanosomes, which are localized in the iris, retinal pigment epithelium (RPE) and choroids. Ommochromes represent the products of tryptophane oxidation and, similar to melanins, are encapsulated in small granules, localized in pigment and photoreceptor cells of the complex eye of the invertebrates.

The main function of shielding pigments is the role of optical protective screen or a filter, which absorbs excessive ultraviolet and visible light and, therefore, passively deteriorates their effect on cellular structures of the eye. However, recently, there is a plenty of publications indicating that the passive screen function is not the unique and even the main for shielding pigments of the eye. It is found that screening pigments – melanins and ommochromes are able to protect visual cells from the damaging impact of free-radical processes of various etiology [1].

The problem of biological membrane protection from the damaging impact g reactive oxygen species is specifically important for visual cells. This relates to the following reasons: relatively high concentration of oxygen, which diffuses through these cells from choroid to retina, and intensive phagocytosis of wasted external segment disks of photoreceptors. Fragments of apical fragments of human and vertebrate photoreceptor external segments occur between retina and retinal pigmentary epithelium. Phagocytosis of these fragments is one of the main functions of this epithelium. Light intensifies renewal of membranes in external segments and phagocytosis

of their fragments. In this case, a great amount of phagosomes may be accumulated inside the pigmentary epithelium cell. For instance, if at usual cyclic day and night alteration 20 – 30 phagosomes are detected in this cell, then after intensive radiation by light their amount increased by an order of magnitude of higher (to 250 – 400). Therefore, they occupy up to 70% of cytoplasm volume [2].

In some pathological states, when the phagocytosis mechanism is broken, fragments of external segments may be accumulated in a gap between retina and pigmentary epithelium, which is extremely dangerous for the eye. The structure of phagosomes includes easily peroxidizing lipids [3]. Moreover, it is known that phagocytosis is accompanied by activation of oxidative enzymes that increases concentration of active free radical particles, reactive oxygen species, for example [4].

As is known, practically all luminous flux hitting the eye passes through the retina and is absorbed (by 85 – 90%) by RPE cells. Obviously, light absorbed by phagosomes or other pigmented intracellular structures is able, under definite conditions, to initiate degradative photooxidation reactions in a photoreceptor or pigmentary cell.

Toxicity of wasted photoreceptor membranes is also determined by high concentration of retinal and its phototoxic derivatives (A2E), as well as lipofuscin granules – the phagosomes incompletely digested by lysosomal enzymes, in them. Retinal and products of its transformation, as well as lipofuscin granules themselves possess highly manifested photosensibilizing properties. They are able to sensibilize oxidation of polyunsaturated fatty-acid residues of lipids and proteins. Meanwhile, it should be taken into account that photoreceptor and pigmentary cells are saturated with oxygen. For instance, toxicity of the photoreceptor membrane autooxidation products is confirmed by the fact that the fragments of photoreceptor external segments effectively inhibit DNA and RNA synthesis in guinea pig lymphocytes, whereas α-tocopherol and other antioxidants are protectors in this case [5].

Also, renewal and phagocytosis of photoreceptor membranes are typical of rhabdomeres of the complex eye of invertebrates. The fragments of microvilles are phagocyted directly by the photoreceptor cell, where they degrade then [6].

PIGMENTATION AS THE PROTECTION METHOD AGAINST DAMAGING IMPACTS OF FREE-RADICAL PROCESSES

Pigmentation protects visual cells from darkish free-radical peroxide oxidation of lipids.

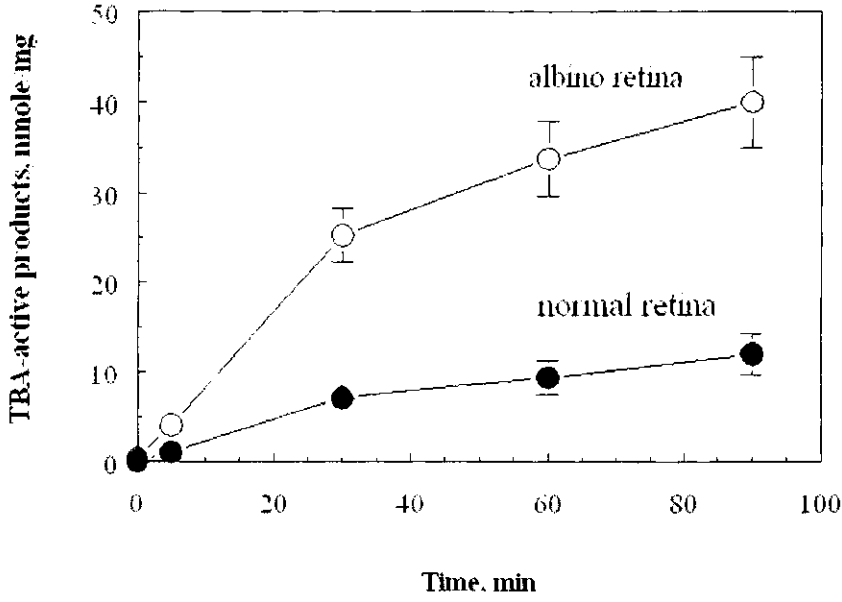

Figure 1. Comparison of peroxide oxidation of lipids in retina of pigmented animals and albinos

It has been shown that shielding pigments possess manifested antioxidant activity e.g. are able to prevent development of oxidative processes in the cell [7 – 15]. It is found that photoreceptor cells of albino animals are highly subject to darkish peroxide oxidation of lipids, induced by prooxidant factors as hyperoxia, reactive oxygen species, hydroperoxides and alternate valence metal ions. Figure 1 shows kinetics of TBC-active products in retina of albino rabbits compared with pigmented animals, induced by bivalent iron ions in the presence of ascorbate. As is observed, albino animal cells are more than three times more sensitive to the effect of this prooxidant. High resistance of

pigmented cells is not caused by high concentration of lowmolecular antioxidants and antioxidative enzymes or low concentration of oxidation substrates in them [16]. It is the consequence exclusively of the presence of shielding pigments in them – melanin-containing melanosomes or ommochrome granules. Elimination of shielding pigments from RPE cells by differential centrifuging method sharply intensified lipid peroxidation processes in them [17].

Injection of lipid hydroperoxides into albino rabbit eyes induced deterioration of electroretinogram (ERG) parameters already on the next day; 12 days after ERG was not detected at all. At the same time, ERG of pigmented rabbits did not changed even 24 days after the injection of hydroperoxides [18].

Experimentally induced uveitis caused two-fold greater increase of peroxide oxidation product concentration in retina of albino guinea pigs compared with normally pigmented individuals [19].

Thus, in the presence of shielding pigments of melanosomes or ommochrome granules, prooxidant factors such as bivalent iron and ascorbate, superoxide anion-radicals of oxygen, hydroperoxides, and increased concentration of molecular oxygen, have not caused any voluminous accumulation of POL products in the eye structures.

Resistance of cellular structures containing shielding pigments to photooxidation

It is commonly known that albino individuals are extremely sensitive to the damaging impact of light. For example, development of degenerative processes in retina of albino rats requires 60-fold lower light intensity compared with pigmented individuals [20]. For some albino invertebrates, for example, white-eyed crustacean mutants, light of even room intensity induces degeneration of photoreceptor cells [21].

In our experiments, bright visible light easily initiated peroxide oxidation of lipids in retina and pigmentary epithelium of albino animals; on the contrary, pigmented tissues were highly resistant to photooxidation [22 – 28]. These differences are directly related to the antioxidant activity of melanosomes. Actually, activities of the main enzymes in the antioxidant system (glutathione peroxidase, superoxide dismutase) in retina and pigmentary epithelium of pigmented animals and albino individuals are identical; moreover, albinos possess 1.5 – 2 times higher concentration of α-

tocopherol [16]. The latter may be considered as a compensatory biochemical reaction of the cell to the absence of melanosomes in it.

We have also demonstrated that shielding pigments of invertebrates (ommochromes) also implement the function of protection against photo-induced free-radical damage of *Mysis relicta* shrimp eyes. There are two populations of this species, which were separated about 9000 years ago, at the end of the glacial epoch. At present, one of the populations inhabits in the sea in shallow waters, where illumination is rather high, whereas another population occupies lakes at high depths, where illumination is very low. Meanwhile, the lake population was found much more sensitive to luminous influence – moderate illumination causes total loss of vision [29]. Our experiments indicate much lower concentration of ommochromes (about 2.5 times) in the eyes of lake shrimps compared with marine population; moreover, marine population is much more resistant to photo-induced accumulation of peroxide oxidation products in the eye structures [30 – 32]. To put it differently, luminous resistance of marine population of *M. relicta* shrimp is generally explained by high content of the shielding pigment ommochrome.

Therefore, resistance to photo-induced damages of cellular structures in the eyes of vertebrates and invertebrates and a man is generally determined by the presence of shielding pigments in them.

For primates and people, the group of shielding pigments also includes carotinoid macular pigments of yellow spot, which similar to melanosomes and ommochromes demonstrate antioxidant activity. Being the light filters, they absorb ultraviolet and, partly, short wavelength visible light; as antioxidants, they inhibit free-radical oxidation reactions.

Pigmentation protects visual cells against free-radical peroxide oxidation of lipids, induced by ionizing radiation

The radioprotector role of melanins under the effect of ionizing radiation on naturally pigmented organisms or biological structures in the presence of exogenic pigment is commonly known [33 – 38]. Moreover, animals and people impacted by ionizing radiation demonstrated additional synthesis of melanins in various organs and tissues. For example [39], liquidators of Chernobyl APP nuclear accident consequences demonstrated accumulation of melanin-containing products in blood serum and bronchoalveolar lavages, and their concentration increased with time of presence at the production site four of APP. Apparently, melanin accumulation is the adaptive response of the organism to intensification of secondary

radiation-induced free-radical processes. Of course, albino animals are much more sensitive to ionizing radiation compared with pigmented individuals. We have performed comparison studies of total X-ray radiation impact on product accumulation dynamics of peroxide oxidation of lipids (dienic conjugates, TBC-active products, Schiff's bases) in retina of pigmented and albino mice [40]. The results indicated rather low accumulation of peroxide oxidation products in dark-pigmented mice compared with the control (nonradiated) individuals (Figure 2). Hence, radiation of albino mice induced a noticeable increase of peroxide oxidation product accumulation in them, which reaches its maximum in the initial period after the impact and is preserved at least during three months after.

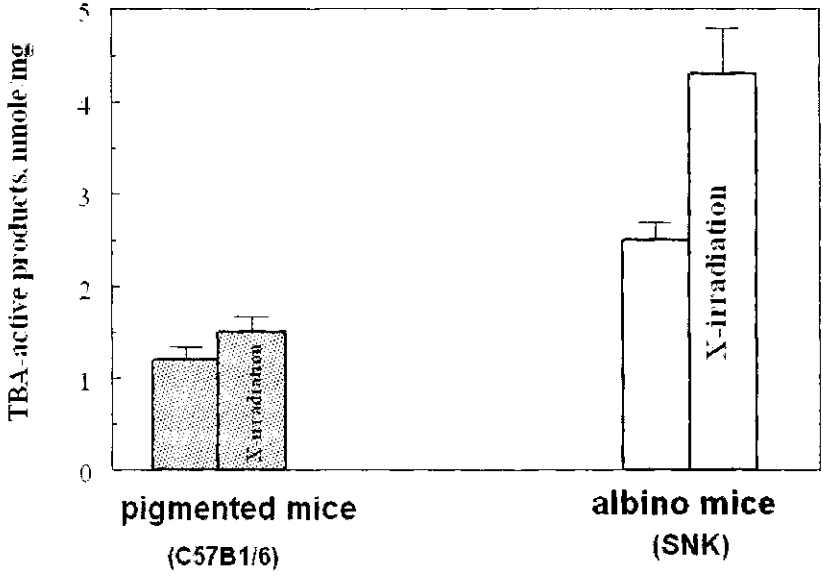

Figure 2. Comparison of the accumulation level of peroxide oxidation products in retina of pigmented and albino mice, impacted by X-ray radiation

Thus, the padioprotector role of melanin pigments in case of ionizing radiation impact is clearly manifested for the eye structures, too.

The role shielding pigments in aging processes

It is known that during aging retinal pigmentary epithelium cells accumulate a significant amount of lipofuscin granules [41 – 42]. As mentioned above, this process is related to the defects in the lysosomal system utilizing wasted external segments of photoreceptor cells. As a result, phagosomes become the subject of free-radical oxidation, and peroxide oxidation products are accumulated, aldehydes and dialdehydes, in particular. The latter induce formation of protein-lipid cross-links, resulted in formation of the so-called senile pigment lipofuscin, which cannot be utilized by hydrolytic systems of the cells. As indicated in multiple experiments, lipofuscin granules are photochemically toxic, able to photoregenerate reactive oxygen species [43 – 45]. They are also able to damage lipids, proteins and nucleic acids, inactivate enzymes, and induce apoptosis of visual cells [46 – 51]. It is the commonly accepted that accumulation of lipofuscin granules in the old age is one of the main reasons for development of senile macular degeneration of retina [52] – the disease causing the loss of central (direct) vision by many people aged over 65 – 70.

It is found that RPE melanosomes significantly inhibit the processes of lipofuscin granule accumulation. For example, it is shown that after two to four weeks of presence in the atmosphere with 40% oxygen saturation, albino and rabbits and low-pigmented bovine RPE cells accumulate much greater amount of lipofuscin granules than the cells of pigmented rabbits and highly pigmented bovine RPE cells [53].

It is also shown that after 4 weeks, RPE cell culture, containing melanin and loaded by oxidized external segments of photoreceptor cells, demonstrated 3 times or more lower level of lipofuscin granules compared with RPE cell culture of albinos [54]. In this case, antioxidant protective action of melanosomes is, apparently, due to direct contact between melanosomes and oxidizing granules. It is known that in RPE cells melanosomes may easily fuse with various phagocyte-affected material, for example, gold particles, latex, fragments of wasted external segments, lipofuscin granules and phagosomes [55 – 57]. For instant, contacting directly with phagosome (Figure 3), melanin intercepts free-radical products and, to a definite extent, suppresses peroxide oxidation and, therefore, inhibits lipofuscin synthesis. This effect explains the known fact of decreased risk of retina macular degeneration for people with high pigmentation [58, 59]. Moreover, it is suggested that in RPE cells melanin may be synthesized directly inside the phagosome [60].

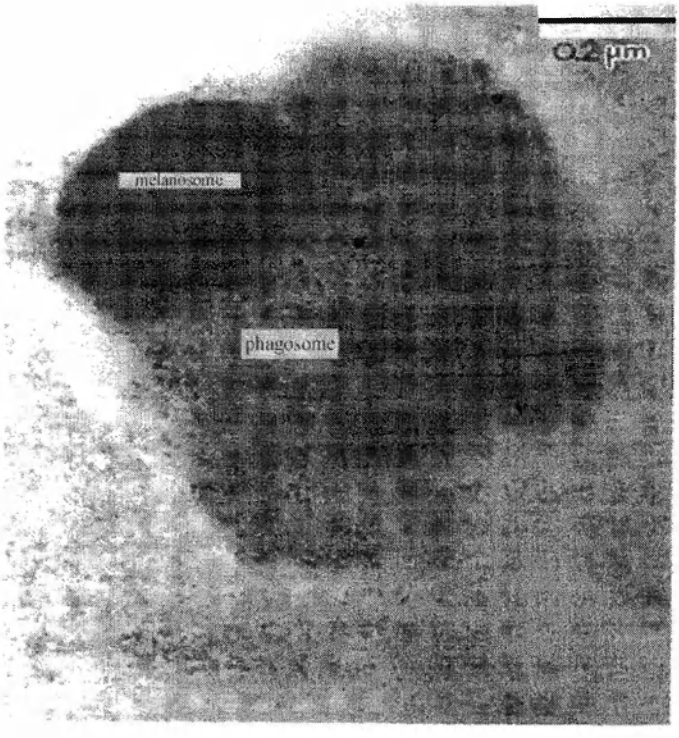

Figure 3. Electron microscopy of retinal pigmentary epithelium showing binding of melanosome and phagosome in a general structure

Isolation of melanin-containing RPE cells from neutral retina, for example, in the case of retinal detachment, may also intensify peroxide oxidation processes in it. In our works, it is shown that detached neutral retina accumulated 3 – 4 times more peroxide oxidation products under the effect of UV-radiation or bivalent iron compared with retina tightly contacting pigmented RPE cells [61, 62].

Unfortunately, usually aging is accompanied by melanin concentration decrease. It is known that with age the number of melanosomes increases [63]. However, it has been shown, that concentration of melanin in the granule does not practically change with the age [64]. Nevertheless, the volume of RPE cell occupied by melanosomes is decreased from 8% typical of 20 year-old individuals to 6% for 40 year-old and then to 3.5% for 41 – 90 year-old donors

[65]. Apparently, this process is one of the factors promoting development of senile macular degeneration of retina.

PHYSICOCHEMICAL MECHANISMS OF THE ANTIOXIDANT ACTION OF SHIELDING PIGMENTS

The antioxidant activity of melanins and ommochromes is based on, at least, three physicochemical properties of these systems:

1) ability to inactivate free radicals;
2) ability to quench electron-excited states of photosensibilizers and singlet oxygen;
3) ability to unite metal ions of alternate valence and organic sensibilizers in inactive complexes.

We have initially indicated the melanosomes, synthetic DOPA-melanin and ommochromes effectively interact with superoxide anion-radicals of oxygen [12, 14, 66, 67]. These data were confirmed by further investigations [68, 69]. We have studied dismutating ability of shielding pigments both in conditions close to physiological (aqueous medium, pH 7.8), and in aprotic solvents (Figure 4). Figure 4 shows that both ommochromes and melanin significantly intensify superoxide degradation in nonaqueous medium, melanin rather than ommochrome being more active. Calculated rate constants of melanin and ommochromes interaction with superoxide were high: $2.2 \cdot 10^5$ $M^{-1}s^{-1}$ per monomeric unit for DOPA-melanin; $6 \cdot 10^8$ $M^{-1}s^{-1}$ for a polymer with molecular mass ~40 kilodaltons; $3 \cdot 10^5$ $M^{-1}s^{-1}$ for ommochromes.

Also, multiple investigations indicate easy interaction of melanins with various free-radical reaction products and their inactivation [70 – 74].

As a consequence, shielding pigments, which concentration in eye cells of both vertebrates and invertebrates is very high, may act as effective traps for cytotoxic radicals and participate in regulating antioxidant status of the cell.

Shielding pigments may also act as quenchers of singlet oxygen [75 – 77]. In this role, melanins are more active than ommochromes: the rate constant of singlet oxygen quenching equals $(2 \pm 1) \cdot 10^8$ $M^{-1}s^{-1}$ and 10^6 $M^{-1}s^{-1}$ per monomeric unit of DOPA-melanin and ommochromes, respectively [77]. Hence, quenching of singlet oxygen and electron-excited states of

photosensibilizers by melanins is, generally, implemented by method of radiation-free transition during times below nanoseconds [78].

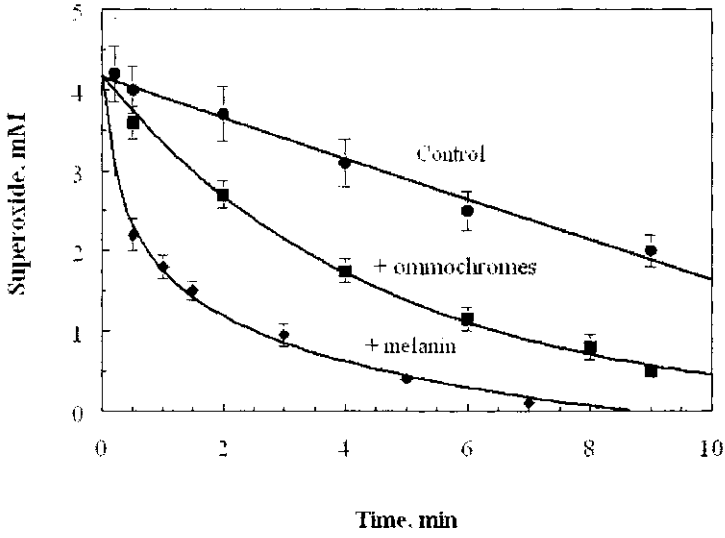

Figure 4. Acceleration of superoxide oxygen radical recombination by shielding pigments in nonaqueous media

Thus, melanins in the animal cell are singlet oxygen quenchers of the same activity as carotinoids in the plant cell.

It is well known that in peroxide oxidation of lipids special role is belonged to bivalent iron ions, which catalyze generation of superoxide and hydroxide radicals in the cell [53, 54]. Melanosomes and ommochromes containing identical set of coordinating groups (COO^-, NH_2, NH, >C=O) were found excellent chelators of iron ions [79 – 82] and other metal ions of alternate valence, such as copper, manganese, iron, lead, selenium, calcium, etc. [71]. According to estimations obtained, the number of binding sites in eumelanins for metal ions may reach 20% of polymeric subunit, which is, approximately, hundred times higher than the concentration of free radical site in melanin [45]. Moreover, at binding of metal ions their redox activity is significantly reduced and becomes less dangerous for cellular components. For example, being bound to melanin, bivalent iron ions are incapable of any effective catalyzing of $OH^{\bullet}$ radical generation in the Fenton reaction [45].

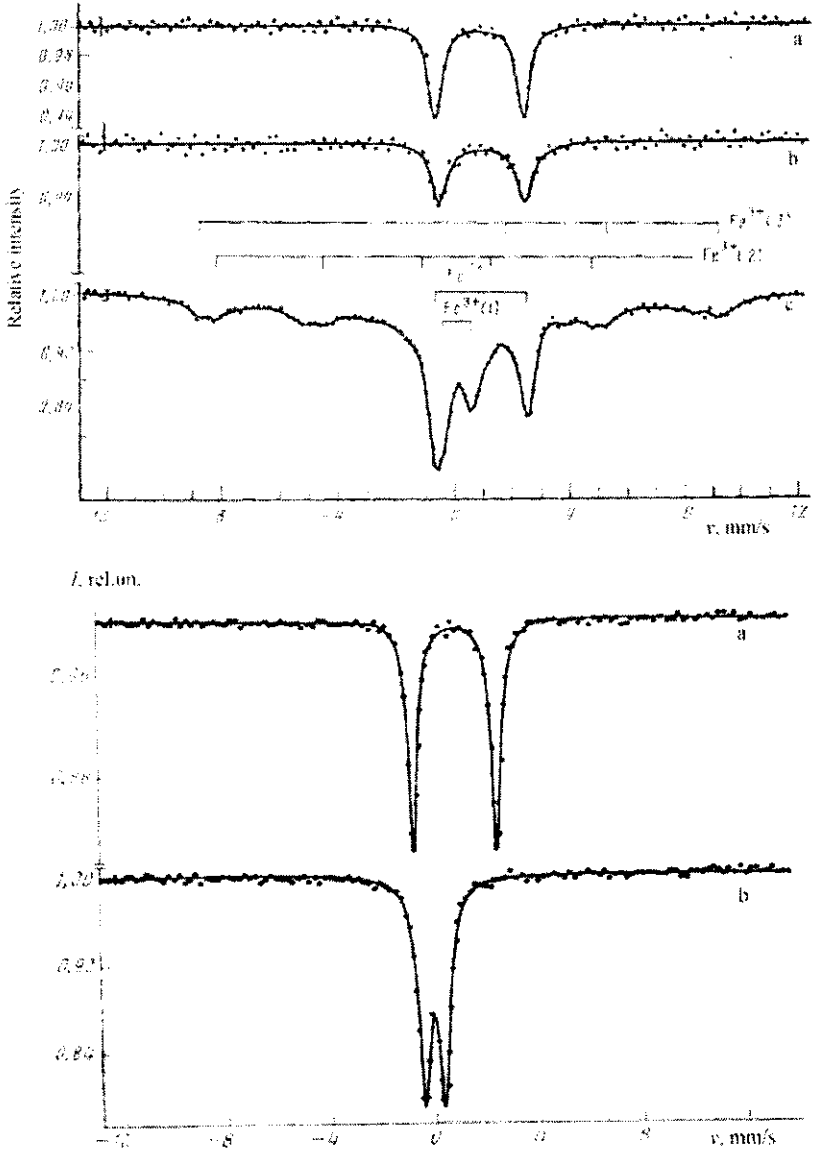

Figure 5. Gamma-resonance spectra of bivalent iron ion binding by:
a) RPE cell melanosomes;
b) ommochromes

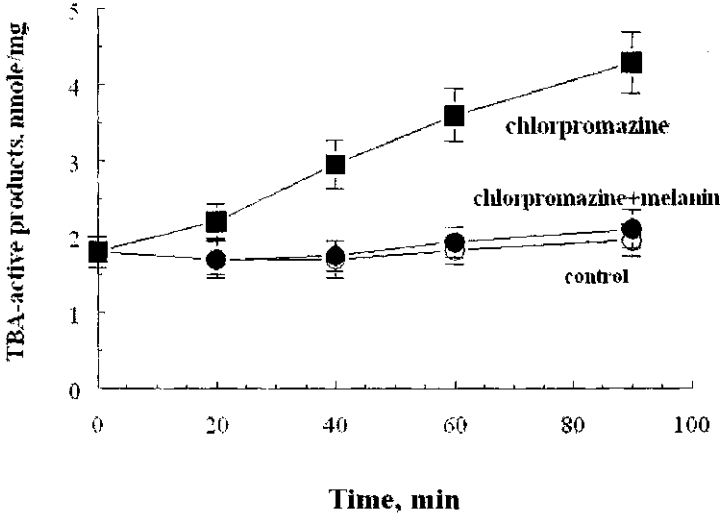

Figure 6. Inhibition of photooxidantion of photoreceptor cells, sensibilized by aminazin and human RPE melanosomes

We have applied the method of gamma-resonance spectroscopy to the comprehensive study of two- and three-valent iron ion binding by shielding pigments [80 – 82]. Melanosomes and synthetic DOPA-melanin form chelate complexes with both Fe^{2+} and Fe^{3+}; meanwhile, they are able to oxidize Fe^{2+} to Fe^{3+} inactive in relation to prooxidants (Figure 5a). Ommochromes bind iron ions much more effectively than melanosomes and DOPA-melanin (Figure 5b). The formation of Fe^{2+} complex with ommochromes causes an abrupt decrease of the redox-potential and oxidation of iron to the three-valent state.

Thus, iron ion binding by melanosomes and ommochromes in prooxidant-inactive complexes represents the important mechanism of the antioxidant action of shielding pigments.

Also, the ability of melanin and melanosomes to bind various photosensibilizers, including photosensitive drugs, is commonly known [83 – 87]. As bound to melanin, these substances usually decrease or even completely lose the ability to induce photodamage of cellular structures. For example, we have shown that anxiolytic aminazin, which may be accumulated in the eye tissues and can sensibilize photoinduced peroxide oxidation processes [88, 89], is bound to melanin [90, 91]. Meanwhile, the process of

photoinduced peroxide oxidation of photoreceptor cells is significantly decelerated (Figure 6). Binding of organic sensibilizers to inactive complexes is one more unit in the mechanism of antioxidant activation of shielding pigments.

Thus, shielding pigments – melanins of vertebrates and ommochromes of invertebrates – can be considered as the important fragment in the antioxidant protection system of visual cells. The presence of such system in highly pigmented animals and people gives apparent advantages before low pigmented individuals and albinos.

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ENZYMATIC CATALYSIS: KINETICS, ACTIVE SITE STRUCTURE, BIOINFORMATICS

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The enzymatic catalysis as an amazing natural phenomenon of chemical reaction acceleration by protein molecules has attracted much attention in classical chemistry since the very origination of catalysis ideas. The catalysis founder V. Ostwald (1853 – 1932) had distinguished four types of catalysts: contact action of "nuclei", homogeneous catalysis, heterogeneous catalysis, and the action of enzymes. The ideas of enzymatic catalysis origin were most actively developed in the last two decades of the previous century. This is associated with invoking modern physicochemical methods, deep structural investigation of enzymes, application of various molecular-biological and molecular-genetic methods to this field.

Possessing the extraordinary catalytic efficiency 10^{10} – 10^{15} times exceeding the efficiency of classical chemical catalysts as hydrogen or hydroxyl ion, in recent decades, enzymes became the objects for comprehensive study using modern physicochemical, structural, and kinetic methods.

Intensive studies of enzymes as the catalysts of chemical reactions results in quite thorough understanding of physicochemical bases of enzymatic catalysis, the origin of enzyme high activity and specificity [1 – 5].

The developed ideas about molecular processes proceeding in active sites of enzymes in the framework of catalytic cycle are based on the combined application of two approaches. On the one hand, these are structural studies of enzymes as proteins with obtaining structural data of the atomic resolution, and on the other hand, at the comprehensive study of kinetics of the enzymatic catalysis.

Chemical kinetics methods are of special importance for the study of enzymatic reaction mechanisms. Chemical kinetics provides for a reliable basis of investigations of the mechanisms of various chemical processes. The application of theoretical and experimental methods of chemical kinetics to the

study of biological catalysis phenomenon was found extremely effective and fruitful.

In recent decades, a great contribution into the study of enzymes was made by the works of Russian school of chemical kinetics, based on the investigations initiated by Academician N.N. Semenov [6, 7].

Decoding of the complex chemical transformation mechanisms mediated by unstable intermediate compounds became possible due to creation, development and consecutive application of various experimental and theoretical kinetic methods [8]. Academician N.M. Emanuel is one of the famous representatives of Russian chemical kinetics investigators. He provided for consecutive application of chemical kinetics to analysis of complex process mechanisms. Enzymatic catalysis is the example of fruitful application of this approach. In the middle 1960 ies, one of the followers of N.M. Emanuel (it was the real member of the Academy Of Sciences of the USSR I.V. Berezin) actively put himself in the study of enzymes and molecular mechanisms of their action [9]. In 1974, he established a special cathedral of chemical enzymology at the chemical department of M.V. Lomonosov Moscow State University. At present, this division provides active investigations in the field of biological catalysis and enzyme application to various chemical tasks.

The Russian scientific school of chemical enzymology leads in the studies of enzymatic catalysis.

ENZYMATIC CATALYSIS KINETICS

The study of enzymes is associated with solving various kinetic problems [10 – 13]. Different methods of kinetic description of enzymatic reactions in pre-stationary and relaxation modes have been studied. In the framework of the study of the origin specificity for enzymatic catalysis, the structure–substrate reactivity regularities have been studied and ideas about the reaction site complexing with nonreactive fragments of the substrate as the factor of reaction intensification in the framework of double-point model of the active site [1, 15, 16].

The developed theory of multisubstrate enzymatic reactions [10, 11, 17] has been applied to the study of complex multisubstrate enzymes, prostaglandin H-synthase – the enzyme limiting prostaglandin synthesis, in particular [18] (see below).

Of the enzymes inactivation processes are rather typical – the losses of catalytic function under one or another conditions. Theoretical and experimental methods of investigation of the enzyme inactivation mechanisms in the framework of the reliability theory application were developed [19]. A kinetic theory of enzyme inactivation during the reaction is developed, and the methods for discrimination of inactivation mechanisms are created [20].

The study of enzymes as electrochemical reaction catalysts has enriched the knowledge of enzymatic catalysis with experimental and theoretical methods of electrochemical kinetics [21].

Enzyme-conjugated reactions in biosystems proceed with participation of polyenzymatic systems. Formal kinetic regularities of enzymatic systems in stationary and nonstationary modes [22, 23] were studied, the models of electron transport processes are described, and the kinetic theory of electron transfer in structurally bound transmitters in the chains [24].

At the molecular level, physiological responses provide for the action of receptor–enzymatic systems. Molecular reception regularities, kinetic methods of receptor discrimination, and the mechanisms of the receptor signal conduction are discussed in the following works [25 – 27]. The neuropeptide–morphine receptor systems was studied comprehensively [28].

Some specific and rather interesting tasks are related to kinetic description of proliferating catalytic systems. The formal kinetic description of the growth and evolution of microbial populations was presented. It included analysis of kinetic models of growing microorganism cultures, exponential phases, and integral shape of the growth equation. Kinetic regularities of the growth inhibition by excessive substrate and the reaction product, and the mechanisms leading to induction periods on the growth kinetic curves are analyzed. Kinetic regularities of replication of plasmide-type genetic elements in growing populations of cells have been studied [30].

The development of modern calculation methods and application of appropriate experimental approaches allowed for solving the question of kinetic description of microbial population behavior, in which a product produced by one microorganism represents a substrate for the growth of another organism. This approach allowed obtaining of a comprehensive kinetic description of the processes proceeding in methane-generating microbial association [29].

BIOINFORMATICS, STRUCTURAL PARADOXES AND PHENOMENON OF THE STRUCTURAL UNITY OF ACTIVE SITES OF ENZYMES

Chemical kinetics forms the grounds for quantitative kinetic analysis of multiple dynamic tasks and provides for the progress in the study of molecular mechanisms for enzymatic catalysis.

Summing up our knowledge about the action mechanisms of enzymes, we can imagine a rather detailed picture of interaction between the substrate and sorptional subsite of the active site and the following processes related to the action of the catalytic site functional groups. Meanwhile, recently, the application of various quantitative approaches allowed determination and bringing up several new questions, which are to be solved in order to deepen our knowledge and understanding of the biological catalysis phenomenon.

Extremely broad structural and kinetic information, obtained in the recent decade, currently should be systematized and generalized. This sort of analysis might be based on bio-informatics method [31 – 33] that would allow for determination of general properties of enzymatic catalysis as the natural phenomenon at the molecular level. The application of approaches and methods of bio-informatics makes possible the discovery of some unobvious structural features of the active sites.

Modern computer databases contain bulky information on the structure of proteins and enzymes, currently meaning both the primary sequence of amino acids in the polypeptide chain and the structure of proteins at the atomic resolution. It is significant that providing for a possibility of operating bodies of information, databases are the measure designed for systematizing structural data and their analysis in the framework of various algorithms specific for each database.

The greatest volumes of information were obtained on the primary structures e.g. the sequences of amino acids in proteins. The studies of genomes supported this branch with gigantic information volumes, which are exponentially increased still.

Commonly, the primary sequence is determining for all structural hierarchies of the protein. Hence, the following scientific problem is obvious: basing on the knowledge about the sequence of amino acids, how could we predict the three-dimensional structure of a protein, including the structure of its active site? At present, two approaches, at least, are applied to solving this

question. The first approach is based on the theoretical calculations of the energy properties of a protein globule with searching for structures with the minimal energy [38]. This path is associated with big volume of calculations. Moreover, in the most cases, the task has no a unique solution, though it may be expected that the progress in this field may lead to practically significant results.

The second approach is much simpler. It is based on the structure composition according to homology. This approach is also based on the fact that many proteins, especially those from various origins but implementing the same or similar function, are rather similar and show high homology level.

Since the full three-dimensional structure of many proteins is strictly determined, an investigator may use these structures for composing the homologue structure. Hence, this significantly simplifies the calculations.

To detect amino acids which form the active site of an enzyme, and structure-forming amino acids which "gather" side radicals of the amino acid to a catalytic active site, the authors have used an approach based on the comparison of amino acid sequences in homological proteins of a large family under consideration [31 – 33, 39].

The procedure for comparing highly homologous (30% or higher) proteins was called the multiple leveling. If any protein in a set is taken for the basis, other proteins-homologues may be somewhat "strung on" it with the help of computer in order to compare the uniformity level of each position in the sequence in the whole set under study. The multiple leveling algorithm suggests automated accounting for "insertions" (the presence of additional, not general sequence) and "deletions" (particular gaps of the chain fragments). As a result, the multiple leveling produces a matrix of locating probabilities for one amino acid or another in each position of polypeptide chain taken for the basis. This approach allows detection of amino acid locations, for which the probability of amino acid preservation is high, detect the so-called conservative locations typical of all proteins in the set. In this case, the probability of the j-th acid in the i-th location approaches 1. On the other hand, this approach gives an opportunity of determining location, in which any of twenty natural amino acids may occur. A tight filter which helps in detecting a "signal" in statistical noise is Shannon's entropy:

$$H = -\sum_i P_i^j \log P^j I,$$

where P_i^j is the probability or relative frequency of amino acid j occurrence in the i-th position.

The remarkable property of this function is that at 100% probability of the event Shannon's entropy equals zero, and the signal can be filtered from the noise.

Data on the multiple leveling of homological proteins are presented in HSSP database [36].

We have used the multiple leveling procedure for homological proteins, the calculation of probabilities for particular amino acid location in particular position, calculation of Shannon's entropy for each position in the sequence for detecting amino acids which compose the active site of the protein. The procedure allows for highly probable identification of the active site groups and structure-forming amino acids basing on the knowledge of just amino acid sequence in polypeptide chain of the protein.

The required applicability condition of this approach is relation of a protein to statistically quite representative group of homological proteins. Statistical selection must include, at least, 50 – 100 proteins. As an example, Figure 1 presents data on multiple leveling and Shannon's entropy calculations for subtilisin – the bacterial protease which hydrolyzes proteins.

For the majority of enzymes, the analysis of such dependencies allows determination of many interesting regularities. Some detected regularities are unobvious and rather paradoxical.

1) Sequences of amino acids in the framework of the same group of proteins possessing well-defined biological function, for example, specific enzymatic activity, are quite various. However, there are locations, in which amino acids are practically invariable, i.e. for the whole group (sometimes up to 1,000 proteins) the same defined amino acids locate in definite positions. In the framework of the approach used, these positions are easily detected, because for amino acids of this kind in located definite positions Shannon's entropy approach zero. Let us call these amino acids and positions conservative.

2) A great part of amino acids in polypeptide chain of allied enzymes is highly variable (Shannon's entropy falls within 1 – 3 range). In the framework of evolution and mutagenesis the Nature has created a broad variety of allied structures having the same function, for example, the ability to carry out enzymatic reaction. The same enzyme may be extracted from various organisms (from *E.coli* to human).

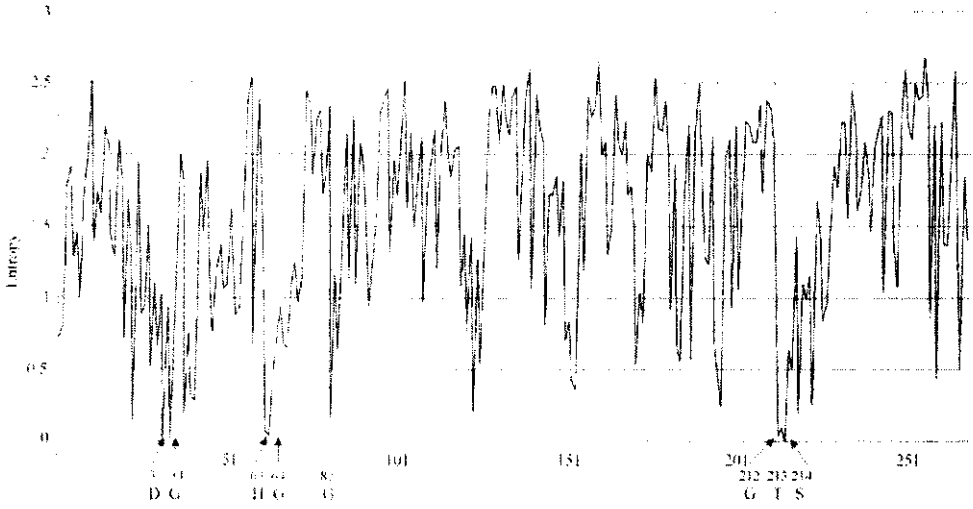


Figure 1. Shannon's entropy calculated for "leveling" a sequence of subtilisin enzymes

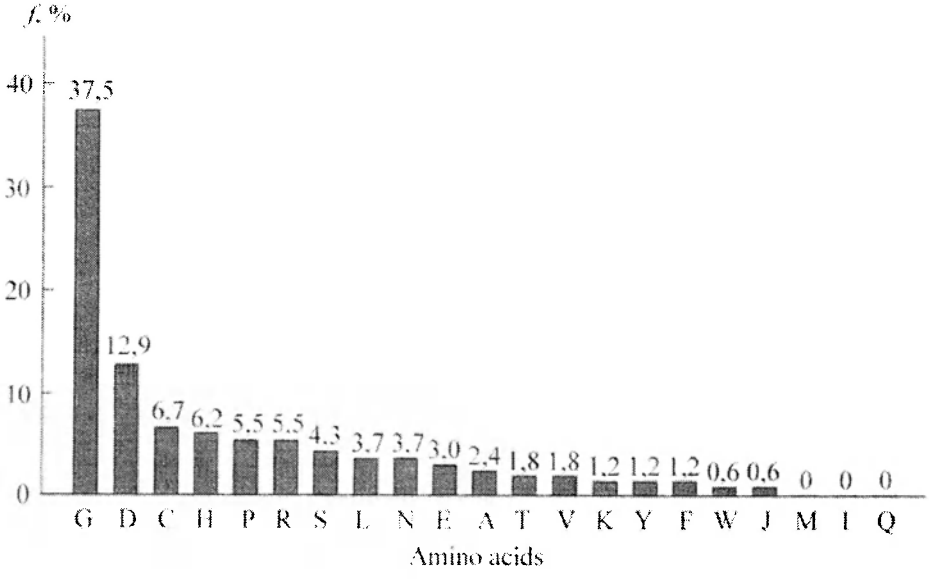
These proteins will have significantly different composition and amino acid sequence, but will possess practically identical structure of the catalytic site. It is of importance that the active site of any enzyme is represented by two sub-structures: sorption site providing for sorption and orientation of the substrate in relation to catalytically active groups and the catalytic site itself which carry out elementary acts of bond redistribution. A broad variety of sequences of polypeptide chains at practically equal spatial structure of the catalytic site indicates that formation of a catalytic site strictly depends on the limited number of amino acids in definite positions of the polymer chain. Natural variation of amino acids indicates that the most part of them can be replaced by others. In many positions Shannon's entropy reaches 2.5 – 3 that indicates a possibility of any of 20 natural amino acids location in it. Meanwhile, some characteristics of the protein such as stability, isoionic point, hydrophilicity, and hydrophobicity will be changed. However, the main function, which is catalytic activity, and structure of the catalytic site is preserved invariable for the whole family.

3) Amino acids composing an active catalytic site always represent conservative amino acids with Shannon's entropy approaching zero. This is checked on a broad selection of enzymes, for which the catalytic site structure is known. This group of amino acids composing catalytic sites includes aspartic acid, histidine, arginine, serine, and glutamic acid e.g. amino acids possessing significant acid-base function capable of molecule activation by the nucleophilic-electrophilic mechanism.

4) What are amino acids most frequently acting as conservative ones? The occurrence of amino acids are conservative ones is ranked. This ranking is shown in Figure 2 [39, 40]. The experimental fact was unexpected that the most important amino acid most frequently determined as the conservative one is glycine. It is paradoxical that being the simplest amino acid having no expressed chemical function of the polypeptide chain, glycine is the most significant amino acid for the protein structure formation and, consequently, in formation of the active site.

The second important place in the ranking of conservativeness belongs to aspartic acid, which is most frequently observed as nucleophile (deprotonated) or electrophile (protonated) in active sites. For hydrolases, histidine with imidazole group is extremely important, whereas for oxidoreductases – arginine possessing positively charged guanidine group.

The structure-forming amino acids such as proline and cysteine also show conservative properties rather frequently.



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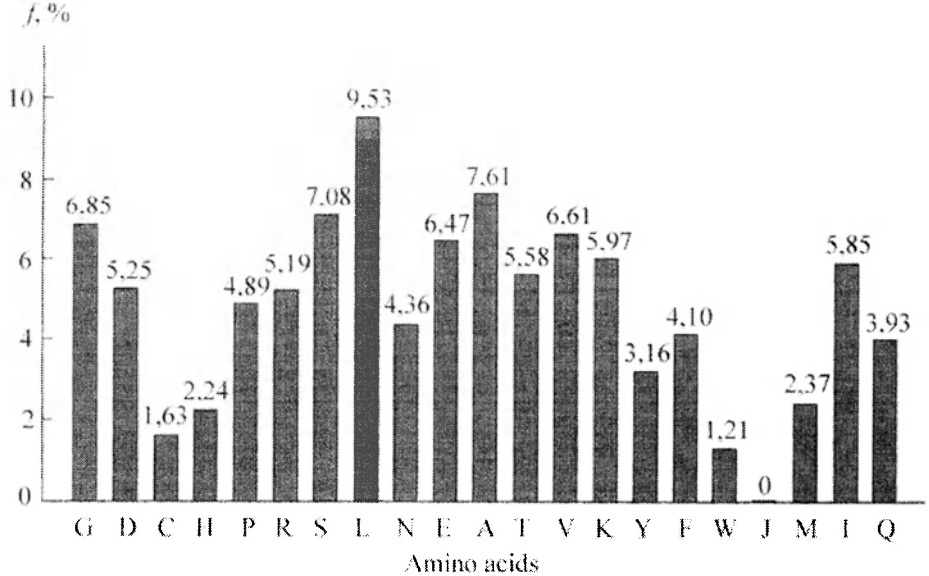


b

Figure 2. The frequency of conservative element amino acid occurrence in the enzyme structure (a) and in nature

The distribution of functions between conservative of amino acids is quite obvious. Carboxylic groups in aspartic and glutamic acids, imidazole group in histidine, and guanidine group in arginine form three-dimensional catalytic structures implementing coordinated nucleophilic-electrophilic catalysis. The elementary act of catalysis is connected to relatively short proton transfer by hydrogen bond (by 1 – 1.5 Å) or bond polarization by an electrophilic agent. Meanwhile, reactivity of the substrate molecule or active site group increases by 10^6 – 10^7 times. For example, proton transfer from water approaches the reagent structure in the transition state to hydroxyl ion and increases its reactivity in the reaction of nucleophilic substitution by 10^7 times. If the process proceeds in the coordinated mode and each substrate is activated so that general increase of the reaction rate by 10^{12} – 10^{15} times may be expected (see below).

5) Catalytically valuable amino acids are distributed by polypeptide chain of the protein. During folding and three-dimensional structure formation catalytically important functional groups are drawn closer in space and, thus, form three-dimensional structure of the catalytic site. The factor providing for appropriate folding of the polypeptide chain is occurrence of structure-forming amino acids glycine, proline, and, finally, cysteine are the appropriate position. Meanwhile, catalytically valuable amino acids can be located according to different orders in the polypeptide chain. For example, two catalytic proteases chymotrypsin (animal protein) and subtilisin (S-protein of microorganisms, see Figure 1) have the following sequence of amino acids forming catalytic triad His57 Asp102 Ser195 (chymotrypsin) Asp32 His64 Ser221 (subtilisin). Nevertheless, three-dimensional structures of the catalytic sites formed from absolutely different polypeptide chains possess structurally identical active sites. Two different sequences of catalytic groups produce two different secondary structures. All serine proteases with catalytic groups located according to α-chymotrypsin type represent β-proteins. In the framework of secondary structure classification, all proteases having subtilisin–type location of catalytic groups represent α/β-proteins [39].

6) The main structural paradox of enzymatic catalysis is that a tremendous number of enzymatic reactions involving practically infinite number of proteins, which possess different primary structures, is based on the application of rather short number of catalytic sites.

For example, the broadest class of enzymes – hydrolases, gives about one third of currently known enzymes (about 1100 enzymes of 3700 existing according to the enzyme classification). Each of these enzymes is followed by

a biological variety, because in biological systems these proteins are presented by almost infinite set of amino acid sequence alternatives. However, there are only four basic catalytic sites which activate molecules and the catalytic cycle [39]. The variety of reactions (about 1100 for hydrolases) is related to the action of the sorption site which forms a complex from the substrate and orienting the reaction site in relation to catalytic groups of the protein. Thus, catalytic sites of enzymes are the unique structures occurred during the evolution, apparently, because of a great search of intermediate alternatives of the structures and genetically fixed, catalytically valuable and structure-forming amino acids at definite positions, which compose functional groups of catalytic amino acids in the desired point of space with the required steric correspondence.

7) The phenomenon of the structural solidity of catalytic site composition is displayed, when we compare active sites of enzymes different from hydrolases with hydrolases [5].

The analysis shows that active sites of enzymes of other classes different from hydrolases are composed from nucleophilic–electrophilic elements similar to active sites of hydrolases. The reaction sites of molecules are activated by the same nucleophilic–electrophilic mechanisms, which often use the same three-dimensional structures.

For example, the charge transfer chain including a pair of imidazole–carboxylic group very frequently may be identified in active sites of oxidoreductases. Similar to many NAD^+–dependent dehydrogenases, the active site of lactate dehydrogenase usually possesses His194–Asp165 pair, which is responsible for nucleophilic activation of lactic acid. The proton transfer by hydrogen bond between imidazole group of histidine 194 and hydroxylic group of lactic acid induces negative charge on the reaction site (α–carbon atom) that promotes hydride transfer to NAD^+ molecule. Positively charged guanidine group in Arg105 is the electrophilic mediator of this transfer.

In peroxidases – the enzymes which use hydrogen peroxide for oxidizing various molecules and containing hemin as a prosthetic group (see below) – imidazole group represents iron ion ligand, for example, His175 for cytochrome C–peroxidase. Positively charged iron ion interacts with unshared electron pair in the imidazole group and forms a strong coordination bond. Meanwhile, the second nitrogen atom of imidazole group is linked by hydrogen bond to carboxylic group of arpartic acid Asp135; hence, similar to the case of hydrolases, imidazole is the mediator of electron transfer, currently implementing polarizing partial charge transfer from negatively charged

carboxylic group to metal ion. Similar to the case of serine hydrolase and dehydrogenase triad, dynamically mobile nucleophilic agent acts in peroxidases. This agent represents structurally arranged pair consisting of imidazole group of histidine and carboxylic group of aspartic acid. Similar to the case of dehydrogenases, guanidine group of arginine (Arg48) participates in the catalytic cycle thus promoting for electron transfer from substrate to peroxide via oxidized iron ion.

8) The phenomenon of structural unity of active sites in enzymes becomes more evident, if we compare structures of active sites in hydrolases with corresponding sites in synthases which form bonds not using water molecule as a reagent. Hence, transition from hydrolytic to synthetic reactions is performed by replacement of water activation by, for example, carbon hydroxyl group activation with full preservation of the active site structure.

The catalytic site of exonucleases, which hydrolyze nucleic acids, represents a complex of magnesium ions formed using carboxylic groups of aspartic and glutamic acids (pyrophosphatase type). The active site of enzyme synthesizing DNA – DNA-polymerase – is highly homologous to the active site of nuclease which is the enzyme hydrolyzing DNA. The reaction site of necleophile in DNA-polymerase is activated by the same functional groups, which do it in pyrophosphatase or exonuclease. However, instead of water activation, in DNA–polymerase carbon hydroxylic group is activated. Nucleophilic attack on the reaction site of phosphate group provides for phosphodiester bond formation and DNA chain propagation. Therefore, this is the example of transition from enzymes which hydrolyze bonds to enzymes which form bonds. Structural substitution of water by other possible nucleophile in the active site is enough for forming an active site able to carry out a synthetic reaction.

According to the above-described identification of catalytic and structure-forming amino acids using the data on the primary sequence of amino acids in the polypeptide chain, the phenomenon of structural unity of enzymes from different classes has many consequences.

It is obvious that catalytically active proteins can be composed by amino acids, much less in the number than twenty amino acids existing in the nature. The greater part of locations in the polypeptide chain may be substituted by other amino acids, which will not cause any significant change of catalytic function of the protein. The presence of several critical amino acids (possessing acid-base properties) forming a catalytic site or structure-forming

amino acids gathering catalytic groups in some defined point of space in definite positions is of great importance.

The traditional method of enzyme property modification is the site-targeted mutagenesis or directed evolution method. The results of analysis performed show that free variation of amino acids is possible in the only positions, which are not of the conservative type. Otherwise, enzyme modification has high probability of its loss the enzymatic activity.

It is obvious that the type of catalytic reaction of enzyme is strictly defined by the sorption site structure, which provides for location for implementing an attack by catalytic groups. This creates the prospects for developing protein catalysts to be used in reactions, not observed in biology. This may significantly broaden the application abilities of enzymes to solving chemical tasks.

MOLECULAR MECHANISMS OF CATALYSIS

Chemical kinetics and structural chemistry have made the determining contribution into understanding of molecular mechanisms and structure of the enzymatic catalysis.

Usually, there are two purposes for studying kinetics of a chemical or biological process:

1. Prediction of the system behavior in future. If we know kinetic law of the system development (usually, a differential equation describing the process dynamics is formally known) and initial conditions (e.g. the system state at the initial moment of time), we can predict the system state at any time in future. This class of tasks is also added by optimization tasks e.g. those, solution of which allows for transiting from one state to another during the shortest (or the longest) time period, getting maximal output of the product, etc.
2. Understanding of the process mechanism e.g. presentation of separate elements and stages providing for currently desired process proceeding with time. Any idea about a process mechanism is a model. The main task of chemical and biochemical kinetics is investigation of reaction mechanisms. In the framework of chemical kinetic approach, the reaction mechanism is usually taken for a sequence of molecular

transformations of the initial reagents to final products. The mechanisms of chemical reactions are rather complicated. They include formation of labile and highly reactive intermediates. Detection of these intermediates, study of their structure and properties, determination of correlations between their structure and reactivity are highly essential for investigations of the process mechanism.

For many enzymes, experimental determination and chemical modeling allowed for obtaining of justified ideas on the substance transformation mechanism in the active site of any enzyme.

Let us show several examples.

"Blue" copper-containing oxidases. The enzymes of this class are responsible for muctielectronic oxygen reduction to water without intermediate formation of hydrogen peroxide. In particular, structures of this type provide for oxygen reduction during respiration of humans and animals.

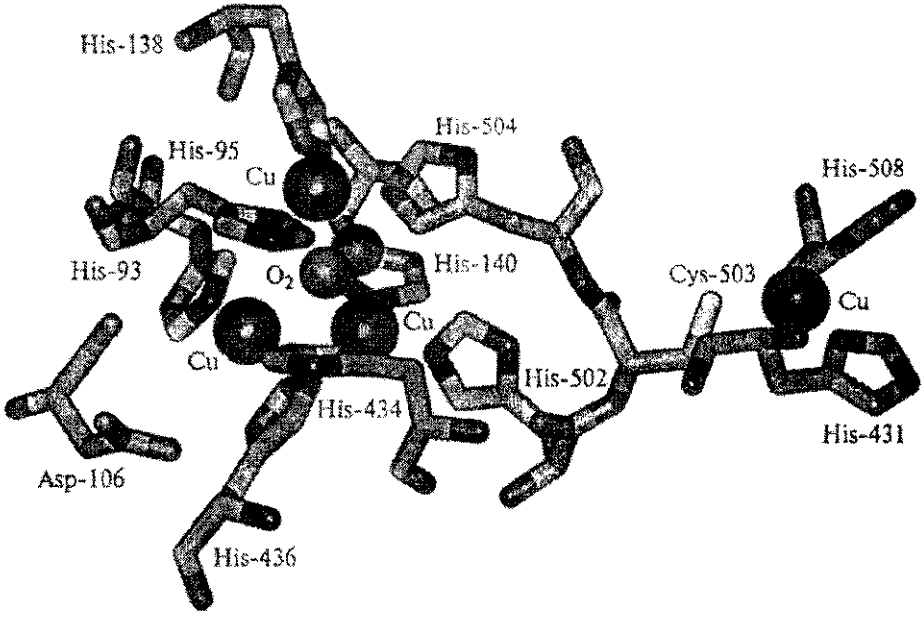


Figure 3. Laccase active site

The structure of laccase active site – the enzyme of microorganisms, is shown in Figure 3. Similar structure is observed for other "blue" copper–containing oxidases.

The studies of electrochemical kinetics of the enzyme action have significantly contributed to clearing up the mechanism of copper-containing oxidases [41 – 51]. It is found that copper (II) ions of laccase active site in a complex with oxygen molecule are capable of quick and effective electron exchange with the electrode. This allowed for comprehensive electrochemical study, determination of the number of electrons transferred at different stages of the process and suggestion of experimentally justified kinetic scheme of the catalyst action. In accordance with the data on kinetics of enzyme action in solution, reduction of molecular oxygen to water not mediated by hydrogen peroxide formation is carried out by the following scheme:

$$E + O_2 \rightleftarrows EO_2$$

$$EO_2 + e \xrightarrow{\text{Quickly}} EO_2^-$$

$$EO_2^- + H^+ \xrightleftharpoons{pK_a\ 5.5} EO_2^-H^+$$

$$EO_2^-H^+ + H^+ \xrightleftharpoons{pK_a\ 5.5} EO_2^-H_2^{2+}$$

$$EO_2^-H_2^{2+} + 2e \rightarrow EO^- + H_2O$$

$$EO^- + H^+ \xrightleftharpoons{pK_a\ 5.5} EO^-H^+$$

$$EO^-H^+ + H^+ \xrightleftharpoons{pK_a\ 5.5} EO^-H_2^{2+}$$

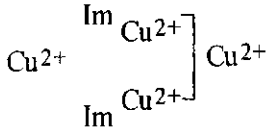
$$EO^-H_2^{2+} \rightarrow (EO^-H_2^{2+})$$

$$(EO^-H_2^{2+}) + e \xrightarrow{\text{Quickly}} E + H_2O$$

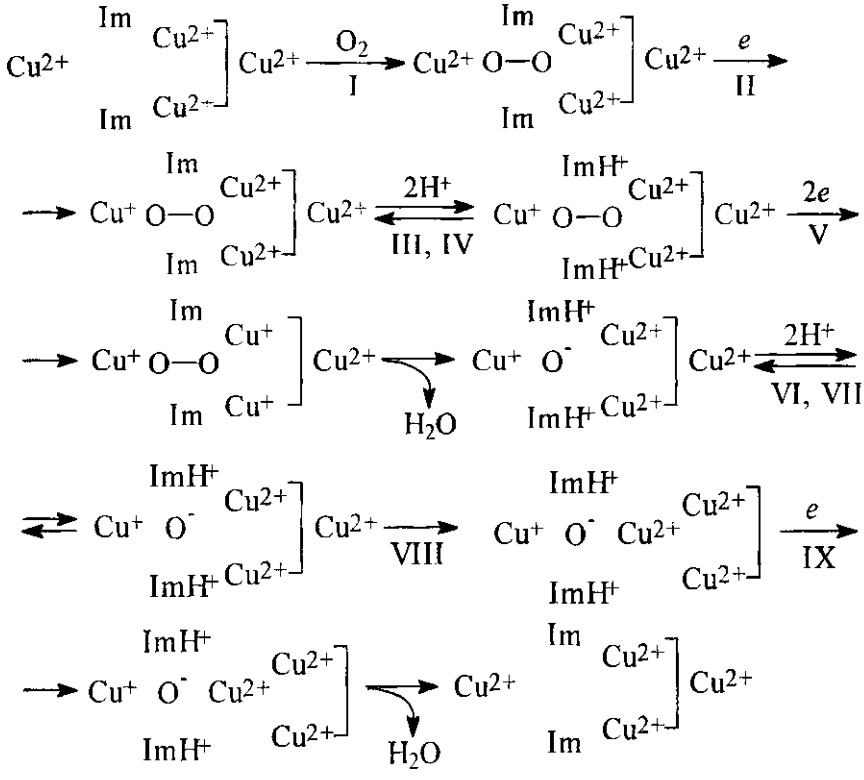
The results obtained at comparison with the data on the composition and structure of laccase active site allow for a suggestion of oxygen reduction mechanism using an enzyme.

It is known that the laccase active type includes four copper ions of three types (Figure 3). Hence, two copper ions are united and may represent

two–electron donor–acceptor. Two ionogenic groups with close values of pK_a about 5.5 participate in the catalysis mechanism. Structural data clearly show that these ionic groups are imidazole groups (Im) of histidine His-60 and His-448. The structure of the active site can be presented as follows:



Possible scheme of molecular transformations proceeding in the laccase active site during catalytic act of oxygen reduction, corresponded to the kinetic scheme, is the following:



The stage (II) (single-electron reduction of the active site of enzyme) is, probably, required for making the complex of oxygen and the active site stronger. Stages III, IV and VI, VII implement protonation of bases participating in the active site of the enzyme. Two equal symmetrically and synchronously operating imidazole units in the mechanism of catalysis with oxidase are principally important. On the one hand, in the protonated form they simplify the electron transfer from the donor (electrode) to two–electron acceptor. On the other hand, they intensify electron transfer and are donors of two protons at the stage of water formation.

The stage VII relates to some structural change of active site of the enzyme. Possibly, this stage represents a conformational change accompanied by substitution of two-electron acceptor by single-electron one in the immediate vicinity of activated oxygen atom.

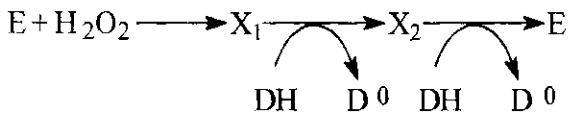
The comparison of kinetic and structural data allows for suggestion of the self molecular structure for each of the active site shapes presented in the scheme.

Two copper ions united in the single electronic system, capable accepting two electrons and transferring them to the oxygen molecule with quick protonation at reduction, are of the greatest importance for the active site structure. These processes then produce water molecule. Superoxide radical as thermodynamically absolutely unfavorable intermediate takes no action in the current mechanism. It is also of importance that oxygen is reduced to water without hydrogen peroxide formation.

Therefore, specific structures formed during evolution of enzymes and capable of implementing two-electron redox processes are favorable due to avoiding formation of thermodynamically unfavorable intermediates.

Peroxidases; prostaglandin–N–synthase. Peroxidases represent a large group of redox enzymes, which use hydrogen peroxide as the substrate. Peroxidases have good representation in plants, bacteria, fungi, and mammal cells. Peroxidases also play the principal role in development of nonspecific immune response by generating highly reactive oxidants in macrophages and neutrophils which destroy agents alien for the current organism. Peroxidase, extracted from horse radish roots, is the one most famous and well-studied.

The kinetic scheme of peroxidase action includes formation of several reactive intermediates:



where E is a free shape of the enzyme (ferriperoxidase); X_1 is compound I (λ_{max} = 410 and 655 nm); X_2 is compound II (λ_{max} = 420, 527, and 554 nm); DH is the substrate, which id the donor of electrons of hydride-ions.

In peroxidases, hemine–iron(III)–protoporphyrin complex plays the role of prosthetic groups. Imidazole group of histidine plays the role protein ligand for iron in the enzyme–hemine complex. Figure 4 shows structure of the active sites of three peroxidases: extractable from animal cells (a), from peanut (b), and fungal peroxidase oxidizing lignin (c). Obviously, these active sites are very similar. Hence, similar to the case of hydrolytic reactions, nucleophilic and electrophilic components of the active site play the principal role in the catalytic site structure and, consequently, are important for catalysis.

Imidazole group for from the following histidines are ligands for Fe^{3+} ion: His-175 (Figure 4a); His-184 (Figure 4b); His-176 (Figure 4c). Positively charged iron(III) ion represents a powerful electrophilic agent and interacts with unshared pair of electrons in nitrogen of imidazole group, forming a strong coordination bond. Imidazole group forms hydrogen bond with carboxylic group of aspartic acid (see Asp-235, Figure 4a; Asp-246, Figure 4b; Asp-238, Figure 4c). Meanwhile, similar to hydrolases, here imidazole in the intermedite in the electron transfer from negatively charged carboxylic group to metal ion.

The important participant of the catalytic cycle is imidazole group of histidine (see His-52, Figure 4a; His-56, Figure 4b; His-47, Figure 4c). It is apparent that this group represents a proton acceptor at peroxide activation stage and, therefore, significantly increases oxidability of peroxidase. Moreover, imidazole group may play the role of proton donor at the stage of water molecule formation.

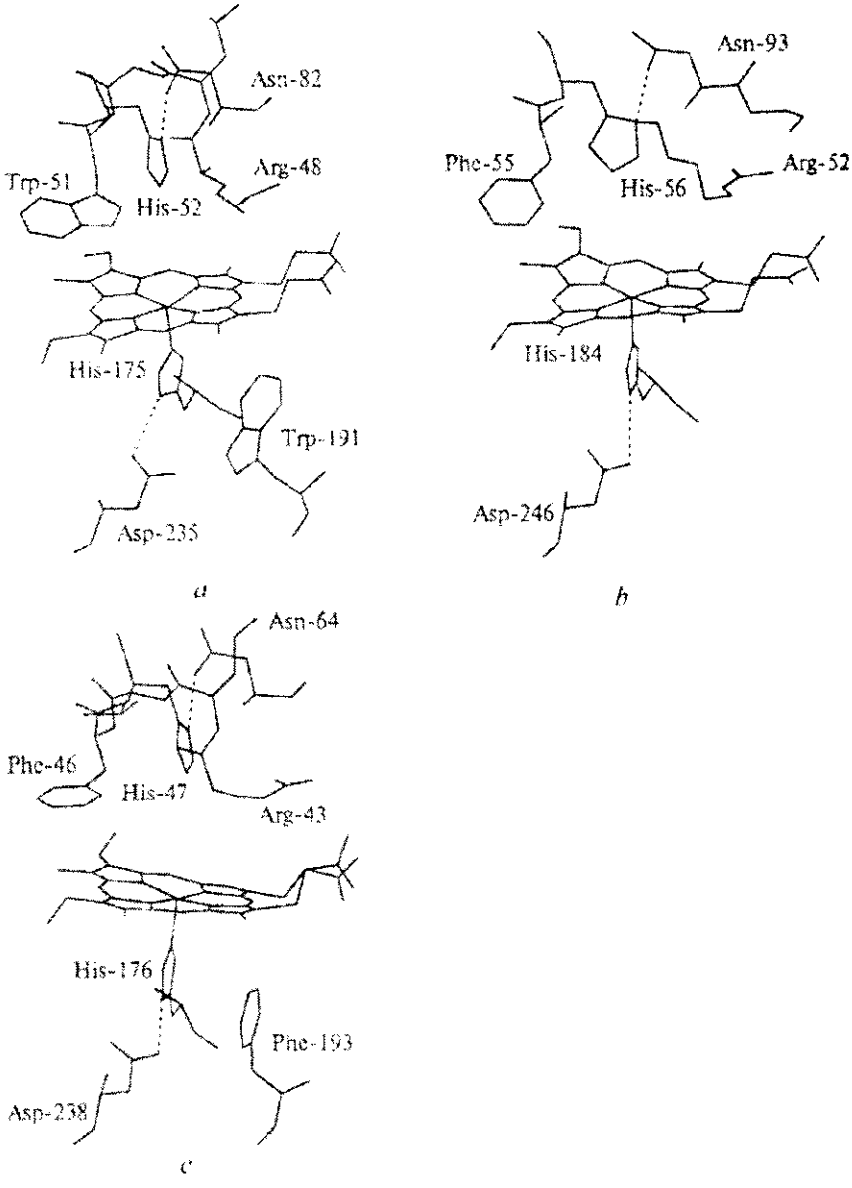
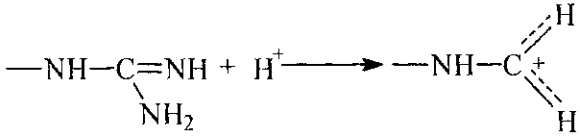


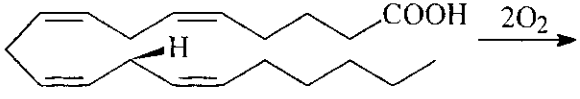
Figure 4. Presentation of the active sites of: *a* – cytochrome c oxidase; *b* – peanut peroxidase; *c* – lignin peroxidase

As components of the active site, peroxidases have guanidine group of arginine (see Arg-48, Figure 4a; Arg-52, Figure 4b; Arg-43, Figure 4c). As is known guanidine group of arginine possesses an original electron structure:

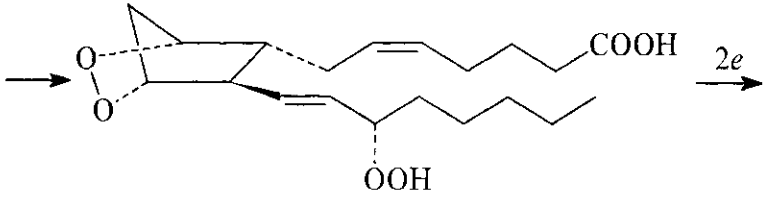


In protonated form, both nitrogen atoms are equivalent and, therefore, in the case of guanidine group of arginine we have an effective electrophilic agent with delocalized positive charge. This group may play an important role in electron and hydride-ion transfer from the substrate–electron donor to iron(III) ion during reduction of compounds I and II. It is of importance that guanidine group plays the same role in the mechanisms of both peroxidases and dehydrogenases (see above).

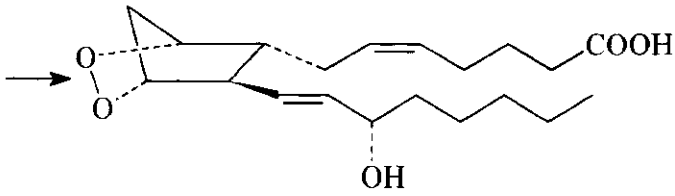
Peroxidases are similar to enzymes capable of using organic peroxides as a substrate. Of great importance for animal and human molecular physiology is prostaglandin-synthase endoperoxide. Other common names of this enzyme are prostaglandin-H-synthase (PGH-synthase) or cyclooxygenase. The active site of the enzyme carries out extremely complicated sequence of reactions transforming polyunsaturated fatty arachidonic acid into intermediate prostaglandin H_2:



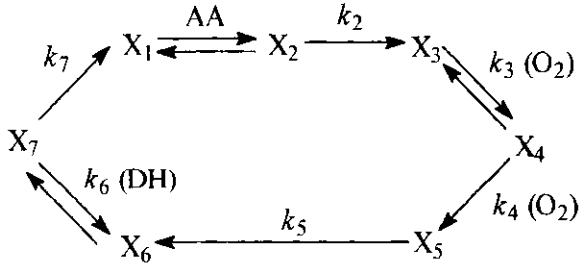
Arachidonic acid



PGG_2



The catalytic cycle includes four substrates: arachidonic acid, two oxygen molecules and reducing agent – the electron donor. Chemical transformation includes oxygen molecule addition, isomerization of hydrocarbon chain and pentatomic ring formation, hydroperoxide formation (PGG_2), and hydroperoxide group reduction to oxy-group with PGH_2 formation. The enzyme is of the greatest physiological importance. It is known that a great part of anti-inflammatory preparations such as aspirin, analginum, indomethacin, ubiprophen, etc. applied in medicine, are effective inhibitors of this enzyme. Formal kinetic study of the stationary reaction rate dependence on concentrations of all four substrates gave the following kinetic model:



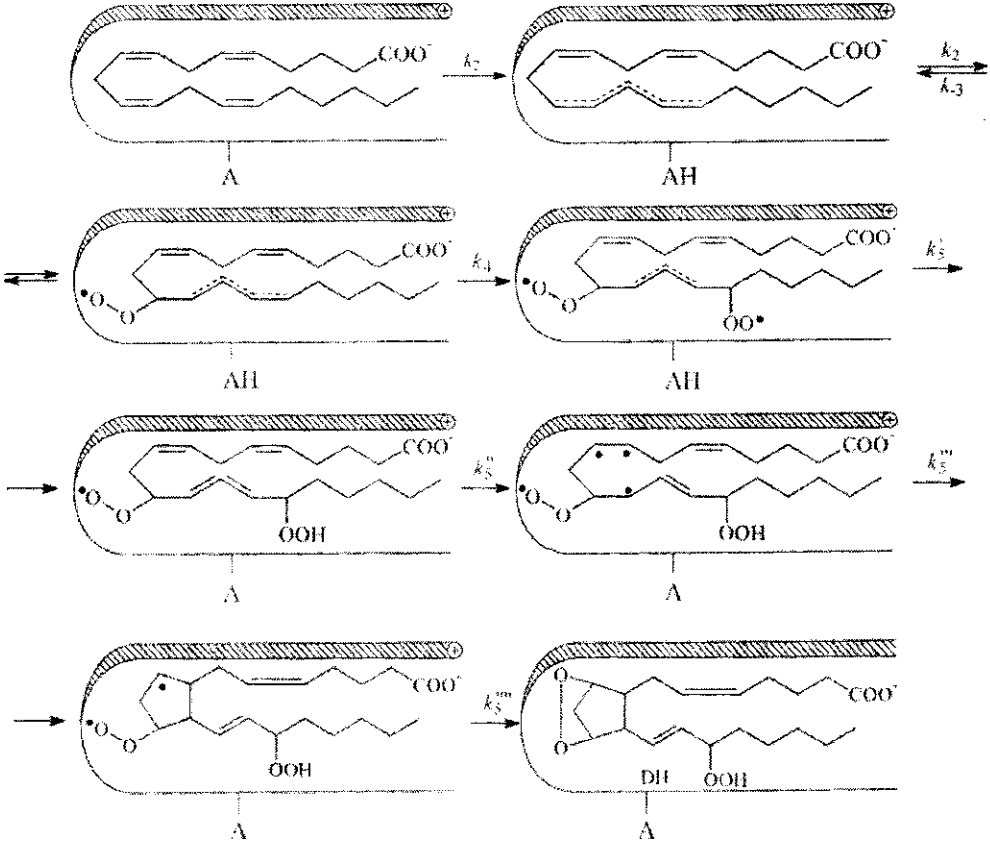
The process is four-substrate (arachidonic acid, two oxygen molecules, and electron donor). The model includes four stages of substrate injection to the catalytic cycle. As is shown by catalytic data, the stages of injection of arachidonic acid and oxygen, oxygen and electron donor, and electron donor and arachidonic acid to the reaction are separated irreversible stages.

The data from the literature about structural changes accompanying arachidonic acid conversion into prostaglandin H_2 and their comparison with kinetic results give us a molecular model of PGH-synthase action [52]. The first step in arachidonic acid activation formation of a complex from the substrate molecule and active site of the enzyme. For this process of great importance are hydrophobic interactions and the presence of carboxylic group

of unsaturated acid. Arachidonic acid carries out an electrostatic interaction with positively charged guanidine group Arg-120 from the active site of the enzyme.

It is apparent that hydrogen atom transfer from C(13) is the limiting stage of the whole reaction, and the maximal rate observed at enzyme "saturation" with all substrates will characterize the elementary act of hydrogen from C(13) bound to arachidonic acid enzyme. The following reactions represent an attack and addition of oxygen molecule at 11th location, addition of the second oxygen molecule to C(15) atom, isomerism of double bond in 13th location, C–C bond formation between C(8) and C(9) atoms, and intramolecular attack of oxygen radical on C(9). These quick radical processes may proceed spontaneously, without participation of a protein molecule.

The most probable sequence of arachidonic acid transformations in the active site of the enzyme is illustrated by the following scheme:



The stage characterized by constant k_2 represents hydrogen atom removal from arachidonic acid by an endogenic acceptor. This stage initiates further restructuring of arachidonic acid molecule. It should be outlined that kinetic data show regulated addition of oxygen molecules: the first equilibrium stage and the second bimolecular stage are mediated by the proton transfer and conformational changes of the active site, but are not mediated by such irreversible reactions as isomerism of hydrocarbon skeleton and formation of new C–C bonds.

The final product of the transformation sequence is the intermediate prostaglandin G_2 complex with active site of the enzyme. This complex may dissociate, and if electron donor DH is absent in the system, this hydroperoxide derivative occurs in the solution. This compound has been successfully detected and characterized. In the presence of electron donors hydroperoxide group is quickly reduced with formation of the final product – prostaglandin H_2.

The following question is of the principal importance for clearing up PGH–synthase mechanism: what is the initial acceptor of hydrogen atom at carbon atom C(13), which initiates the following complex sequence of radical reactions and restructuring of molecule backbone? This question is answered by comparison of PGH-synthases and peroxidases.

Prostaglandin–H–synthase possesses clearly expressed peroxidase activity. In the presence of hydrogen peroxide we may observe catalytic oxidation of all substrates – electron or hydrogen atom donors typicl of peroxidases (see above).

Catalytically active groups implementing this complicated cycle of transformations are shown in Figure 5. As mentioned above, arachidonic acid is fixed at the active site via electrostatic interaction with positively charged guanidine group of Arg–120. PGH-synthase possesses the structure and the surrounding of heme typical of peroxidases. The fundamental difference is the following: in the case of PGH–synthase, the donor of hydrogen atom required for heme reduction in the catalytic cycle is intramolecular intraprotein donor Tyr–385. Active tyrosyl radical formed due to hydrogen atom detachment (or electron with further proton detachment) is capable of compensating its nonsaturation by detaching hydrogen from carbon atom C(13) in arachidonic acid. It is known that the intermediate prostaglandin G_2 is capable of leaving the enzyme site and can be extracted as a chemical compound. Further transformation of it into PGH_2 is performed by hemine site due to classical

peroxidase reaction. Meanwhile, the independent reducer DH is hydrogen donor. If this donor is not added to the reaction mixture, the reaction is terminated at prostaglandin PGC_2, which is the compound of the hydroperoxide type. If the same reaction is carried out with H_2O_2 and a hydrogen donor, the same active site proceeds with the classical peroxidase reaction.

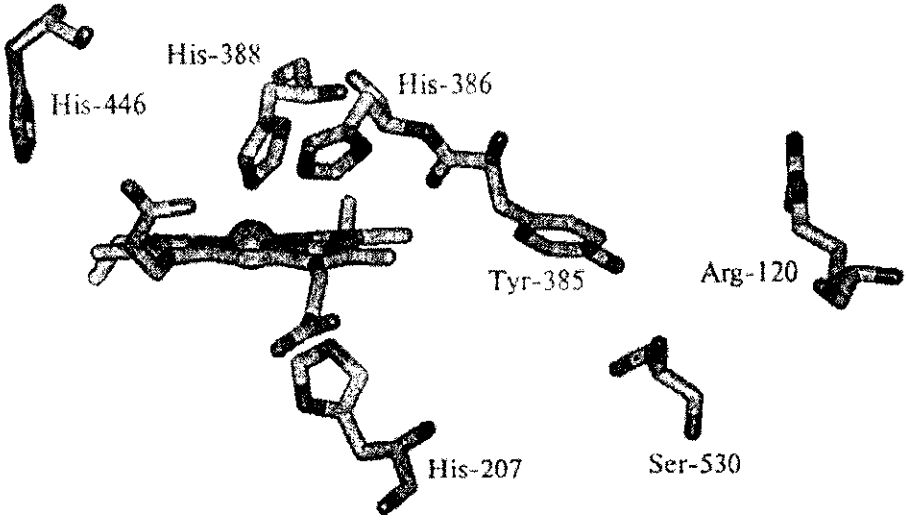


Figure 5. Functional groups in the composition of prostaglandin H–synthase

The comparison of Figures 4 and 5 shows that the catalytic site of PGH–synthase is almost identical to peroxidase catalytic site. Apparently, imidazole group His–388 accepts a proton from hydroperoxide and, therefore, increases oxidative reactivity of hydroperoxide. The main difference is that for PGH–synthase arginine is replaced by imidazole–386 group. It is apparent that protonated imidazole shape may represent an electrophilic intramolecular mediator of the electron transfer from donor to iron ion.

THE ORIGIN OF CATALYTIC EFFICIENCY OF ENZYMES

Presently, we possess a broad information about structures of the active sites of enzymes and catalytic cycle mechanisms. This allows for discussion of general principles of active site formation and physicochemical nature of their catalytic efficiency [5, 40].

What are the factors defining high selectivity (specificity) and efficiency of the enzymatic catalysis? Recently, we are able to give justified answers to this question.

Specificity is the fundamental important property of enzymes, which provide for a possibility of chemical reaction proceeding in biological systems with minimum losses for secondary processes.

For many enzymes, the substrate reactivity is defined by structural groups remote from the reaction site.

To explain the feature of enzymes giving them an opportunity to "select" molecules of definite structure, several model ideas have been developed and experimentally supported. Specificity of enzymes is based of the effects of steric correspondence ("key – lock"), induced correspondence, stresses and deformations appearing at the interaction of the substrate with enzyme [5]. The two-site model has got a quantitative confirmation. It binds selection of the substrate with its specific fixation by the sorption site of the enzyme.

Active sites of enzymes represent structurally heterogeneous compositions which carry out two consecutive processes:

1) sorption and fixation of the substrate, its orientation relative to catalytic groups;
2) the act of chemical transformation of the substrate induced by electrophilic and nucleophilic agents.

The analysis shows that these two processes are carried out by two components of the active site, structurally and functionally different. These components may conditionally be presented as sorptional (complex forming) and catalytic sub-sites of the active site.

The idea about two-site origin of the active sites of enzymes is fundamental for the theory explaining the ability of enzymes to "select" molecules of higher reactive substrates [1, 5].

A complex between enzyme and the substrate is formed due to relatively weak but kinetically quickly manifesting forces (hydrophobic and electrostatic interactions, hydrogen bonds). These interactions are relatively quick so that in most cases formation of a complex may be considered as the nonequilibrium process.

Let the active site A – C of an enzyme consists of two functionally different zones: catalytic (C) and sorptional (A). Two zones may also be outlined in the substrate structure: reactive (X) which is subject to chemical transformation and chemical inert (R) capable of forming a complex with sorptional zone of the active site. Specific substrate may be presented as $X - R_s$ and nonspecific as $X - R_n$. The difference between R_s and R_n zones is that the former is capable of effective interaction with sorptional zone A of the enzyme, whereas structural features of R_n zone do not allow it for such interactions.

Let us consider enzyme reaction with specific $X - R_s$ and nonspecific $X - R_n$ substrates in the framework of absolute reaction rates.

For specific substrate, the enzyme–substrate complex and the transition state may be presented as follows:

$$\begin{array}{c}C\\|\\A\end{array} + \begin{array}{c}X\\|\\R_s\end{array} \underset{}{\overset{\Delta G_s}{\rightleftharpoons}} \begin{array}{c}C\\|\\A\cdots\end{array}\begin{array}{c}X\\|\\R_s\end{array} \overset{\Delta G_X^{\neq}}{\rightleftharpoons} \left[\begin{array}{c}C\cdots X\\|\quad\;\;|\\A\cdots R_s\end{array}\right]^{\neq} \longrightarrow \text{Products}$$

As the equilibrium process, complex formation is described by the rate constant K_s and free energy $\Delta G_s < 0$. Chemical reaction passes through a transition state, preceded with the equilibrium formation of $A\cdots R_s$ bond.

For nonspecific substrate, the activation process may be presented by the following scheme, showing no interaction between R_n and the sorptional site.

$$\begin{array}{c}C\\|\\A\end{array} + \begin{array}{c}X\\|\\R_n\end{array} \overset{\Delta G_X^{\neq}}{\rightleftharpoons} \left[\begin{array}{c}C\cdots X\\|\quad\;\;|\\A\quad R_n\end{array}\right]^{\neq} \longrightarrow \text{Products}$$

If we accept that the interaction between catalytic (C) and reactive (X) zone of the substrate proceeds identically for both cases, then, in the first case, structural feature of the transition state represents formation of additional

A····R_s bond. This must lead to stabilization of the mentioned transition state and, of course, to decrease of free activation energy of the catalytic process. For two cases under consideration, energy levels of the system states are shown in Figure 6. As suggested, the bond structure in the transition state C····X is the same for both reaction paths. As a consequence, both cases of activated complex formation show identical activation free energies $\Delta G_S^{\neq}$. The activation barrier of the reaction at transition from the initial substrate to the activation complex is independent of the reaction path.

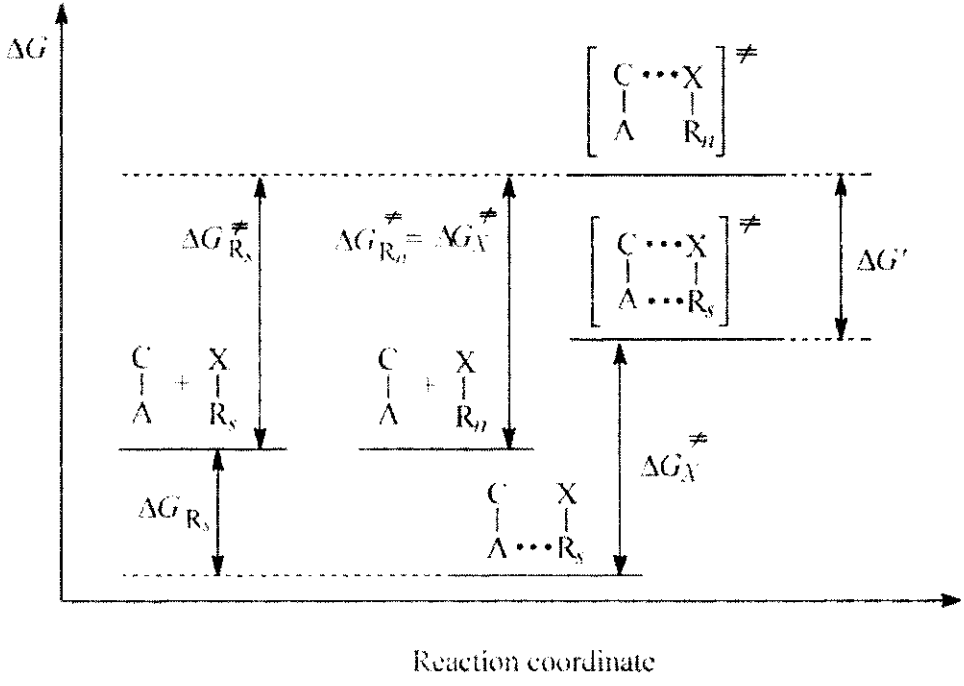


Figure 6. The energy diagram of a system participating in enzymatic reaction with specific and nonspecific substrate

Then for specific substrate, we get:

$$\Delta G_{R_S}^{\neq} = \Delta G_X^{\neq} - \Delta G_{R_S};$$

for nonspecific substrate:

$$\Delta G^{\neq}_{R_n} = \Delta G^{\neq}_{X}.$$

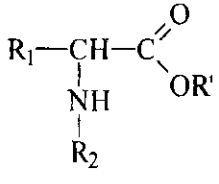
In the case of specific substrate compared with nonspecific one, a decrease of activation free energy equals:

$$\Delta G' = \Delta G^{\neq}_{R_s} - \Delta G^{\neq}_{R_n} = \Delta G_{R_s},$$

i.e. for the specific substrate the activation threshold is lower by sorption free energy of nonreactive part of the substrate in the substrate–enzyme complex.

Therefore, simple kinetic–thermodynamic consideration of enzymatic reaction in the framework of the two-site model forecasts that there should be a correlation between the substrate ability to form an intramolecular complex with the sorptional sub-site of the active site and its reactivity. This forecasting was made for several enzymes and then strictly confirmed in the experiments. The fullest picture was obtained for α-chymotrypsin. For this enzyme, reactivity of substrates of various structures was studied at both acyl-enzyme formation and deacidylation stages.

Similar to many enzymes, many model substrates for α-chymotrypsin are known. In particular, kinetics of N-acidylated L-amino acid ester hydrolysis has been studied:



where R_1 is the side group of amino acid; R_2 is N–acyl substituent; R′ is the removable group substituent. The enzyme selects substrate using interaction of L–amino acid side group and N–acyl substituent R_2 with sorption groups of a sub-site of the active site.

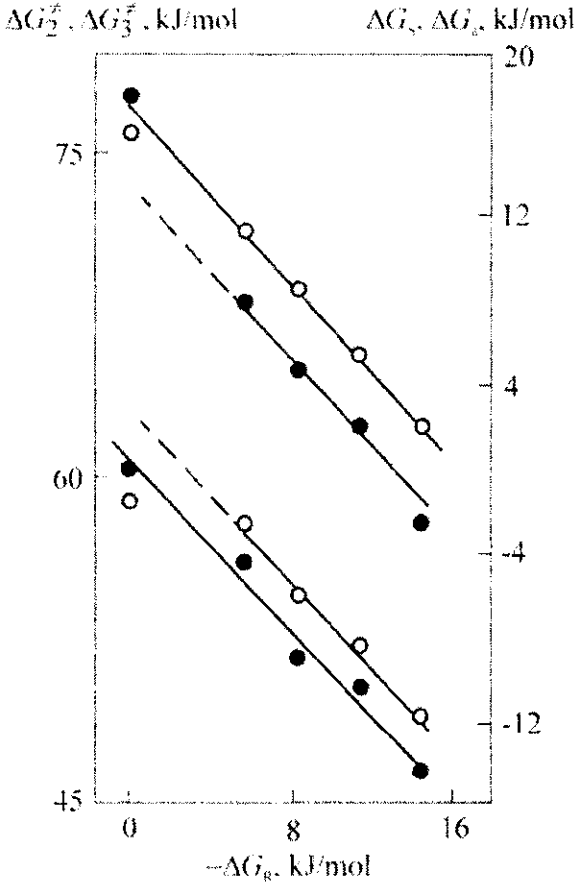


Figure 7. The variation of free energy at formation of the enzyme–substrate complex ΔG_s (1), acyl enzyme ΔG_a (2), activation free energy for catalytic stages of acidylation $\Delta G_2^{\neq}$ (3), and deacidylation ΔG_3 (4) for the reaction with α–chymotrypsin in relation to hydrophobicity of R_1 radical in various compounds of aliphatic or aromatic origin

In accordance with the two-site model on the mentioned sequence of α-chymotrypsin substrates, reactive enzyme substrate is sorbed by "recognizing" participant of the active site via hydrophobic interactions and due to decrease of the activation threshold of the reaction. The kinetic mechanism of enzymatic reaction includes the equilibrium stage of the complex formation (K_s), Ser-195 acidylation stage (k_2) and acyl enzyme hydrolysis (k_3).

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES \xrightarrow{k_2} EA \xrightarrow{k_3} E + P.$$

The study of dependence of radical R_1 hydrophoby on the substrate reactivity for all reaction stages (Figure 7) shows increasing sorption of the substrate with the substituent hydrophoby, as well as the increase of its acidylating ability (k_2) and reactivity of acyl-enzyme in deacidylation reaction (k_3). Tangents of dependencies shown in the Figure approach one [15, 16, 53].

Therefore, α-chymotrypsin represents the ideal example of hydrophobic sorption of the substrate enzyme for reduction of the activation threshold of the chemical stage of the process.

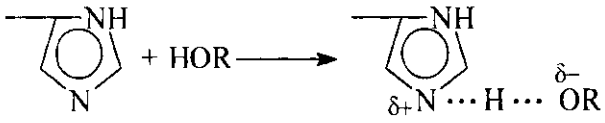
To "fix" the substrate in active site of the enzyme, the whole set of weak but "quick" interactions is used: hydrogen bond, ionic and dipole interactions, hydrophobic "extraction" of the substrate enzyme associated with thermodynamically favorable water displacement. For enzymes with three-dimensional structure may quite strictly and reliably identify interaction groups of the substrate and the enzyme. There are several examples discussed in some reference [5]. It should be noted that the change in specificity e.g. ability to recognize the "native" substrate is often related to relatively small structural change of the sorptional site. Hence, the transition from selectivity by hydrophobic amino acids (phenylalanine, tyrosine, tryptophane, leucine) to positively charged amino acids (lysine, arginine) is related to substitution of Ser-189 by aspartic acid. This provides for proteinase translation from chymotrypsin to trypsin sequence.

Reactivity increase. Aspartic acid and histidines are the agents most frequently used in the processes of molecule activation in enzymatic catalysis (refer to Figure 2 above). It is obvious that catalytic function of aspartic (glutamic) acid and histidine in active sites of enzymes is related to their acid-base properties.

In the catalysis, induced by enzymes, acids and bases participating in active sites play very important role due to their activation of substrates. Meanwhile, as two substrates participate in the reaction, reaction sites of both substrates may be activated.

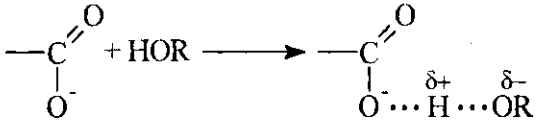
Several activation schemes may be presented [5].

1. Imidazole group acts as a nucleophilic agent:



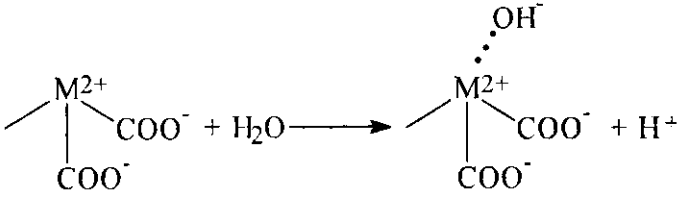
Negative charge $\delta-$ is induced on the reaction site.

2. Carboxylic group acts as a nucleophilic agent:

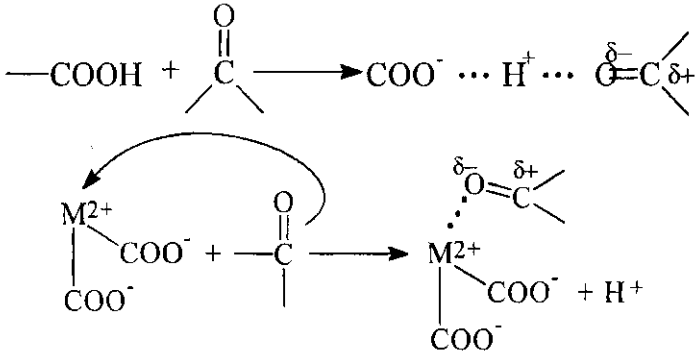


Metal ions (Zn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Mn^{2+}) present in active sites of enzymes in complexes with carboxylic groups of aspartic or glutamic acid may participate as electrophilic agents in the catalytic cycle.

3. Electrophilic activation of water by metal ion M^{2+} via substitution of proton in water molecule and formation of reactive and mobile hydroxyl ion:



4. Electrophilic activation of the reactive site may be performed both under the effect of carboxylic group (the proton donor) or metal ion as follows:

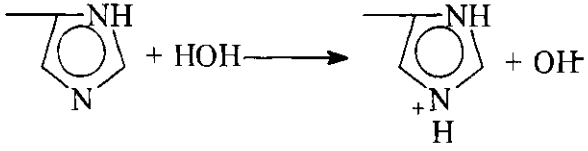


This reaction produces deficient electron density on the reaction site.

The above-shown activation schemes for molecules with the help of acids and bases provide for the greater part of mechanisms proceeding in a catalytic sub-site of the active sites. Frequently, water molecules may be replaced by different particles. However, in the most cases, the catalytic act includes nucleophilic–electrophilic activation of molecules.

The above-shown activation mechanisms of the reagents are based on classical statements of organic chemistry, developed on I. Broenstedt ideas. In the transition state, reactive sites accumulate excessive positive ($\delta+$) or negative ($\delta-$) charge. The shift of electron density induced by nucleophilic or electrophilic agent depends on acid or base strength.

For limited cases of hydrolytic reactions, the stage of water activation at nucleophilic catalysis may be presented as follows:



$$—COO^- + HOH \rightarrow —COOH + OH^-$$

The water reactivity at its dissociation with formation of hydroxyl ions increases by 10^7 times. If one imagines that electrophilic activation of the reaction site also increases reactivity of the active site by 10^6 – 10^7 times,

therefore, in the first approximation high efficiency of enzymatic catalysis and its ability to $10^{12} - 10^{13}$ time increase of the reaction rate can be explained.

The investigation of the charge transfer in the transition state of enzymatic reactions is not complete yet. It is not clear, if a high dipole moment is induced in water molecule or the reactive particle is represented by solvated and relatively stabilized hydroxyl ion. However, it is obvious that activation is carried out on the reaction sites of definitely oriented and fixed molecules in relation to one another. This provides for high acceleration of the reaction.

Thus, acids and bases participating in active sites of enzymes represent the main motive force providing for activation of substrates and high reaction rates.

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ENZYMES AND THEIR REGULATORS IN MODERN DRUGS

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By the middle of 20th century, the laws of chemical physics and kinetics have found their application in medicine. Academician Nikolai M. Emanuel (1915 – 1984) and the Corresponding Member Ilya V. Berezin (1923 – 1987) were in the first cohort of scientists who headed the researches in this direction. Their deaths have not stopped the initiated works.

Breakdown of any enzymatic system supporting vital activity of the organism induces pathological state. Normalization of the health status depends on successful actions of biologists, chemists and technologists in their work on the problems put forward by medical doctors. To-date, the success of protein preparations as drugs provides for solving the main problems:

1. Development of methods for extraction and purification of proteins;
2. Creation of methods for their stabilization and processing to compounds suitable for operation in the organism;
3. Development of drug targeting methods;
4. Increase of efficiency and reduction of secondary effects by development of prolonged drugs.

The work is started with searching for preparation sources, their extraction and maximum possible purification using methods, which may be transformed to a technological process.

We followed this way in our laboratory, when creating drug substances for protein proteinase inhibitors, which promote the repair of disorders induced by proteolytic disbalance in the organism. These disorders are shocks of various etiologies, ischemias, inflammatory states caused by infections, burns and other traumas.

This phenomenon is known to occur in response to exhaustion of damaged organism ability to eliminate the problem by, as now proved, synthesis of additional quantities of inhibitors.

The abundance of natural protein proteinase inhibitors is explained by the necessity to protect proteins of the organisms against proteolytic attack and control the elimination rate of denatured proteins. Catalytic activity is controlled by reversible enzyme–inhibitor interaction:

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftarrows}} EI$$

This process is used in vivo more frequently than others: the reaction reversibility makes it polyfunctional, because the equilibrium may be shifted in the presence of compounds which are bound with either enzyme or inhibitor, i.e. both components can enter other interactions [1].

The association reaction rate obeys the equation of the second order:

$$[v_i] = k_i[E]\cdot[I]$$

To be effective, the inhibitor must bind target proteinase quickly and practically irreversibly in doses (about 10^{-6} M) i.e. high enough. As a consequence, the protein preparation must be highly purified.

The studies of the research group headed by Prof. N.I. Larionova were devoted to development of protein proteinase inhibitors preparations highly effective in vivo, which could be the basis of novel methods of patient treatment. A process schemes for preparation of protein inhibitors for medical purposes from bovine pancreas and soybean flour were elaborated [2]. Both proteins were 100% pure, which was confirmed by the amino acid analysis. We were the first who measured both inhibition constants and association rate constants for basic pancreatic trypsin inhibitor and Bowman-Birk soybean inhibitor with proteinases [3, 4]. Both inhibitors have low molecular weight (6.5 and 8.2 kDa, respectively) and are quickly excreted from the organism by kidneys. This seriously decelerated their application in medicine because of high expense of the inhibitors.

Chemical modification and immobilization of these proteins on biocompatible carriers resulted in increase of mean residence time in circulation (Figure 1) and targeting to desirable organs and tissues (Figure 2) [5, 6].

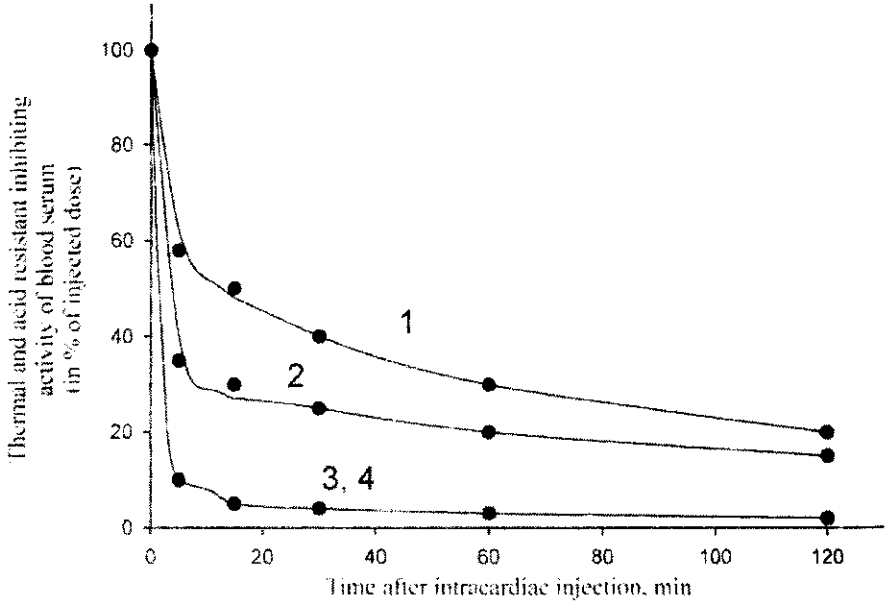


Figure 1. Elimination of drugs containing trypsin pancreatic inhibitor from blood stream of rats. Dose: 10,000 KIE per 1 kg of body mass.
1 – native inhibitor; 2 – lactose-modified inhibitor; 3 – carboxymethyl dextran–modified inhibitor; 4 – inhibitor modified by high molecular D-galactosamine derivative

Our investigations in the field of new forms of protein delivery, resulted in the novel method of micro- and nano-particles preparation by consecutive adsorption of oppositely charged biocompatible polymers on formed protein aggregate. Such method is also prospective for new peroral insulin shapes. Figure 3 indicates [7] the possibility of producing hormone preparations, which leave the carrier at different rates in the required area of intestine without any loss of physiological activity.

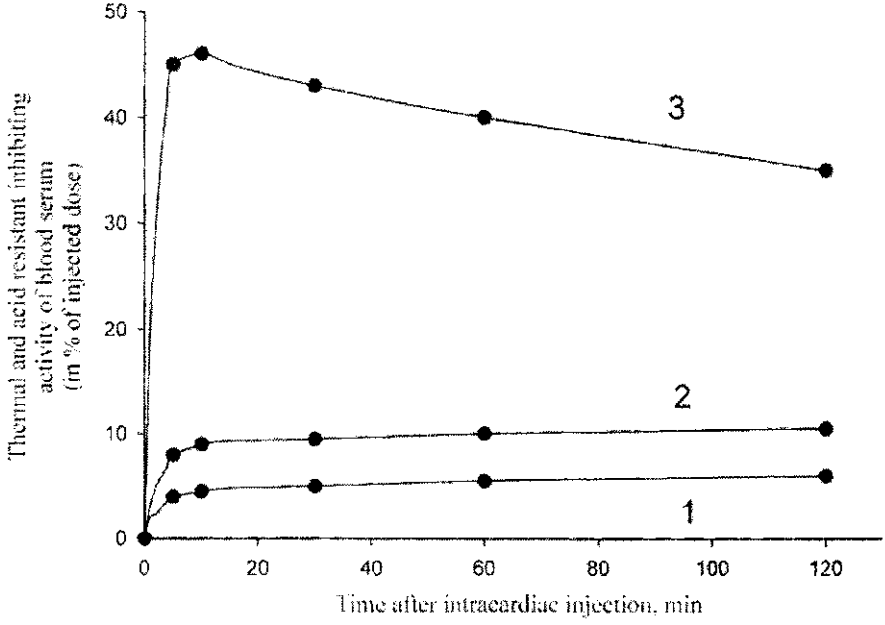


Figure 2. Accumulation of drugs containing trypsin pancreatic inhibitor in liver of rats:
1 – native inhibitor; 2 – carboxymethyl dextrane–modified inhibitor; 3 – inhibitor modified by high molecular D-galactosamine derivative

The development of ophthalmic drugs for the treatment of eye diseases is of special interest. One of disadvantages of aqueous solutions used in eye drops is the limited time of the drug contact with cornea and conjunctiva of the eye globe, because over 80% of the preparation is washed off with tears. Therefore, treatment of eye diseases often requires either increased concentration of the preparation (which is sometimes limited by allergenic potency of the active substance) or increased number of instillations that deteriorates the patient's life. Recently, we have suggested a perspective method for solving this problem by creating a non-traumatic gel carrier capable of phase transition at physiological temperatures. Thermosensitive polymers in

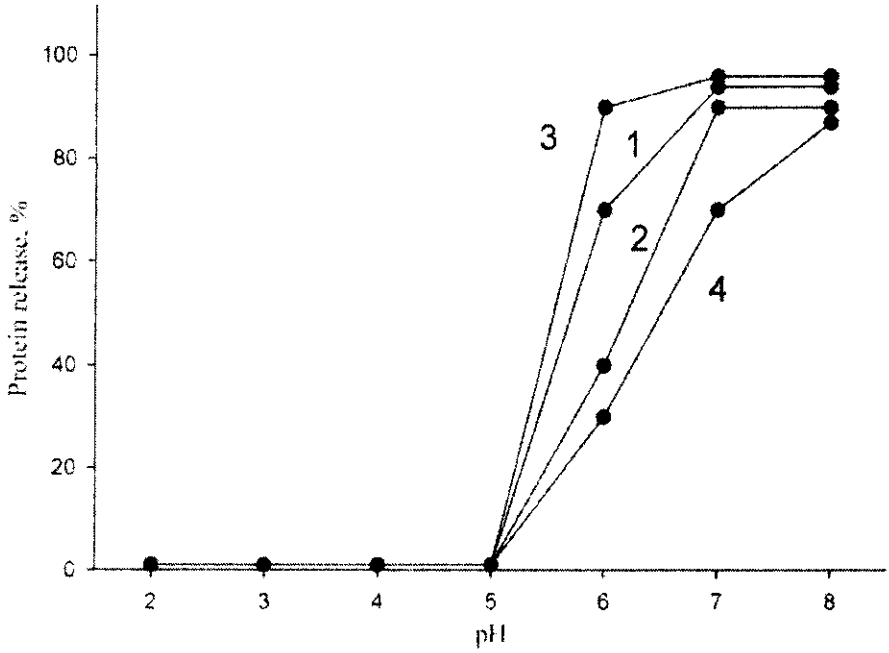


Figure 3. Influence of pH on protein release from microparticles with insulin with one (1, 2) and three (3, 4) cycles of polyelectrolyte sorption

aqueous solutions have the so-called lower critical solution temperature (LCST). It is the critical temperature at which polymer is subjected to reversible phase transition accompanied by a change of hydrophilic–hydrophobic balance of polymeric chains: below LCST the polymer displays hydrophilic properties, e.g. is water-soluble, whereas above LCST is becomes hydrophobic, e.g. water-insoluble. We used poly-N-isopropylacrylamide gel (poly-NIPAA) which LCT equals 32°C. This temperature falls within temperature range between room temperature (~20°C) and human body temperature (37°C). This fact allows a suggestion that thermosensitive poly-NIPAA suspension will display relatively good sorption to the eye surface, and the drug solution preliminarily included into the gel will contact with cornea for relatively long time.

We studied physiological effect of proxodolol (hybrid $\beta_{1\text{-}2}$–α_1 adrenoceptor blocking agent) included into thermosensitive gel carrier on

intraocular pressure in comparison with the effect of proxodolol applied as eye drops.

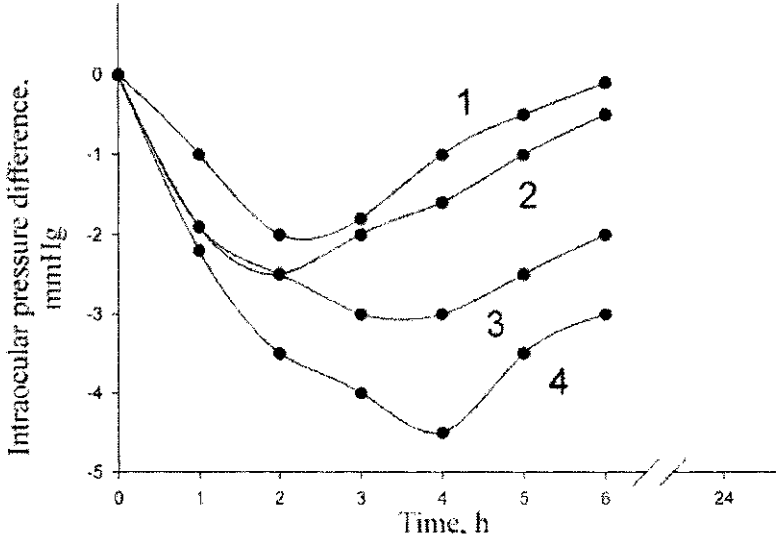


Figure 4. Comparative influence of aqueous solutions of proxodolol and proxodolol suspension in poly-NIPAA gel on intraocular pressure in intact rabbit eyes.
1 – 0.5% proxodolol; 2 – 1% proxodolol; 3 – poly–NIPAA + 0.5% proxodolol; 4 – poly–NIPAA + 1% proxodolol

The results indicate the difference between traditionally applied and suggested drug form of proxodolol expressed in the value and duration of hypotensive effect. Figure 4 shows that poly-NIPAA suspension can be used as the carrier of ophthalmologic drug. It will significantly increase time of its action (hypotensive effect) and decrease proxodolol concentration in the active agent [8].

Enzymes are wider applied as drugs, because they catalyze with high specificity and catalytic efficiency a broad variety of biochemical reactions important for good health of the organism.

One of the most important fields of enzyme application is therapy of cardiovascular diseases. In developed countries these diseases give 36 – 39% of lethal outcomes in total mortality of the population. Acute myocardial infarction (AMI) is the greatest contributor into this index. AMI and insult therapy requires intravenous injection of agents activating plasminogen to plasmin, which then dissolves thrombus fibrin. Thrombolytic therapy applies such plasminogen activators as streptokinase (SK), two-chain urokinase type plasminogen activator (tcu-PA), single-chain urokinase type plasminogen activator (scu-PA), tissue plasminogen activator (t-PA) and acylated plasminogen–streptokinase complex (APSAC). The plasminogen activators have different mechanisms, catalytic efficiency and fibrin-specificity. The general disadvantage of these agents is their quick clearance from the blood flow ($\tau_{1/2}$ = 3 - 18 min). Therefore, therapeutic effect is reached by long-term infusion of high doses of these expensive agents to the blood flow that induces activation of systemic plasminogen. Plasmin formed in plasma may degrade fibrinogen, exhaust α_2-antiplasmin and effect on some factors of the coagulation system in plasma that increases risks of such complications as hemorrhages and rethromboses. The absence of an ideal thrombolytic agent requires optimization of risk/benefit and price/benefit ratios. To solve the urgent problem of increasing the thrombolysis efficiency and reduction of side effects scientists in the group headed by R.B. Aisina, the leading scientist, PhD, apply the following approaches:

1) investigation of plasminogen activators from new sources;
2) modification of the known agents;
3) optimization of therapy regime.

The first approach includes study of new thrombolytic agent properties – recombinant staphylokinase (STA). Similar to SK, this agent represents protein of bacterial origin, possesses no enzymatic activity, but forms an equimolar complex with endogenous plasminogen (Pg). However, contrary to Pg–SK complex which is capable of activating excessive plasminogen, Pg–STA complex become an activator only after transformation to plasmin(Pm)–STA complex. The comparison of kinetics of plasma clot lysis immersed in human plasma by Pg–STA and Pm–STA complexes (Figure 5a) and the dependence of thrombolysis rate on the agent concentration (Figure 5b) indicated that STA is much more effective thrombolytic agents rather than SK.

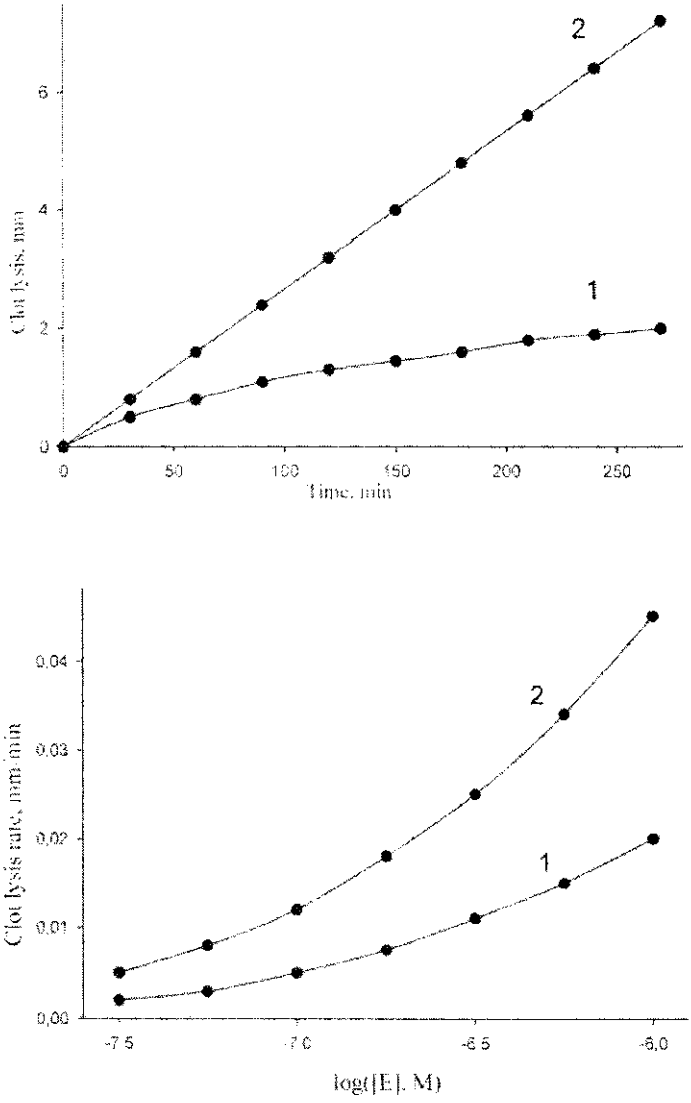


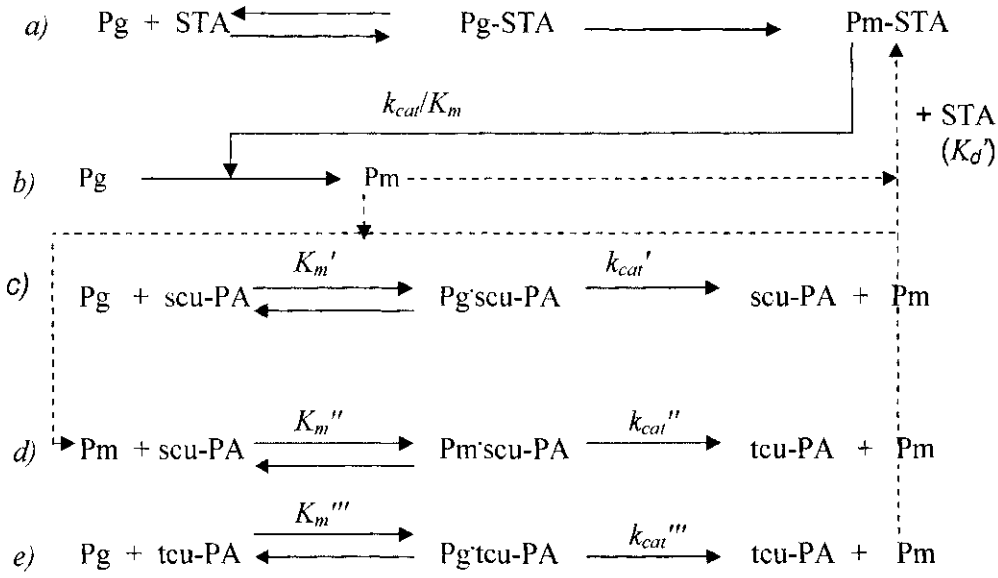
Figure 5. a) Kinetics of plasma clots submerged to human plasma (37°C) lysis by equimolar Pg–SK (1) and Pm–STA (2) complexes
b) Dependence of thrombolysis rate on Pg–SK (1) and Pm–STA (2) concentration

A detailed study of stability of two complexes was carried out and the mechanism explaining these differences was suggested [9].

In the second approach to the studies the authors applied modification methods to some plasminogen activators used in clinics. Methods of reversible acylation of active sites of Pg–SK and tcu–PA complexes by various low molecular weight titrants of proteases were designed. The properties of obtained acyl-derivatives (Acyl–Pg–SK's and Acyl–tcu–PA's), reactivating at different rates, were studied. It was shown that Acyl–Pg–SK's and Acyl–tcu–PA's in the presence of plasma inhibitors, from the action of which their active sites are protected by acyl-groups, possesses several advantages over free SK and tcu–PA. As the reactivation rate decreased, duration and degree of thrombolysis by acyl-activators and their stability in plasma increased, and level of their side effects in plasma surrounding the plasma clot was reduced [10, 11]. Diffusion of SK, tcu-PA and their acyl-derivatives into fibrin and plasma clots was studied [12, 13]. As indicated, Acyl–Pg–SK's, which are bound to fibrin due to lysine-binding sites of plasminogen, support a long-term surface lysis of the plasma clot during reactivation, whereas Acyl–tcu–PA's having no affinity to fibrin penetrate deep inside the plasma clot and, slowly deacylating in it, proceed with prolonged thrombolysis from inside.

Irreversible chemical modification of arginine residues in inhibitor-binding cluster of scu–PA was also implemented. It was shown that this modification increases stability of scu–PA in plasma and its thrombolytic efficiency [14].

To improve therapy regime, in the third approach thrombolytic action of various STA and scu–PA combinations was studied, and synergism of their actions in a narrow range of combinations of their doses ([STA] < 30nM and [scu–PA] < 75 nM) was determined. For example, as concentrations of 15 nM of both agents were combined, a 3.8-fold increase in thrombolysis in 4 h was observed comparing with the expected total effect of the same doses of individual agents (Figure 6). Side effects of synergic combinations were lower than expected total effects of doses of individual agents, used in combinations. A mechanism explaining synergism of action of these agents is suggested (see the Scheme below). Trace initial concentrations of Pm–STA formed from Pg–STA initiate reactions (a) and (b):



Plasmin (Pm) formed in the reaction (b) splits fibrin that leads to formation of increasing COOH–terminal lysines on its surface. These lysines represent sorption sites for Pg molecules, which are the preferable substrate for scu–PA. Thus, STA accelerates plasminogen activation by scu–PA (c). Plasmin also converts one-chain scu–PA to two-chain tcu–PA (d), which is more effective activator of plasminogen (e) rather than scu–PA. As a result of reactions (b – e), plasmin concentration sharply increases that has a positive inverse effect. Plasmin binding to free STA molecules in plasma induces increase in Pm–STA concentration. This complementary action of two activators significantly increases the rate of fibrinolysis. Detected synergism of STA and scu–PA actions allows for reduction of their total therapeutic dose for 3 – 4-fold and, consequently, side effects and expenses for therapy.

In the majority of cases, heterogeneous form of biocatalysts complicates kinetics of substrate or inhibitor interaction with the active site of enzyme due to diffusion hindrances induced by the carrier. The reaction rate in the particle of heterogeneous bioctalyst depends on the enzyme concentration, particle diameter, pore size and penetrability of its cover, for example, microcapsule cover. Transport of reagents to the particle or from it may have the rate lower than the rate of enzymatic reaction inside, but usually a relation between mass transfer and enzymatic reaction rate is not considered as a

particular transformation. We have discovered this phenomenon on particular example of microcapsulated α–chymotrypsin [16]. The analysis showed full conformity to classical regularities used in chemical macrokinetics.

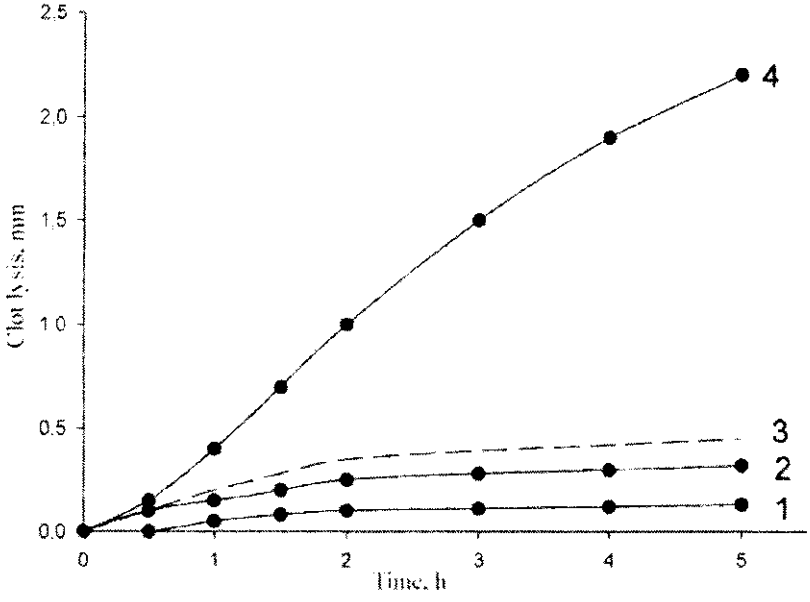


Figure 6. Kinetics of plasma clots lysis affected by 15 nM scn–PA (1) or 15 nM STA (2) and their combination (4). Dashed line (3) shows their expected total effect

A group of scientists headed by O.A. Kost, the leading scientist, PhD, is working on creation of a fundamental basis for designing inhibitors of angiotensin–transforming enzyme (ATE) – the drugs of the new age.

Angiotensin–transforming enzyme inhibitors (ATEI) become more and more important for clinics. Due to high efficiency, good tolerance and ability to prevent development of changes in organs, ATEI become the leading compounds in the sequence of hypotension substances. The main ATE function is in C-end dipeptide detachment from angiotensin I and angiotensin II formation – one of the most powerful pressor compounds in the organism.

Moreover, the target for ATEI is kallikrein–kinin system, where the role of ATE is in degrading bradykinin having a vasodilatory action. For example, one of new perspective directions is ATEI application as supporter at cancer chemotherapy, because Ac–Ser–Asp–Lys–Pro (the hemopoesis factor) is one more ATE physiological substrate.

Modern data indicate the existence of local tissue factors in various organs, and the action of these factors is provided by the renin-angiotensin system (RAS). For example, in tissues and fluids of eye many components of RAS are detected that may by significant for pathogenesis of eye diseases. Angiotensin-converting enzyme (ACE) is the key enzyme of RAS, the activity of which is controlled by the inhibitors of angiotensin-converting enzyme (ACEI).

Broadening the spectrum of pathological states for which application of ACE inhibitors can be recommended demandes urgent development of new effective drugs, based on ACEI.

Besides β-blockers, ACEI became the agent of choice for patients who postponed myocardial infarction. Nephroprotector effect of ACEI and correction of metabolic disturbances are of special importance in case of insulin–resistance syndrome, therefore, they became the first choice drugs for patients with arterial hypertension accompanied by pancreatic diabetes and obesity. ACEI is also applied to patients with diabetic nephropathy, including patients with normal arterial blood pressure. Absolutely unexpected reports also occur which indicate possible reduction of cancer frequency and prevention of Alzheimer's disease upon ACEI therapy.

The unique feature of ACE is the presence of two catalytically active sites within single polypeptide chain of enzyme molecule. These sites hydrolyze the same group of peptide substrates; however, quantitative characteristics of these sites are different. Physiologically valuable substrates may preferably be hydrolyzed on one or another ACE active site that, in turn, brings up the task of selective suppression of one or another active site, which are usually assumed as independent ones.

Our recent investigations of bovine ACE mechanism in the hydrolysis of peptide substrates of different structures indicate that, generally, active sites in the enzyme cannot be considered to be independent. It is found that the hydrolysis of "*long*" nona– and decapeptides (for example, bradykinin and angiotensin I) really proceeds independently on the two active sites of ACE, i.e. both active sites can simultaneously bind and hydrolyze the substrate. However, in the hydrolysis of shorter substrates (tri-pentapeptides) active sites

of the enzyme demonstrate strong negative cooperativity. In this case, binding of one molecule of the substrate with any ACE active site suppressed binding of the second molecule with the second active site of the enzyme [17, 18].

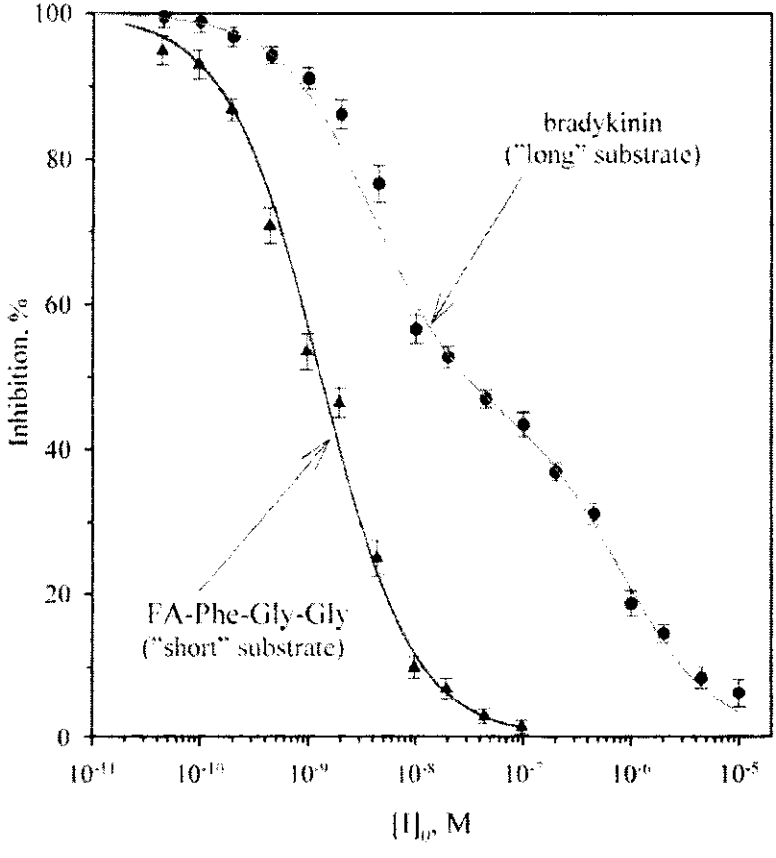


Figure 7. Inhibition of two-domain ACE activity by captopryl

Similar phenomenon is observed for inhibitor binding, i.e. the ability to bind one or two inhibitor molecules on the two-domain ACE molecule is defined by the ligand structure. Moreover, binding of a "*short*" inhibitor (for example, captopril or lisinopril used in hypertension therapy) on one of ACE active site makes the second active site incapable of binding and hydrolyzing

"*short*" tripeptide substrate, but allows it for binding and hydrolyzing the "*long*" substrate (Figure 7).

The data obtained allow a conclusion that modern screening technique for new inhibitors based the estimation of their efficiency in suppressing enzymatic activity towards "*short*" synthetic substrates is incorrect and gives only incomplete idea about potential efficiency of inhibitor as a drug.

Among eye pathologies, inflammatory processes are the most widespread. Their etiology is extremely variable and includes various traumas (burns, mechanical trauma, surgical operation consequences, etc.). Proteolytic enzymes play an important role at all stages of inflammation, participating in degradation of structural proteins and limited proteolysis of biologically active compounds that define the type of inflammation process. Therefore, targeted activity adjustment of these enzymes during inflammation is of importance for preserving structural organization of the eye tissues and, consequently, vision itself.

The department team in cooperation with experts from Helmholtz Moscow Research Institute of Eye Diseases have for the first time determined a correlation between ACE activity in tear fluid, which metabolism reflects proceeding of biochemical processes in the eye, biochemical parameters (trypsin-like proteinase and antitrypsin activity, α_2–macroglobulin concentration) in a tear, and clinical picture of disease at eye burn trauma of different localizations [19, 20].

Positive influence of local instillations of ACEI, used for treatment of hypertension and cardiovascular disease, on clinical proceeding of posttraumatic period was demonstrated. The development of deep ulcers of cornea was highly suppressed (by 50%), edemas were reduced, ischemia and eye tissue necrosis development in test animals were diminished or even prevented (Figure 8).

The data obtained allowed us to suggest a new drug derived from ACE inhibitors, designed for local application in therapy of eye pathologies associated with microcirculation disorders and inflammation [21, 22].

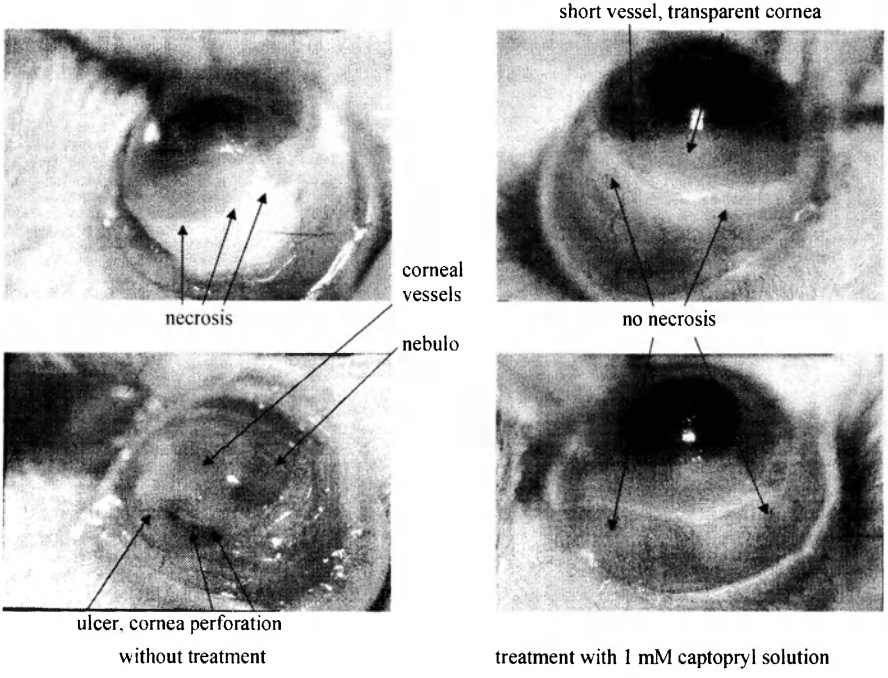


Figure 8. Influence of ACE inhibitors on microcirculation in conjuctiva of the 15th day after trauma

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STRUCTURE AND FUNCTIONS OF FIREFLY LUCIFERASE

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Bioluminescence is light emission from the living organisms based on chemiluminescent reaction, catalyzed by specific enzyme – luciferase. Bioluminescence originates from oxidation of an organic substrate called luciferin in the presence of enzyme luciferase. Luciferin and luciferase are collection-functional rather than structure-functional types that indicate substrates and enzymes, interacting with one other and emitting light, simultaneously. The emission of light energy in chemical and biological processes is observed rather frequently, especially in the reactions with free radicals, however, in the most cases, the quantum yield (the number of light quanta emitted on the oxidation of one molecule of a substrate) is not higher than tenth or thousandth percent. The distinctive feature and the great advantage of bioluminescent systems is the high quantum yield (from 1 to 100%), which is explained by the participation of special proteins and enzymes in the reaction. On the one hand, luciferases act as a matrix, which is covalently or non-covalently bound to emitting chromophore, and, on the other hand, they play the role of biocatalysts that makes possible formation of electron–excited product. In most cases, the exclusively specific organization of protein structures in bioluminescent systems makes strict demands on the structure of luciferase substrates. Apparently, this is necessary for the maximum concentration of the enzymatic reaction energy on chromophore and formation of the electron–excited reaction product, which then returns to the basic state with the emission of the quantum of visible light.

The luciferases have been isolated from a large number of organisms (insects, fish, bacteria, jellyfish, protozoa, etc) and characterized. All of them are oxidation-reduction enzymes. Air oxygen or hydrogen peroxide acts as an oxidizing agent. The structures of oxidizing substrates, luciferins, are rather different. In the most cases, luciferins are heterocyclic compounds. The products of their oxidation have rather low excitation energy and this is indeed the reason for the energy of enzymatic reaction appears to be enough for electron–excited product formation. Therefore, it may be concluded that

luciferin-luciferase systems are unique, high-performance energy converters in biochemical reactions to light, and the interest in the study of structures and functions of luciferases and elucidation of their biological role in nature does not diminish.

The interest to bioluminescent systems is also arisen due to their practical importance. For a series of luciferases, such important metabolites as flavin mononucleotide (FMN) or adenosine triphosphate (ATP) are necessary participants (co-substrates) of the enzymatic reaction. High quantum yield of bioluminescence in the luciferase-catalyzed reactions simplifies the light registration procedure. Due to practically absolute specificity of luciferases to the substrate structure, they are widely used as highly efficient analytical reagents. The intensity of light emitted in the course of the reaction (bioluminescence intensity) is proportional to the rate of electron–excited reaction product formation and, hence, proportional to the concentration of substrate provided for lower than K_m the substrate concentration. Since every molecule of the substrate entering the reaction gives from 0.3 to 1 quantum of light, the sensitivity of bioluminescent methods of substrate detection is 10^{-13}–10^{-18} moles of substrates in test solution that is by many orders of magnitude higher than sensitivity of other detection methods. In addition, the construction of instruments for light measuring (luminometers) is much easier than that of spectrophotometers or spectrofluorimeters also simplifying practical application of bioluminescent systems.

The present review presents the analysis of physicochemical regularities for the functioning of the most important bioluminescent system, which id bioluminescent system of fireflies, with *Luciola mingrelica* firefly luciferase as an example. This enzyme was for the first time isolated in our laboratory more than 20 years ago, and our investigations allowed for obtaining new interesting data on the structure and functions of firefly luciferases.

THE SCHEME OF LUCIFERASE-CATALYZED REACTION

Firefly luciferase [luciferin 4-monooxygenase (ATP hydrolysis); luciferin: oxygen 4-oxidoreductase (decarboxylation, ATP hydrolysis), EC 1.13.12.7] catalyzes firefly luciferin oxidation by air oxygen in the presence of MgATP [1, 2]. The reaction is accompanied by emission of visible light with

the quantum yield about 90% [3]. As shown in Figure 1, the enzyme at the first step binds its substrates luciferin (1) and ATP.

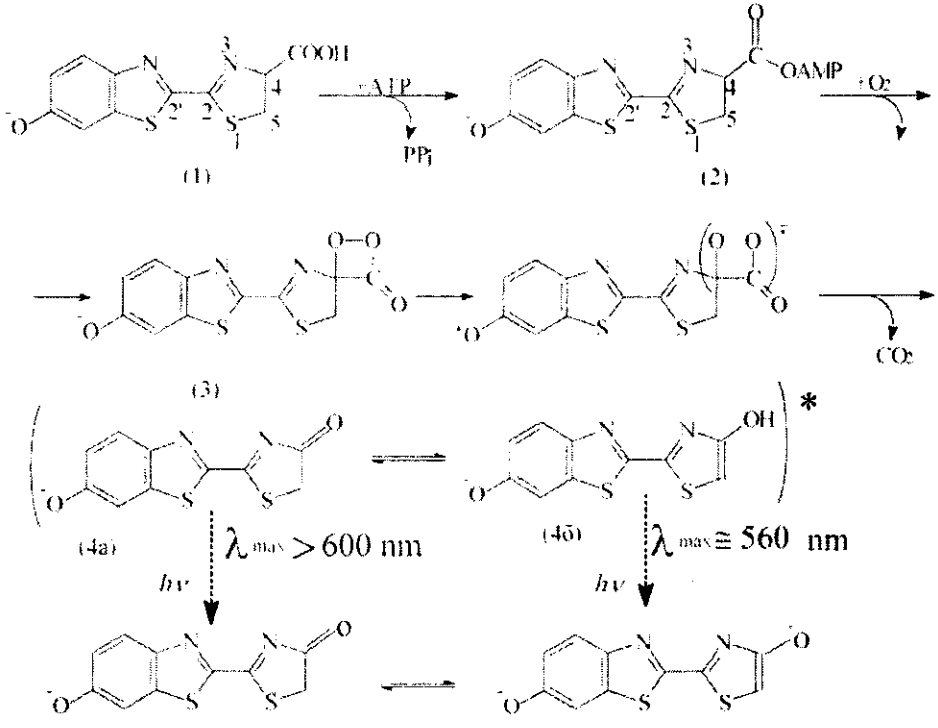


Figure 1. The reaction scheme with firefly luciferase as the catalyst [2]

After the triple complex formation, the luciferin interacts covalently with ATP to form mixed anhydride of carboxylic and phosphoric acids, luciferyl adenylate (2) and pyrophosphate (PP_i). Luciferyl adenylate is oxidized by air oxygen to form cyclic peroxide, dioxytanone (3), through a series of intermediate steps. The dioxytanone molecule has a remarkable feature: one portion of the molecule is a readily oxidizable heterocyclic structure (luciferyl) with low ionizing potential and another portion (peroxide) has a high to affinity to electron. Due to the intramolecular electron transfer from the phenolate group to the peroxide, a resonance charge-transfer structure is formed. The break of O-O bond causes decarboxylation of dioxytanone and biradical (ketone anion-radical and phenolate cation-radical) formation. As a result of intramolecular recombination of the radicals, the reaction product

oxyluciferin (4) in a singlet electron-excited state is formed. Depending on the properties of microenvironment, oxyluciferin may exist in the form of ketone (4a), enol (4b), or enolate-anion. The electron-excited oxyluciferin deactivates with the emission of quantum of light with λ_{max} from 540 to 612 nm depending on the firefly species and pH.

STRUCTURE OF FIREFLY LUCIFERASE

By the middle 1980ies, the studies were carried out with natural firefly luciferases, which were isolated from desiccated firefly lanterns. As a rule, the composition of enzyme preparations was not homogeneous due to the presence of different modified forms of enzyme, which, probably, appeared during the functioning of the enzyme. For this reason, for a long time even primary amino acid sequence of luciferases remained unknown. A new phase began in 1985, when cDNA of the American *Photinus pyralis* firefly luciferase was isolated [4]. In 1987, the amino acid sequence of this luciferase was determined [5], and in 1989 four luciferases from Jamaican click beetles were cloned [6]. In the early 1990ies, luciferase from the East-European (North Caucasian) *Luciola mingrelica* fireflies was cloned and homogeneous reagent of recombinant *L. mingrelica* luciferase was produced [7]. At present, the primary structures of more than 20 luciferases isolated form different species of fireflies, beetles, and their glowworms inhabiting in the USA, Russia, Japan, Latin America, etc. are known. The chemical scheme of the reaction catalyzed by these enzymes and the structure of the emitter are identical for all firefly luciferases. Luciferase molecules consisting of one polypeptide chain (542 – 552 residues) do not contain cofactors and have similar amino acid composition. More than a half of amino acids are nonpolar or ambivalent. The number of charged residues is also practically identical for all firefly luciferases and the main difference between them is in the number of Trp and Cys residues. The amino acid sequence homology for different luciferases corresponds to the relationships based on their biological classification. For the *Luciola* fireflies, the homology is about 80% and starting from the 200th residue, it exceeds 90%. The structure of *L. mingrelica* luciferase is the most similar to Japanese *Hotaria parvula* luciferase (98% homology). The *Luciola* luciferases have less homology with luciferase of American fireflies *Photinus pyralis* (67%) and luciferases from

click beetles (43%) [8]. At the same time, the homology between luciferases isolated from Jamaican and Brazil beetles reaches 80% [9].

Firefly luciferase belongs to a superfamily of enzymes catalyzing formation of acyladenylates from ATP and compounds with carboxylic group. This superfamily includes families of peptide synthetases, CoA-ligases, *etc.* Searching for conserved motifs in the amino acid sequences of proteins within this superfamily has been carried out using computerized analysis [10]. In addition to previously known motif 1 (residues 197 – 210 in *L. mingrelica* luciferase), another conserved motif 2 (residues 410 – 460) has been found. It was proposed that the conserved amino acid residues in motifs 1 and 2 implemented the key functions in catalysis, and both motifs belonged to a single conserved structural element. It was also proposed that the 197 – 220 residue range formed an ATP binding site and the 410 – 460 area was important for the interaction between luciferase and its activator coenzyme A [10].

In 1996, the spatial structure of luciferase isolated from American fireflies *Photinus pyralis* was determined by X-ray diffraction analysis [11]. This provided for a new stage in the studies of firefly luciferases. The X-ray data confirmed the above proposal about the important role of motifs 1 and 2. Both motifs represent loops with undefined electron density indicating high mobility of these fragments and the motif 2 is partly incorporated in polypeptide connecting two domains of the protein globule. As shown below, the arrangement of domains is sufficient for catalysis.

SPATIAL STRUCTURE OF LUCIFERASE AND ITS COMPLEXES WITH SUBSTRATES

The spatial structure of luciferase unbound to a complex with substrates was determined from X-ray diffraction data; therefore, one may only assume the possible binding sites for luciferin and ATP [11]. In 1997, a group of scientists (UK) published some data on the crystal structure of another enzyme belonging to the superfamily of adenylating proteins [12]. In this case, they managed to obtain crystals for the complex of the enzyme with its substrates AMP and phenylalanine (an analogue for the complex of enzyme with the adenylation product). As one may expect, spatial structures of adenylase and

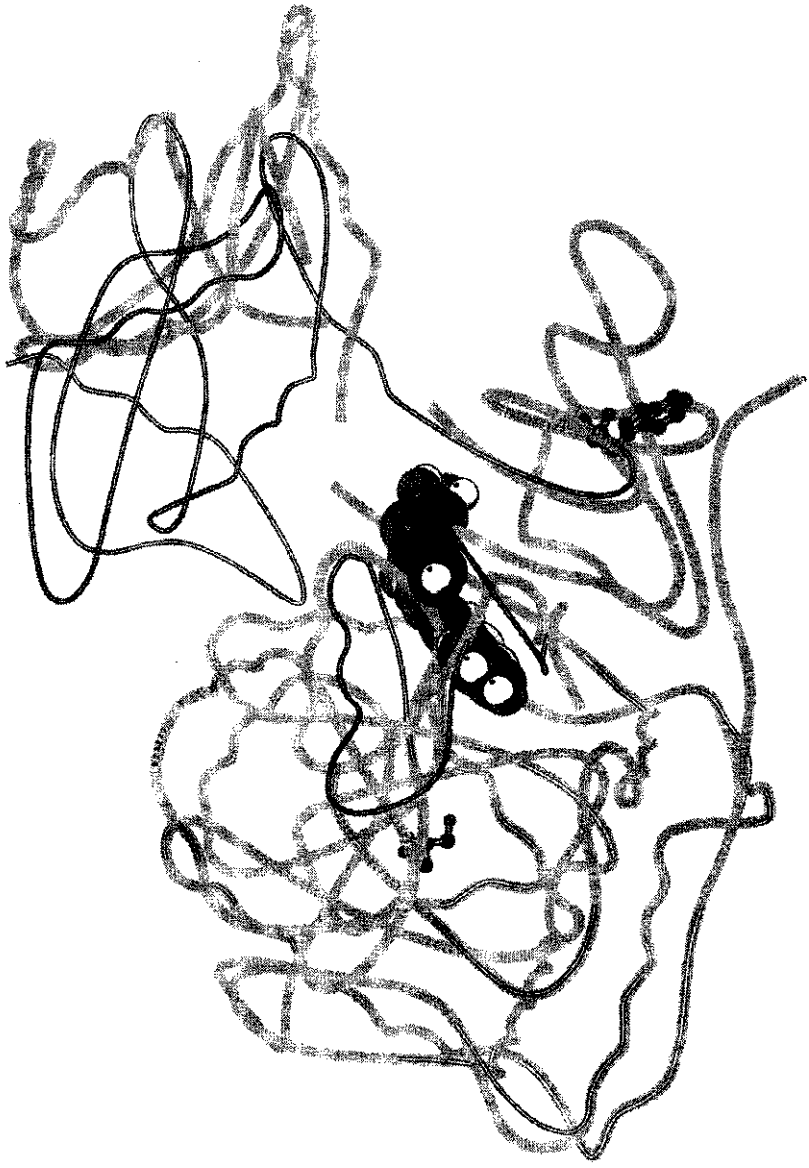


Figure 2. Spatial structure of firefly luciferase without substrates (gray ribbon) and the luciferase-luciferin-ATP complex (black ribbon). The substrates are shown as CPK models [13]

luciferase, which both possessed equal ability to adenylate carboxyl group and ATP substrate, appeared to be rather similar, although the amino acid sequence homology of these enzymes was low. Both enzymes are composed of two domains, large N-domain, which, in turn, may be divided into three sub-domains, and a small C-domain. Two domains are connected to a very flexible and disordered loop. Each domain and even sub-domain of adenylase has similar topology with the corresponding domain or sub-domain of the luciferase. The only significant difference is mutual rearrangement of N- and C-domains. The adenylase domains are drawn together and rotated by 90° with respect to their orientation in the firefly luciferase [12]. We proposed that the observed rotation of domains relative to one another is a consequence of a change in the globule conformation upon binding of the substrates. In this case, the three-dimensional structure of the luciferase-luciferin-ATP complex should be similar to that of the adenylase-phenyl alanine-AMP complex. Computerized simulation based on this hypothesis allowed us to compose a model of an enzyme–substrate complex for *Photinus pyralis* luciferase [13]. The structures of the luciferase before and after binding of the substrates are shown in Figure 2. Neither ATP, nor luciferin is able to bind the enzyme without rotation of the domains. Catalytically important residues of C-domain (Lys529 and Thr527) turn out to be near the substrate molecules only after the domain rotation. The computerized analysis has shown that the spatial structures of *P. pyralis* and *L. mingrelica* luciferases are practically indistinguishable, although they are 67% homologues. All the residues directly contacting the substrates are invariant.

The ATP molecule is bound to luciferase so that the adenine moiety is buried in the protein and «*tightly packed*» between residues 315 – 318 and Ile434 (Figure 3). Adenine amino group forms a hydrogen bond with oxygen atoms in Gly339 and the side chain of Gln338. The existence of multiple contacts between ATP and the protein explains the absolute luciferase specificity to ATP. For example, etheno-ATP, the fluorescent analogue of ATP, is unable to bind luciferase [14]. Ribose hydroxyl groups of ATP are bound to Asp422 and Tyr340 residues by hydrogen bonds and phosphate groups are bound to Ser198, Thr343, and Glu344 via Mg ion. In addition to the residues composing the N-domain, there are at least two residues (Thr527 and Lys529) from highly conservative portion of the C-domains belonging to the active site. Thr527 may form hydrogen bonds with β– and γ–phosphates in ATP, and Lys529 is located at equal distance from carboxyl group of luciferin

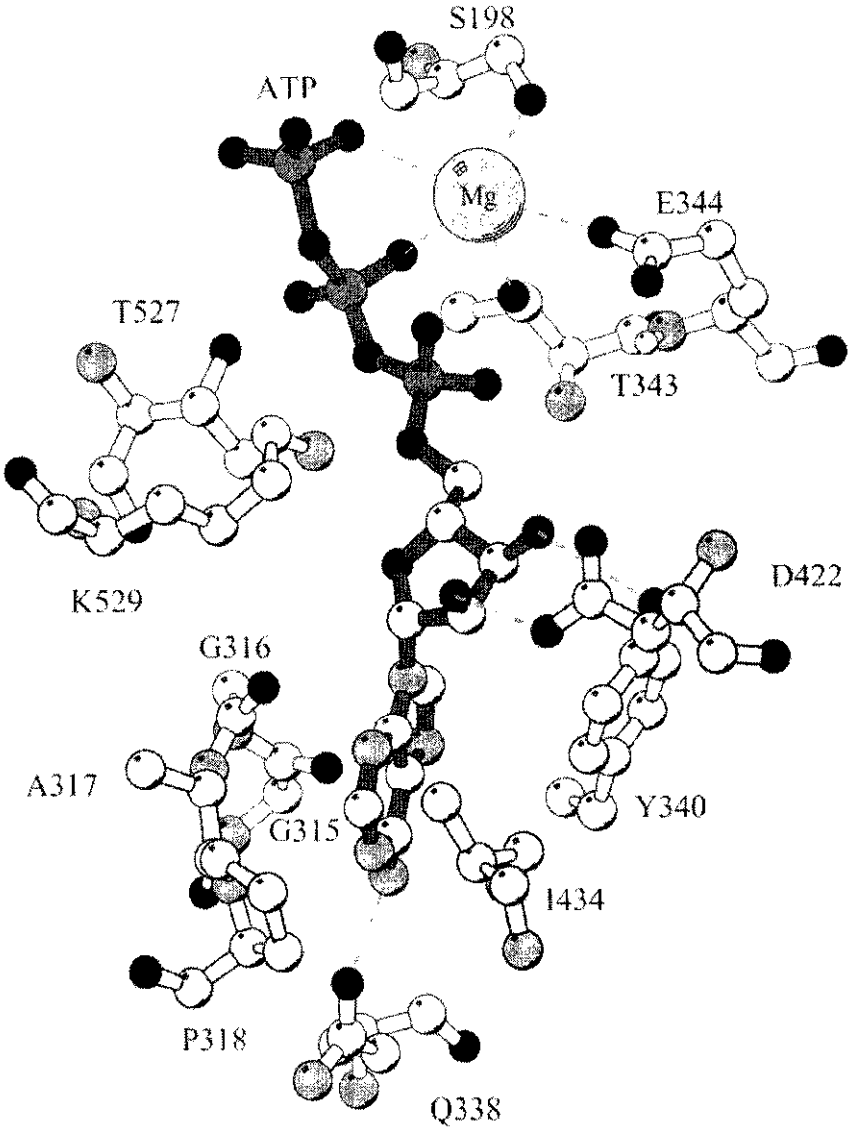


Figure 3. ATP-binding site of firefly luciferase. ATP is colored dark gray. Dotted lines show hydrogen bonds between ATP and protein, and bonds formed by Mg^{2+} [13]

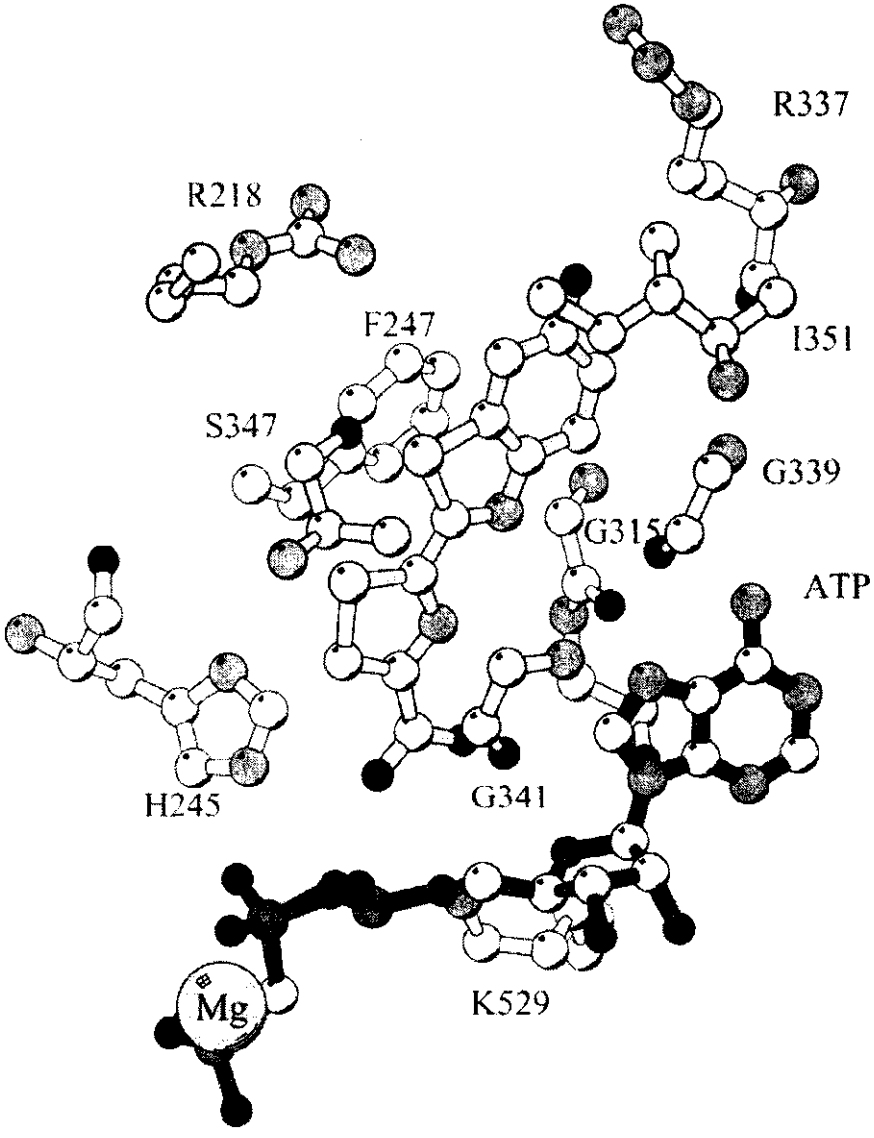


Figure 4. Luciferin-binding site in firefly luciferase. The luciferin molecule is colored light gray, and ATP molecule – dark gray [13]

and α–phosphate group of ATP. The latter residue favors in the approaching of phosphate and carboxylic groups of two luciferase substrates at the rate-limiting stage of luciferyl adenylate formation. This hypothesis was then confirmed by independent studies: the residual activity of a luciferase mutant, in which Lys529 was replaced by Ala, was below 0.1% [15].

Luciferin molecule is buried in the protein globule and the ribose-phosphate moiety of ATP shifts luciferin from the solvent (Figure 4). Nevertheless, the luciferin environment contains both hydrophobic and hydrophilic residues. The phenyl cycle of Phe247 is almost parallel to the aromatic ring of luciferin and, together with Ala313, Ala348 and Ile351 residues, forms the hydrophobic surrounding of the latter. The side chains of Arg218 and Arg337 shield phenyl group of luciferin from the solvent. The imidazole group of His245 is transversal to the luciferin cycle and, probably, plays an important role in binding and oxidizing luciferin. The luciferin-binding site contains a great number of glycine residues (228, 246, 315, 316, 339) and atoms of polypeptide backbone. The absence of bulk side residues provides tight fixation of luciferin molecule at the active site. The presence of a many hydrophilic groups at luciferin explains luciferin and oxyluciferin fluorescence in different solvents and complexes with luciferase: spectra of a complex with luciferase are most close to spectra in aqueous solutions rather than to spectra of organic solvents [16, 17].

A CORRELATION BETWEEN BIOLUMINESCENCE SPECTRA AND THE STRUCTURE OF FIREFLY LUCIFERASE

As mentioned above, chemical scheme of the reaction catalyzed by firefly luciferase and structure of the emitter are identical for all known insect luciferases. The main difference is in color of the originating bioluminescence. Fireflies *in vivo* emit yellow-green light (540 – 580 nm), bioluminescence of click beetles falls within the range from green to orange, and bioluminescence of Brazilian beetle glowworms varies from green to red (536 – 638 nm). For the reaction *in vitro*, the observed color of bioluminescence coincides with color *in vivo* at neutral pH, but shifts to red with pH decrease in the reaction mixture [8]. For a series of mutant luciferases, red shifts are observed at neutral pH, too. Thus, the main factor that determines bioluminescence color is properties of microenvironment of the emitter, which is located in the enzyme

active site. Recently, a correlation between the luciferase structure and bioluminescence spectrum parameters became the subject of continuous scientific discussions [8]. To explain variations of the bioluminescence λ_{max} for the natural and mutant luciferases, several mechanisms were proposed in the literature. According to the prevalent mechanism, changes in the bioluminescence spectra, for example, at pH variations, result from the keto-enol tautomerization of oxyluciferin [18].

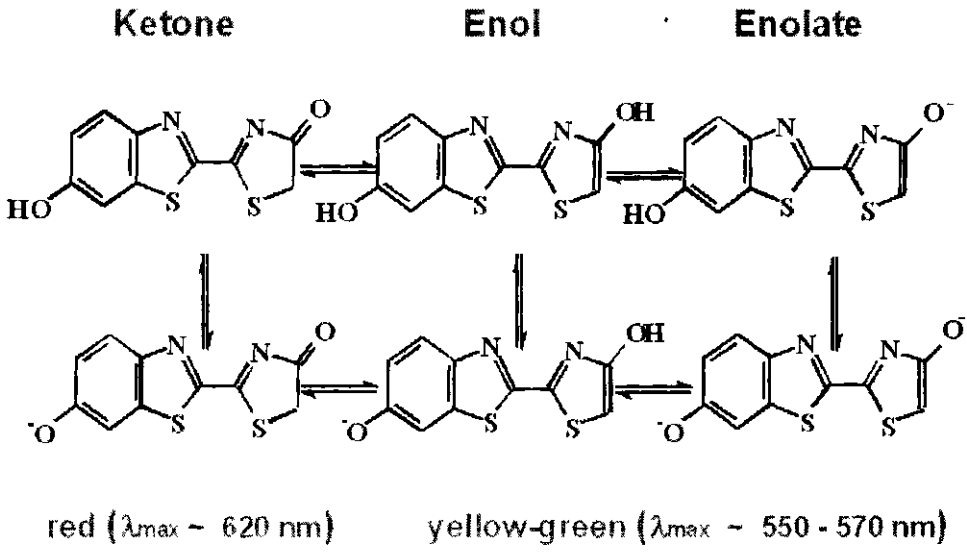


Figure 5. Different shapes of electron-excited oxyluciferin: ketone, enol, and enolate-ion [19].

As shown in Figure 5, C_5 proton of the thiazole cycle forms a hydrogen bond with the side residue of amino acid (B_1) acting as a base and removes C_5 proton. Oxygen atom of ketone group interacts with protonated base (B_2) and accepts a proton from the base. Thus, the keto-enol tautomerization is a synchronous transition of two protons: one proton from C_5 atom of oxyluciferin to B_1 base and another proton from B_2 base to oxygen atom of the carbonyl group. The tautomerization results in the enol formation. The efficiency of this process depends on correctness of B_1 and B_2 fixation in the proximity of the oxyluciferin thiazole cycle. The interaction between enolic hydroxyl group and B_3 base results in formation of enolate ion. If B_1 base (independently of pH) is absent or protonated (for example, at pH < 6.0), the

keto-form of oxyluciferin possesses bioluminescence maximum in the red area of the spectrum. At intermediate pH, both spectral components, ketone and enol, are observed in the bioluminescence spectrum. All authors were generally interested in location of bioluminescence maximum and had not analyzed changes in the spectrum shape. Moreover, it was tacitly assumed that the emission spectra of the enol and the enolate-ion are practically identical [8].

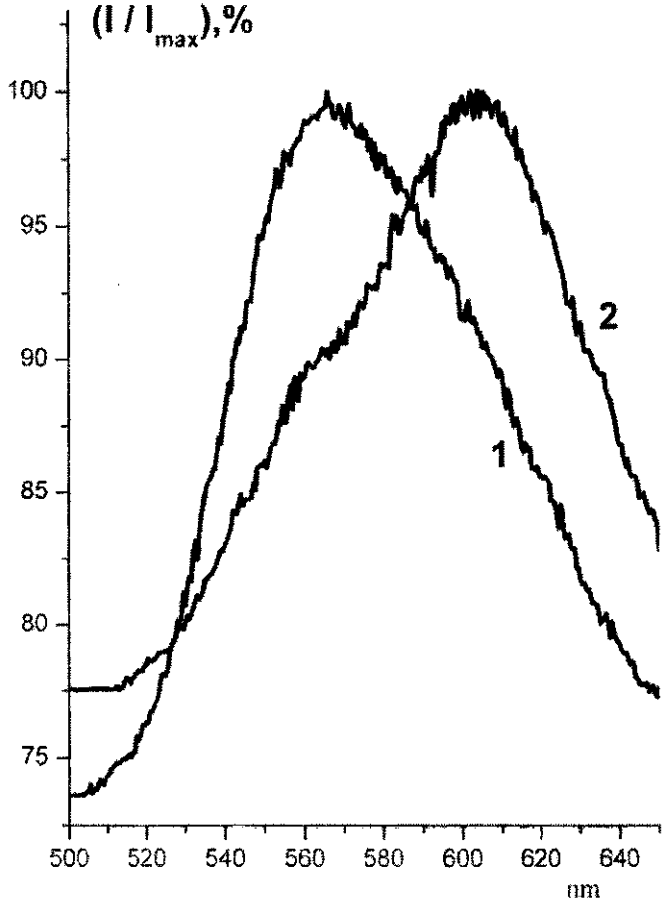


Figure 6. Bioluminescence spectra of the wild type (*1*) and mutant luciferase (*2*) from *L. mingrelica* fireflies at pH 7.8. Intensities are normalized by maximum [19]

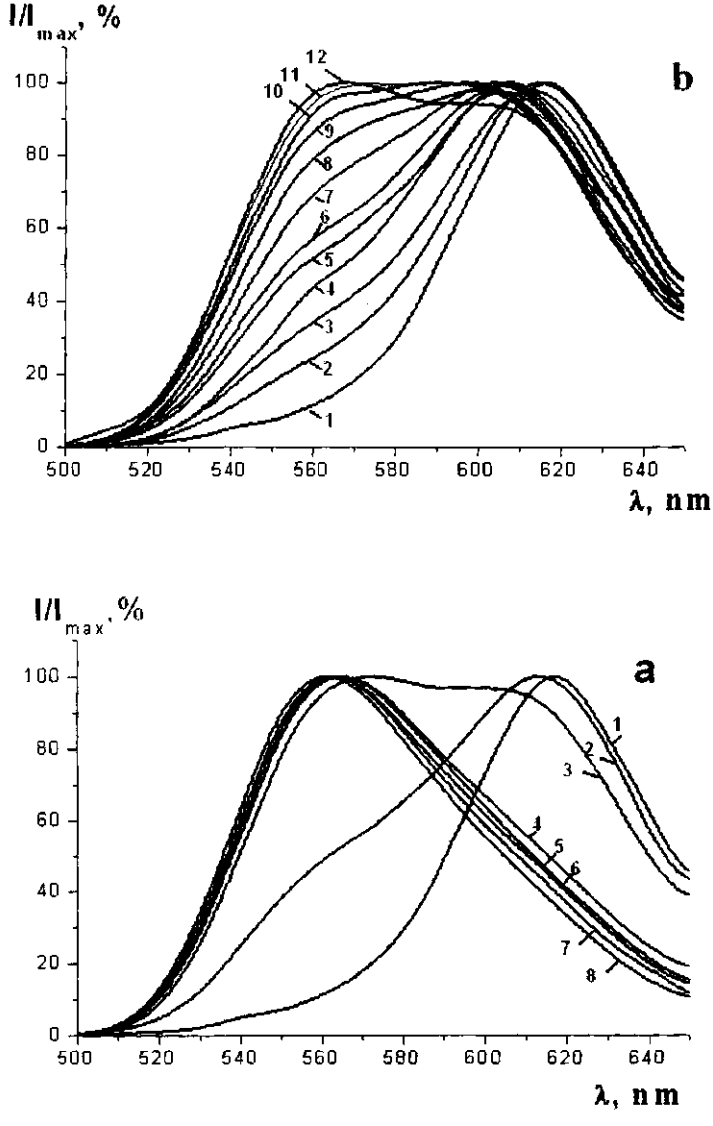


Figure 7. Bioluminescence spectra of the wild-type (a) and mutant (b) *L. mingrelica* luciferase at different pH:
(a) *1* – 5.6; *2* – 6.4; *3* – 6.8; *4* – 7.0; *5* – 7.6; *6* – 7.8; *7* – 8.0; *8* – 8.5;
(b) *1* – 5.6; *2* – 6.1; *3* – 6.4; *4* – 7.2; *5* – 7.6; *6* – 7.8; *7* – 8.0; *8* –8.6; *9* – 8.9; *10* – 9.2; *11* – 9.6; *12* – 10.2. In each spectrum, the intensities are normalized by maximum [19]

The studies of pH-dependence of bioluminescence spectra of the wild-type recombinant *Luciola mingrelica* firefly luciferase with bioluminescence (λ_{max} = 566 nm) and its mutant form with the His433Tyr point mutation (bioluminescence λ_{max} = 608 nm) and quantitative estimation of contribution of different oxyluciferin forms into bioluminescence spectra allowed for elucidating the role of the protein in observed differences in bioluminescence spectra of the wild-type and mutant luciferases [19]. The His433Tyr mutant retains high catalytic activity since the His433 residue is located at the distance of 12 Å from the enzyme active site, and its mutation cannot have strong effect on the catalytic properties of the enzyme. Mutations of the amino acid residues in the luciferase active site described in the literature [20], resulted in changes in bioluminescence spectra. At the same time, they decreased the luciferase catalytic activity by orders or tens of orders of magnitude that impeded the comparative analysis of the spectral characteristics of wild type and mutant enzymes.

At pH 7.8 (pH optimum for the natural enzyme activity), bioluminescence λ_{max} equals 566 nm for the wild-type luciferase and 606 nm for the mutant luciferase (Figure 6). Thus, the single His433Tyr point mutation resulted in the λ_{max} shift by 40 nm to red. For the wild-type luciferase, bioluminescence spectrum in the pH range within 5.6 – 10.2 is yellow-green (pH ≥ 7.0) and red (pH = 5.6). Both shapes are present at intermediate pH (Figure 7a). For mutant luciferase, red bioluminescence is observed at pH ≤ 6.1. A threshold in the yellow-green area appears with increasing pH, and bioluminescence intensity in it also increases pH. The yellow-green bioluminescence dominates over the red one at pH ~10.2 only (Figure 7b). The comparison of pH dependences of bioluminescence spectra shows that the shift of λ_{max} for mutant luciferase at optimal pH of the catalytic activity (pH 7.8) is explained by changes in the ratio between the different forms of oxyluciferin.

The bioluminescence spectra at different pH were so far considered as a superposition of spectra of two oxyluciferin forms, ketone and enol, with λ_{max} of bioluminescence at 550 – 570 nm and 618 nm, respectively [8, 16]. If fact, the observed spectra are a superposition of the three forms of electronically exited oxyluciferin rather than the two ones, namely, enolate-ion (λ_{max} = 556 nm), enol (λ_{max} = 587 nm), and ketone (λ_{max} = 618 nm). Gauss multi-peak fitting for decomposition of spectra allowed us to determine the relative content of each spectral form at different pH. The relative content of the ketone, enol, and enolate-ion for the wild type and mutant luciferases varies with changes of pH due to shift in the ketone ↔ enol ↔ enolate equilibrium. A

change in the relative content of different emitter forms with pH variation results in the changes of both bioluminescence maximum and form of the bioluminescence spectrum [19].

THE MECHANISM OF CHANGES IN BIOLUMINESCENCE SPECTRA UPON MUTATIONS

To elucidate the role of amino acid residues of the enzyme active site in the keto-enol tautomerization, a computer model of the structure of the triple luciferase-oxyluciferin-AMP complex was constructed (Figure 8) on the basis of a minimized structure of the luciferase-luciferin-ATP complex [13].

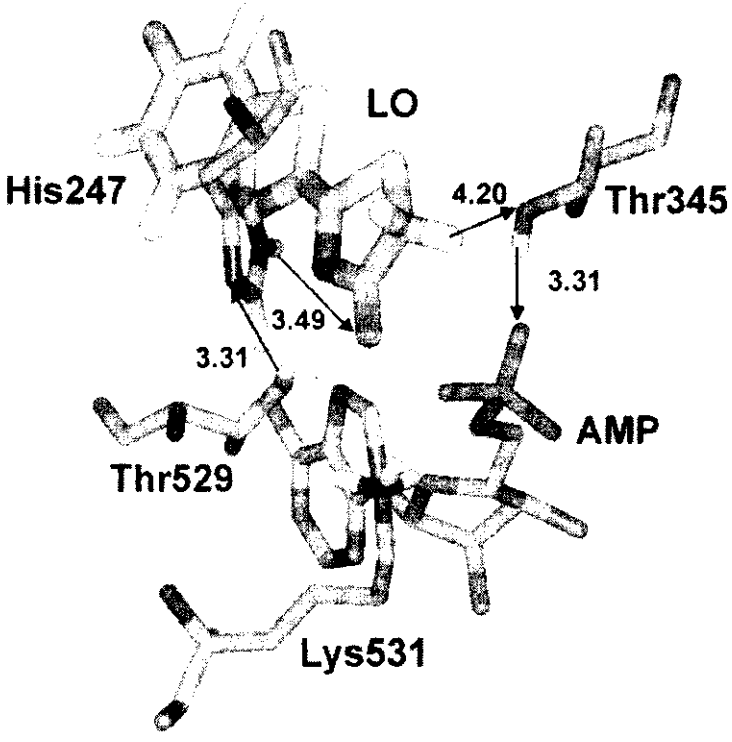


Figure 8. Computerized model of the luciferase-oxyluciferin-AMP complex for *L. mingrelica* firefly luciferase [19]

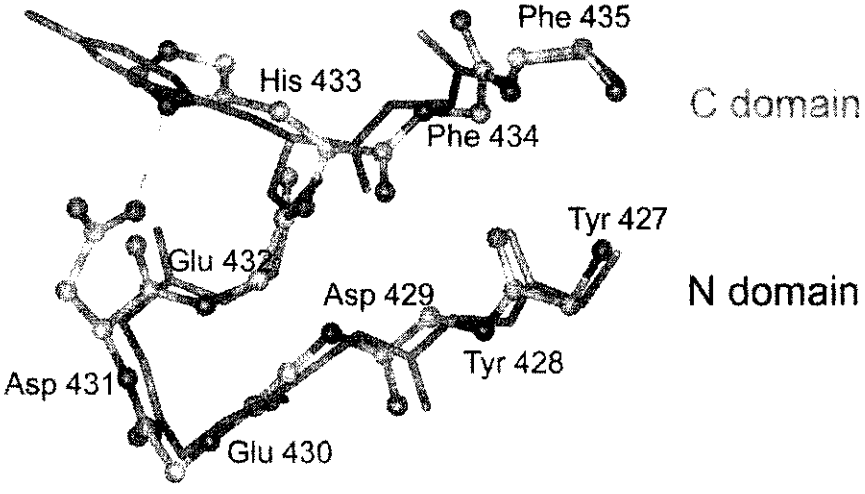


Figure 9. Computerized model of residue His433 microenvironment structure (Tyr427 – Phe435) in the wild-type (ball-and-rod model) and mutant (rod model) *L. mingrelica* firefly luciferase. Dotted line shows hydrogen bond between His433 and Asp431 residues of the wild-type luciferase [19]

In this complex, amino acid residues of *Luciola mingrelica* firefly luciferase His247, Thr345, and Lys53, which are absolutely conserved in all luciferases, are located at the distance of 5 Å or shorter from the thiazole ring of oxyluciferin. Oxygen atom of the Thr345 hydroxyl group is located at C_5 proton of oxyluciferin, whereas the imidazol ring of His247 is located at oxygen atom of ketone group. Apparently, these two groups are directly involved in the keto-enolic tautomerization process. This supposition is in good agreement with data from the literature on mutagenesis of these residues in *P. pyralis* firefly luciferase [20]. A mutant with Thr343Ala replacement (according to numeration for *P. pyralis* luciferase) had bioluminescence maximum in the red spectrum (λ_{max} = 617 nm) independently of pH, whereas His245Arg, Phe, Ala, Gln, and Asn replacements resulted in significant broadening of bioluminescence spectra and the change of their pH dependencies. His247 and Thr345 residues form hydrogen bonds with Thr529 and Lys531 residues of luciferase C domain that are also necessary for the tautomerization process. Thus, the change in localization of key amino acid residues and some loosening of the protein structure in the vicinity of thiazole ring of oxyluciferin result in the shift in the equilibrium towards enol and ketone.

There a question is brought up: how could point mutation (His433Tyr) of a residue located by 12 Å far from the active site affect bioluminescence spectra? The comparison of computer models of the environment structure of His433 residue (Figure 9) for the wild type and mutant luciferases reveals an essential change of His433 residue conformation. Tyr phenyl ring in mutant luciferase is rotated by 60° relative to His imidazol ring location in the wild-type luciferase, where His433 residue is exposed to protonated solvent and forms a hydrogen bond with Asp431 residue [13]. Upon His-Tyr replacement, hydrogen bond is broken, and Tyr phenyl ring is rotated by 60° in conformity with the local force balance. The analysis of X-ray diffraction data for luciferases and other adenylating enzymes shows that highly conserved His433 residue is located in a flexible loop formed by Tyr427 – Phe435 residues binding N- and C-domains of luciferase [11, 12]. This loop can be considered as a hinge binding two luciferase domains. The imidazol ring of His433 residue forms hydrogen bond with the carboxyl group of Asp431 residue, that increases rigidity of a "*hinge*", decreases thermal fluctuation amplitudes of N- and C-domains with respect to one another, and sufficiently ensures rigid fixation of Thr529 and Lys531 amino acid residues of the C-domain, which are involved into the nearest environment of oxyluciferin thiazol group. Upon the

His433Tyr replacement, the rigidity of Tyr427 – Phe435 "*hinge*" decreases, which results in the increase of thermal fluctuation amplitude of the domains with respect to one another. For this reason, the emitter microenvironment becomes looser that impedes the keto-enolic tautomerization. This results in a shift of the equilibrium (Figure 5) to ketone and enol. A strong effect of the C-domain on the form and λ_{max} of bioluminescence spectra is confirmed by data from the literature: the mutant luciferase in the absence of small C-domain generates only red and very week bioluminescence [21].

DYNAMICS OF LUCIFERASE INTERACTION WITH ADENOSINE TRIPHOSPHATE (ATP) AND LUCIFERIN STUDIED USING THE STEADY-STATE AND TIME-RESOLVED FLUORESCENCE SPECTROSCOPY METHODS [14, 16, 17]

Binding of *L. mingrelica* luciferase to substrates causes quenching of intrinsic fluorescence of the enzyme (fluorescence of a single tryptophan residue). The absorption spectrum of ATP does not overlap with the fluorescence spectrum of tryptophan in luciferase, therefore, the energy transfer from tryptophan to ATP is impossible. Fluorescence of both free tryptophan (buffer solution of the amino acid) and luciferase denatured with 2 M guanidine chloride is not quenched in the presence of ATP. This means that the quenching of natural luciferase results from the interaction with ATP. The analysis of fluorescence decay curves of free luciferase and luciferase in the presence of ATP showed that the decrease in the fluorescence intensity obeys two-exponential law. Characteristic times and relative amplitudes of the quenching decay, which were calculated from the decay curves, indicated the dynamic type of the fluorescence quenching. The analysis of the obtained data allowed us for a conclusion that the formation of ATP-luciferase complex results in the decrease in the quantum yield of the tryptophan fluorescence by 10% due to the changes in the microenvironment of the tryptophan residue, i.e. partial quenching of the tryptophan fluorescence with polar groups of the protein (there are seven mobile charged groups in the microenvironment of luciferase Trp-419).

Binding of luciferase to luciferin is also accompanied by quenching of tryptophan fluorescence of the natural protein. Similar to ATP, denatured luciferase is not quenched. The average lifetime of luciferase fluorescence ($<\tau>$

= 2.9 ± 0.1 ns) does not change at formation of the enzyme-luciferin complex indicating the stationary type of quenching. At the same time, an efficient energy transfer from the tryptophan to luciferin in the luciferase-luciferin complex is observed that is typical of dynamic fluorescence quenching. This contradiction is resolved by a proposition that tryptophan residue Trp-419 approaches luciferin upon binding of luciferin with luciferase and the energy transfer efficiency is so high that the fluorescence quantum yield of the protein-luciferin complex may be neglected. In this case, the fluorescence decay curve of the complex should be identical to that of free enzyme (that's what actually observed). The comparison of the experimental data on the quenching time of the tryptophan fluorescence of free luciferase and its complex with luciferin with model decay curves allowed us to conclude that on the formation of the complex, the tryptophan residue and the luciferin molecule come close to each other at a distance of about 12 Å. This value is comparable with linear dimensions of the interacting molecules (L_{TRP} ~ 8 Å, L_{LH_2} ~ 12 Å). In this case, in addition to the dipole-dipole interaction, the exchange-resonance interaction becomes of importance, and electron clouds of tryptophan and luciferin may overlap.

CORRELATION BETWEEN CONFORMATION DYNAMICS AND FUNCTIONAL ACTIVITY OF LUCIFERASE [22]

As mentioned above, approaching of luciferase domains and other conformation changes occur in the spatial structure of the enzyme on the formation of the luicferase-luciferin-ATP complex (see Fig.2). The hypothesis of changing conformation state of the system at electron-excited product formation, namely, about dissociation of the luciferase–electron-excited oxyluciferin complex was proposed in 1989 [23]. To check the hypothesis, the studies of dynamic processes in the luciferase–luciferin complex using time-resolved fluorescence spectroscopy and fluorescence spectrochronography have been carried out. In fact, the increase in fluorescence intensity of the single tryptophan residue in the luciferase was recorded upon photoexcitation of the luciferase-luciferin complex with picosecond laser pulses in accordance with the pump-and-probe scheme. These data confirmed the hypothesis and allowed us for estimation of the conformational change propagation rate via protein molecule ($v \geq 20$ m/s). The distance at which electron-excited luciferin

molecule dissociates from the complex with the luciferase appeared to be much shorter than the average distance between molecules in solution. The luciferin-luciferase system used in the experiment was different from the natural bioluminescence system. However, it is reasonable to believe that the results obtained support the dissociative mechanism of the interaction between luciferase and the electron-excited product of bioluminescence reaction [22].

REGULATORY FUNCTIONS OF LIPIDS IN FIREFLY LUCIFERIN-LUCIFERASE SYSTEM

An important role of the protein–lipid interactions in the control of bioluminescence has been for the first time indicated by the firefly luciferin-luciferase system, as an example. In 1982, the activating effect of non-ionic and zwitterionic solvents on *P. pyralis* luciferase was described. The authors proposed that the activation is due to luciferase complex formation with detergent molecules, and the complexes manifest higher catalytic activity. At the same time, luciferase was completely inhibited by cationic and anionic detergents [24]. In 1983, an unusual behavior of natural (isolated from the firefly lanterns) *L. mingrelica* luciferase in reversed micelles formed from octane, aqueous buffer solution, and poly-10-oxyethylene oleinate (Brij-96) was found [25]. No luminescence was observed at water content below 9%. Increasing water content resulted in gel formation and, therefore, bioluminescence appeared possessing much higher intensity than that for buffer solution at the same concentration of the reagents. The observed enhancement of bioluminescence intensity is explained by ATP and enzyme accumulation in the water phase; the ratio of water phase is small as compared with the total volume. In addition, the catalytic constant of the luciferase-catalyzed reaction increased by 2 times. The phenomenon of remaining constant bioluminescence signal for several minutes in contrast with the peak signal in aqueous solution is of the greatest interest. Therefore, enzyme transition from the aqueous solution to a membrane-like structure resulted in, approximately, four orders of magnitude increase of the electron-excited reaction product yield [25].

Further studies show that firefly luciferase belongs to lipid-dependent enzyme class. Luciferase is spontaneously built in phophatidylcholine liposomes and the luciferase activity and stability in liposomes increase. The

increase in lipid concentration did not lead to the change in k_m for ATP and luciferin, but the maximum reaction rate is 2-fold increased and can be described by the Michaelis dependence [26]. Luciferase is localized on the external surface of liposomes. If luciferase was added to liposomes, in which both luciferase substrates were incorporated, bioluminescence appeared only after destruction of liposomes with Triton X-100 [27]. Embedding of luciferase into liposomes resulted in 20-fold increase in the enzyme stability and, in this case, as with reversed micelles, constant bioluminescence signal was observed. Apparently, the specific interactions of luciferase with phospholipid bilayer led to a change in the enzyme conformation resulting in the activation and stabilization of luciferase and electron-excited product yield increase. Since micro-pH of the environment on the surface on the lipid bilayer is changed, the micelle charge has a pronounced effect on the luciferase stability. Phophatidylcholine liposomes have no surface charge, but the addition of 5 – 20% of charged lipids (dicetyl phosphate or stearylamine) to the liposomes destabilizes the enzyme [27].

Natural luciferase purified by gel filtration and ion-exchange chromatography is a protein-lipid complex containing phospholipids and neutral lipids; the qualitative composition of lipids in the complex is identical to the lipid content of firefly lanterns. Phosphatidylcholine is the predominant phospholipid. Lysophosphatidylcholine, sphingomyelin, phosphatidylinosite, cholesterol and fatty acid fractions are present in smaller amounts [28]. Salts of bile acids, which are able to replace lipids that are in direct contact with a protein, are most often used for delipidization of the membrane proteins. As a result, active enzyme-detergent complexes may be obtained. Incubation of natural firefly luciferase with deoxycholate results in inactivation of the enzyme, and the higher the detergent concentration, the higher the rate and degree of inactivation. The inactivation process involves several consecutive steps and is described by kinetic Scheme 1 [29]:

$$\mathbf{EL} + \mathbf{D} \xleftrightarrow{k_1} \mathbf{ELD} \xleftrightarrow{k_2} \mathbf{ED} \xrightarrow{k_3} \mathbf{E}^n.$$

Scheme 1

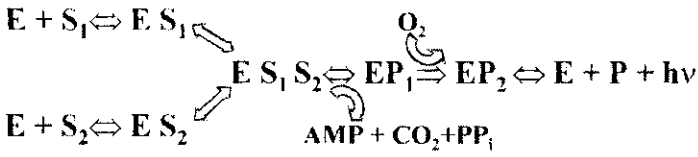
According to Scheme 1, natural luciferase as the protein–lipid complex (**EL**) forms complex with deoxycholate (**ELD**). Deoxycholate (**D**) displaces phospholipids to form a complex with luciferase (**ED**), which then transforms to an inactive enzyme aggregate ($\mathbf{E}^n$). The activity of **ELD** and **ED** complexes

is lower than that of natural luciferase. The individual rate constants (k_1 = $0.83 \cdot 10^3$ $M^{-1}s^{-1}$, $k_2 = 0.185$ s^{-1}, $k_3 = 0.011$ s^{-1}) are effective and characterize the total process, including enzyme delipidization and a change in the enzyme conformation that results in the change of luciferase activity observed. If phosphatidylcholine is added during luciferase inactivation by deoxycholate, the enzyme is reactivated, and the reactivation degree depends on the inactivation level. **ELD** and **ED** complexes are able to reactivate, whereas the protein aggregates ($\mathbf{E}^n$) with the molecular mass above 700 kDa consisting of more than 10 luciferase molecules are enzymatically inactive and are unable to reactivate.

If phosphatidylcholine is added before deoxycholate, a pronounced stabilizing effect of lipid is observed. The effect is so stronger, the higher the phosphatidylcholine concentration is. At a high phospholipids/detergent ratio (equal 4.0), the luciferase activity does not decrease in time, but even increases by ~3 times. Apparently, natural luciferase loses a portion of bound lipids during isolation and purification, and the addition of large amounts of phosphatidylcholine in a mixture with the detergent enriches the enzyme with phospholipid that results in luciferase activation. Binding of the substrates (ATP and Mg) to luciferase also increases the enzyme stability with respect to deoxycholate. For example, **EL·ATP·D** complex stability is 26 times and activity is 10 times higher compared to the **ELD** complex [29]. Other choline-containing phospholipids, such as lysophosphatidylcholine and sphingomyelin, also have a reactivating effect on luciferase delipidized by deoxycholate, and their reactivating effect is 3 and 2 times higher than the effect of phosphatidylcholine, respectively. Thus, choline group is required for a specific interaction of phospholipid molecule with luciferase. This is confirmed by experiments on luciferase inactivation with phospholipase C, which eliminates phosphocholine group from phosphatidylcholine in the protein-lipid complex of natural luciferase. It is very likely that the molecule of choline-containing phospholipid is necessary for maintaining the natural conformation of the enzyme [30].

KINETICS OF FIREFLY LUCIFERASE-CATALYZED REACTION

Bioluminescent reaction catalyzed by firefly luciferase is a multi-stage process described by the following kinetic scheme (2) [31]:



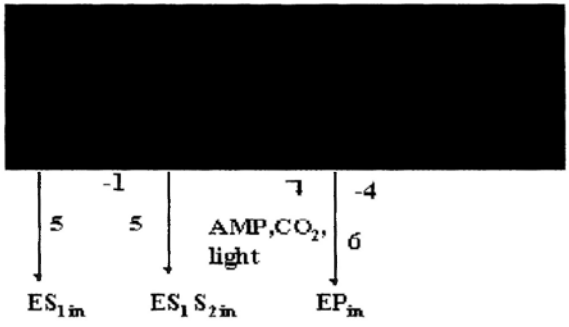
Scheme 2

where **E** is luciferase, $\mathbf{S_1}$ and $\mathbf{S_2}$ are luciferin (LH_2) and ATP-Mg^{2+}, respectively; $\mathbf{P_1}$ is luciferyl adenylate; **P** and $\mathbf{P_2}$ are oxyluciferin in the basic and excited state, respectively. Unlike common enzymatic reactions, where the dependence of final product concentration versus time is recorded and the reaction rate is calculated from the initial slope of the kinetic curve, the rate of bioluminescent reaction is obtained directly from the experimental data as the initial light intensity **(I)** and the kinetic curve is calculated by integration of the time-dependence of light intensity **(I)**. In aqueous solutions, bioluminescence kinetic curves possess a maximum, and the Michaelis-Menten equation is nominally true only at the maximum of these curves [32]. The analysis of bioluminescence kinetics in the initial time period (0.5 – 30000 ms) by the the «stop–flow» method and the analysis of integral kinetic curves down to 99% luminescence decrease indicated an induction period (~10-30 ms) on kinetic curves followed by a monoexponential increase of luminescence to its maximum (~ 0.15 – 0.4 s). Thereafter, prolonged decay in bioluminescence intensity to zero happened during several hours. The induction period decreases with the increase in the concentrations of the enzyme and the substrates and, hence, its existence is explained by the formation of enzyme-substrate complexes at the initial stage of the process. The second stage, which is transformation of $\mathbf{ES_1S_2}$ triple complex into luciferyl adenylate ($\mathbf{EP_1}$), limits the rate of bioluminescence increase up to its maximum. The third stage, bioluminescence decay, is described by a combination of several exponents [32].

The product yield (integral bioluminescence curve area) was determined in a broad range of the enzyme and substrate concentrations (in this case, the second substrate was saturated). For the firefly luciferase, the transformation degree of substrate to the product is very low that is explained by the enzyme inactivation in the course of the reaction. Total amount of the product formed is proportional to the initial content of the active enzyme and

described by hyperbolic dependence on the substrate concentration. Such behavior is typical of the so-called suicidal substrates, when the substrate induces inactivation of the enzyme in the course of the reaction [33]. The addition of pyrophospate (one of the intermediate products of the bioluminescent reaction) to the reaction mixture results in 2–5-fold increase in the yield of the final product that may be explained by luciferase turnover increase in the presence of pyrophosphate due to the acceleration of the enzyme-product complex dissociation. However, an addition of pyrophosphate does not change the overall picture indicating the importance of the inactivation stage in the kinetic mechanism of the luciferase-catalyzed reaction.

Basing on these experimental data, kinetic scheme (2) was supplemented with the stages involving inactivation of enzyme-substrate, enzyme-intermediate, and enzyme-product complexes [32, 34] (Scheme 3).



Scheme 3

Taking into account that in the experiment bioluminescence reaction is initiated by the second substrate injection to a mixture of the enzyme with the first substrate, the starting point in this reaction scheme is $\mathbf{ES_1}$ enzyme-substrate complex. The kinetic calculations were carried out by digital integration method [34] using kinetic constants for the individual reaction steps determined in [32] or obtained from the analysis of experimental and theoretical kinetic curves (Table 1). A good correlation between calculated and experimental kinetic curves was revealed within a broad range of substrate concentrations. The divergence between calculated and experimental kinetic dependencies did not exceed 6 – 8%. The obtained set of kinetic constants (Table 1) appeared to be stable enough: the change of any constant by 30% or

more resulted in a significant divergence between calculated and experimental data [34].

Table 1

Kinetic constants of the reaction catalyzed by *L. mingrelica* firefly luciferase [34] (see Scheme 2 for stage numeration)

Constants	Initiation of the reaction with luciferin	Initiation of the reaction with ATP
k_1, $M^{-1}\cdot s^{-1}$	10^6	10^5
k_{-1}, s^{-1}	10	10
k_2, s^{-1}	30	30
k_3, s^{-1}	10	10
k_4, s^{-1}	1	1
k_{-4}, $M^{-1}s^{-1}$	10^7	10^7
k_5, s^{-1}	2.6×10^{-5}	2.6×10^{-5}
k_6, s^{-1}	1.1×10^{-3}	1.1×10^{-3}
k_7, s^{-1}		2.3×10^7
k_8, s^{-1}		20

THE COMPARISON OF NATURAL *LUCIOLA MINGRELICA* FIREFLY LUCIFERASE AND MUTANT FORM KINETIC PROPERTIES

As mentioned above, mutations of luciferase amino acid residues located in luciferin and ATP binding region lead to an abrupt decrease in enzymatic activity. For a series of luciferase mutants with the changes beyond the active site, catalytic characteristics of mutants do not differ practically from those of the natural (isolated from fireflies) and recombinant enzyme [35, 36] (Table 2). Specific activity changed significantly only for the His433Ser mutant. The analysis of computerized models of the luciferase-luciferin-ATP complex for recombinant and mutant (His433Ser) luciferases showed that this mutation resulted in the change of complex conformation, in particular, to some change in configuration of ATP molecule in the enzyme active site, whereas for other His433 mutants none conformation changes were observed [36].

Table 2

Kinetic properties of the wild type, recombinant, and mutant *Luciola mingrelica* firefly luciferases [35, 36]*

Enzyme	Specific activity, $A \cdot 10^9$ rel.un. per 1 mg of protein	$K_{m,ATP}$, mM	K_{m,LH_2}, μM	K_{s,LH_2}, μM**	k_{cat}, s^{-1}
Natural luciferase	(1 – 1.5)	0.15	20	80	0.010
Recombinant luciferase	1.1	0.16	24	110	0.010
Cys82Ala mutant	1.0	0.60	16	200	0.008
Cys260Ala mutant	1.0	0.80	67	200	0.012
Cys393Ala mutant	1.0	0.58	36	130	0.011
Thr204Asp mutant	1.0	1.20	97	100	0.021
His433Asn mutant	0.75	0.23	20	–	–
His433Tyr mutant	1.0	0.22	20	–	–
His433Ser mutant	0.005	0.29	20	–	–

Notes: * The determination error of the constants equals 10%;
** Determined by fluorescent titration method.

The data on specific activity and k_{cat} for Cys and Thr residue mutants are close to the data for the recombinant luciferase, although $K_{m,ATP}$ and K_{m,LH_2} values are several times higher for the mutants. Hence, mutations of amino acid residues localized beyond the luciferase active site may change the kinetic properties of the enzyme if mutations result in changes of spatial structure of the active enzyme-substrate complex.

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STABILIZATION OF SUBCELLULAR STRUCTURES BY FUNCTIONING: A CREATIVE APPROACH TO SOLUTION OF MANY PROBLEMS

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Let us start from a trivial idea that a general scientific hypothesis frequently marks a way to solving already existing or bringing up new particular tasks. The history of one of such hypotheses has started in the middle 1960ies, when the author of the present article has just defended a candidate dissertation under supervision of the Academician N.M. Emanuel. Thus, he obtained an opportunity to choose self direction of the scientific research. The scene of this action was MSU Interfaculty Problem Laboratory of Bioorganic Chemistry (presently, A.N. Belozersky Institute of Physicochemical Biology, MSU) and the part of "benefactor" was played by Professor I.V. Beresin (afterwards the Corresponding Member of the Academy of Sciences of the USSR and the Director of A.N. Bach Institute of Biochemistry, Academy of Sciences of the USSR).

By that time, a new broad field of knowledge called *molecular biology* has been roughly formed. It united the investigations of replication, transcription and translation processes. The next was the question how various supermolecular (or subcellular) structures implementing various integral functions in the cell metabolism are formed in the cell. The true disposition of scientific priorities of that period was discussed in the book 'Horizons of Biochemistry' [1], in which the opinions of many the most famous authorities in the field biochemistry and molecular biology of that time were presented. The questions concerning formation of subcellular structures were discussed in the unique article, presented by Professor D. Green (University of Wisconsin, USA). In his laboratory, the principles of structural organization and formation of the mitochondrion respiratory system located in the mitochondrial inner membrane. At the initial stage of investigations, fragments of the respiratory system (or chain), capable of catalyzing separate stages of electron transfer from initial oxidizable substrates (e.g. NADH and succinate) to oxygen, have been isolated. These fragments called *respiratory complexes I, II, II and IV* are heterooligomers of hemo- and flavoproteins, as well as Fe/S proteins (Figure

1). As is known now, they include from 4 (complex II) to 46 (complex I in eukaryotes) individual proteins, encoded both in the nucleus and mitochondrial genome. It was shown at the next step, that mixing of individual complexes under definite conditions led to recovery of the integral functions of the system. This fact provided the basis for *the self-assembly hypothesis* according to which, the structure of individual complexes carries all necessary information for their correct binding to one another or, to put it differently, the complexes are *complementary* to one another. However, an attempt to assembly respiratory complexes from individual proteins has failed. Later on, Green has suggested that complexes are assembled by specific templates. The origin of the templates is still unknown, and the principle of *the template assembly*, tentatively, gives nothing new, because it is based on the above-mentioned complementariness idea. Obviously, *the self-assembly hypothesis* should be added or specified.

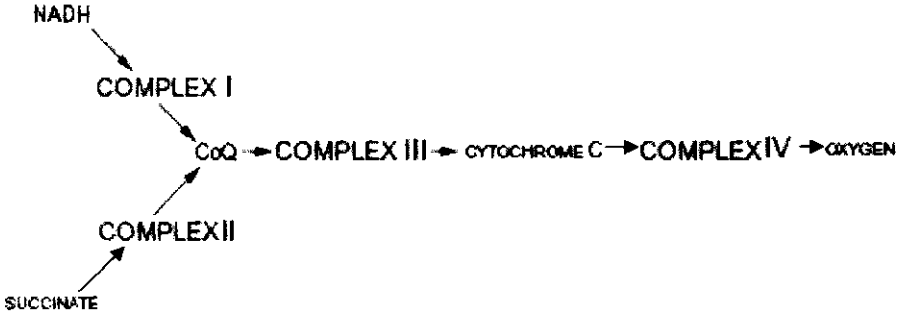

Figure 1. Schematic presentation of the mitochondrial respiratory chain

This point, which became fatal for the investigations by D. Green *et al.*, simultaneously became the start point for our research. Such a dead-lock situation required a new, alternative idea. It has been found then, when the question, if the respiratory chain can spontaneously assemble under physiological conditions, required the answer. For the most approximate simulation of the process, we mixed respiratory complexes under conditions determined by Green *et al.*, and then incubated the mixture at 38°C. It has been observed that integral function of the system (i.e. the ability to transfer electrons from NADH and succinate to oxygen) is quickly suppressed under

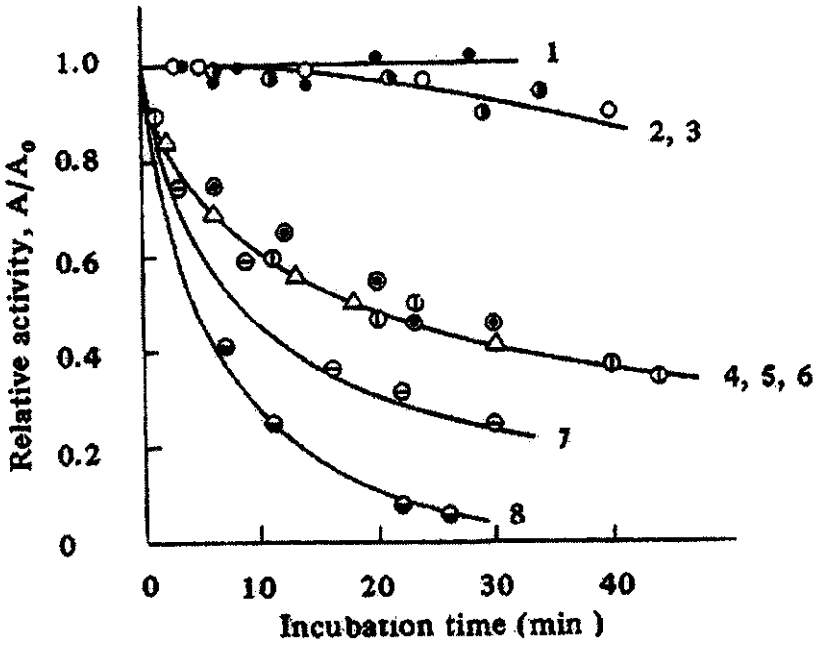

Figure 2. Inactivation of reconstituted respiratory chain and its fragments at 38°C.
1 – complex IV; 2 – complex I; 3 – complex II; 4 – complex III; 5 – complex I - III; 6 – complex II - III; 7 – complex II – III - IV; 8 – complex I – III - IV. Data from [2]

these conditions, whereas partial activities of the complexes (i.e. electron transfer from NAD.N and succinate to coenzyme Q, from reduced to cytochrome c, and then to oxygen) are more heat resistant (Figure 2). At the same time, in the presence of oxidizable substrate, oxygen and cytochrome *c* (the mobile carrier of electrons between complexes III and IV) in the incubation medium, the reconstituted system was stabilized, in general. Interestingly, the fragment I + III of the respiratory chain was unable to be stabilized in the presence of the required substrates. Similar effects were observed at reconstructed system inactivation by exogenous phospholipase A_2, analogous to phospholipase A_2 present in the mitochondrial inner membrane (Figure 3). Experiments carried out with preparations of this membrane

(submitochondrial particles), containing all components of the respiratory chain, produced the analogous results.

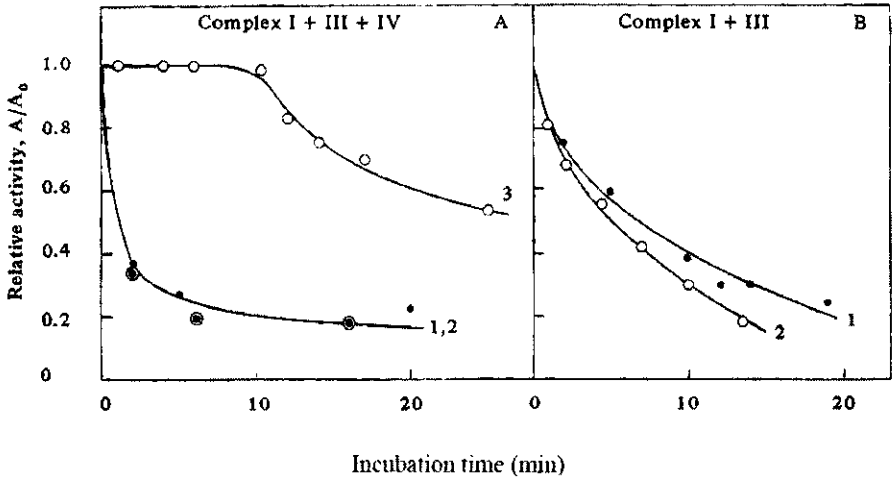

Figure 3. The effect of substrates on inactivation of reconstituted NADH-oxidase (A) and NADH:cytochrome *c*-oxidoreductase (B) by phosphlipase A_2 at 20°C.

A. Complex I + III + IV (+ cytochrome *c*): 1 – in the presence of phospholipase A_2; 2 – in the presence of phospholipase A_2 and NADH (under anaerobic conditions); 3 – in the presence of phospholipase A_2 and NADH (under aerobic conditions).

B. Complex I + III: 1 – in the presence of phospholipase A_2; 2 – in the presence of phospholipase A_2, NADH and cytochrome *c*.

Data from [2].

The more so, these experiments have shown that the electron transfer through some sections of the respiratory chain causes stabilization neither in these sections, nor in the entire system (Figure 4). Taking into account these data and the fact that phospholipase A_2 sensitive sites of the respiratory chain are located at the contact points between the respiratory complexes, it might be concluded that upon electron transfer such multienzyme system assumes a special *operating* form, resistant to various impacts, including high temperature, phospholipase A_2, and proteinases.

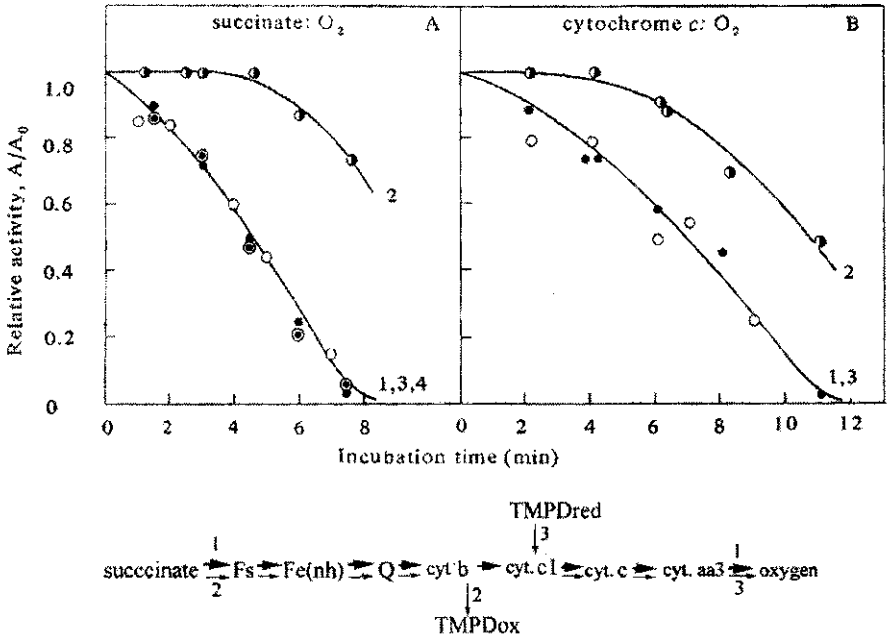

Figure 4. The dependence of resistance of succinate oxidase system and its cytochrome *c*-oxidase section resistance to phospholipase A_2 on their functional state.

A. Succinate oxidase system of submitochondrial particles: 1 – in the presence of phospholipase A_2; 2 – in the presence of phospholipase A_2, succinate and oxygen; 3 – in the presence of phospholipase A_2, succinate and oxidized TMPD (without oxygen); 4 – in the presence of phospholipase A_2, reduced TMPD and oxygen.

B. Cytochrome *c* oxidase activity of submitochondrial particles: 1 – in the presence of phospholipase A_2; 2 – in the presence of phospholipase A_2, reduced TMPD and oxygen; 3 – in the presence of phospholipase A_2, succinate and oxygen.

Data from [2].

Further experiments, carried out with the so-called conjugated submitochondrial particles, show that phosphorylating systems of mitochondria is highly sensitive to proteinases and phospholipases, but is stabilized, when

the incubation medium contains an oxidizable substrate, phosphate ions and ADP e.g. under conditions of ADP phosphorylation coupled to the electron transfer (Figure 5). Interestingly, in this case the better coupling, the higher is the level of phosphorylating system stabilization (Figure 6).

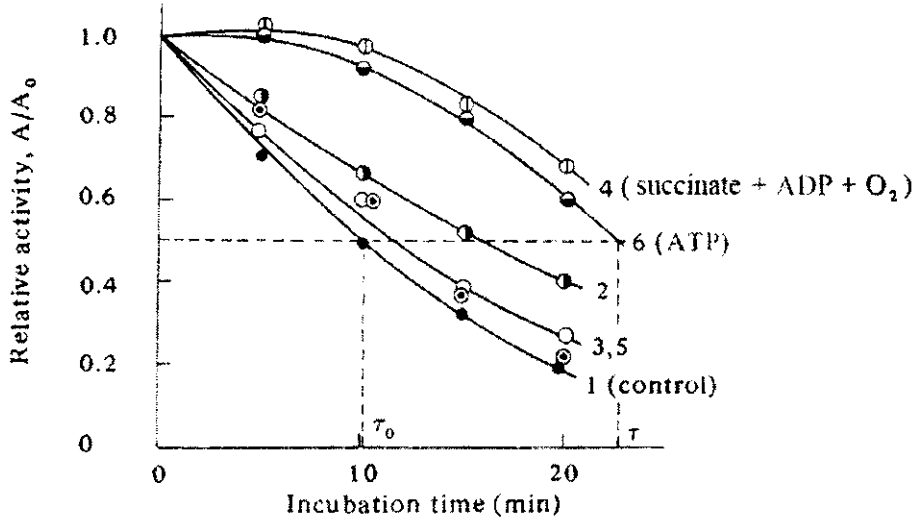

Figure 5. The dependence of resistance of phosphorylating system of submitochondrial particles (ETP_H, Mg^{2+}, Mn^{2+}) to trypsin on its functional state.
1 – in the presence of trypsin; 2 – in the presence of trypsin and ADP; 3 – in the presence of trypsin and succinate (under aerobic conditions); 4 – in the presence of trypsin, ADP, succinate, phosphate, and oxygen; 5 – in the presence of trypsin, ADP, succinate, phosphate, oxygen, and oleate; 6 – in the presence of trypsin and ATP. Data from [2].

In this way, the new principle of subcellular system stabilization – **stabilization by functioning** – was demonstrated on the example of mitochondrial oxidative phosphorylation system. This principle, discussed in detail in several reviews [2, 3] and monographs [4, 5], published in the period of 1973 - 1985, initiated the occurrence of several new directions.

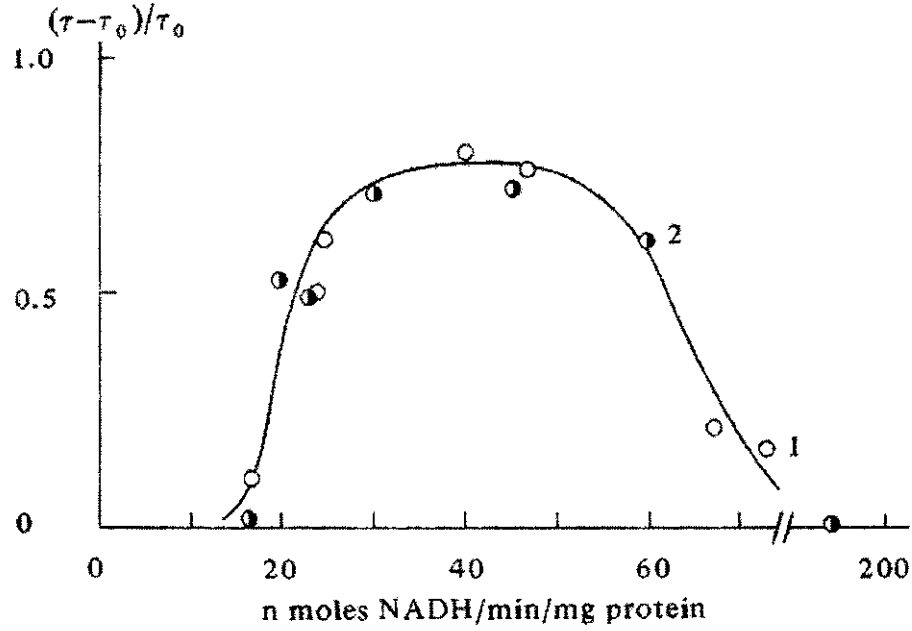

Figure 6. The dependence of resistance of phosphorylating system of submitochondrial particles (ETP_H, Mg^{2+}, Mn^{2+}) to trypsin on the rate of electron transfer through the respiratory chain.
The figure presents a correlation between half-life time of phosphorylating system and P/O ratio at different rates of electron transfer in the coupled respiratory chain. Data from [2]

STRUCTURAL ORGANIZATION OF THE RESPIRATORY SYSTEM OF MITOCHONDRIA

As follows from the data on stabilization of the respiratory chain affected by exogenic phospholipase A_2, upon functioning of the multienzyme system, its phospholipase A_2-sensitive sites, located in contacts between complexes, are shielded from the surrounding medium. This may be possible, if the individual complexes, interacting with one another upon the electron transfer, are assembled into a hyperensemble. In 1968, we published a model

of such hyperensemble reflecting earlier ideas of Green *et al.* [6]. Later on, Hackenbrock *et al.* [7] have suggested an alternative model, in which respiratory complexes do not interact directly with one another, but are functionally bound via mobile electron carriers, namely, coenzyme Q laterally diffusing in the lipid bilayer and cytochrome c playing the role of a shuttle in the intermembrane space.

Recently, as technology of gentle and selective fragmentation of membranes and effective separation of the fragmentation products was developed, it became obvious that the respiratory complexes, at least, when functioning, form hyperensembles [8, 9]. Even the terms of a *respirasome* [9], which means a hyperensemble having properties of complete respiratory system, and a *ATP-synthasome* [10], which includes H^+-ATPase, adenine nucleotide and phosphate carriers, were introduced. Therefore, the principle of stabilization by functioning has made a definite contribution to understanding the principles of organization of the respiratory chain and the phosphorylating system of mitochondria.

SELF-ACTIVATION OF MULTIENZYME SYSTEMS

Studying the features of the functioning state of the respiratory chain, we have found out that the rate of electron transfer in this system increases with this process proceeding [11, 12]. The mentioned effect testified to the increase of amount of *operating* respiratory ensembles, that have kinetic parameters different from those of the initial multicomponent system. Probably, after completion of every act of electron transfer these ensembles do not manage to relax to their basic conformational state. Apparently, the study of the respiratory chain stabilization has revealed a new way of multienzyme system functioning.

PRESERVATION OF MITOCHONDRION ENTITY AND THEIR DEGRADATION *IN VIVO*

Model experiments with the respiratory chain reconstituted from isolated respiratory complexes or with submitochondrial particles, have unambiguously pointed to the critical role of the oxidative phosphorylation in maintenance of mitochondrial integrity in the living cell. In 1970, using facultatively anaerobic yeast *Saccharomyces cerevisiae*, we have shown [13]

that any disturbance of the oxidative phosphorylation (by anaerobiosis or by administration of respiratory inhibitors or uncouplers) induces degradation of the respiratory system (Figure 7), mitochondrial cristae reduction, and other degenerative changes in the morphology of organelles [2].

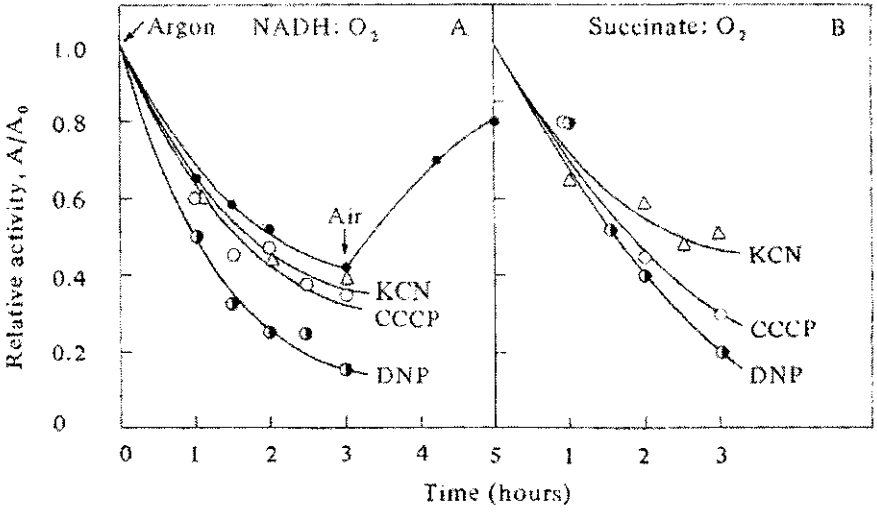

Figure 7. Decrease of specific NADH oxidase (A) and succinate oxidase (B) activities of mitochondrial fraction upon incubation of aerobically grown *S. cerevisiae* yeast under anaerobic conditions, in the presence of cyanide or uncouplers of oxidative phosphorylation (DNP or CCCP). Data from [2].

This observation explains how intracellular content of mitochondria (or oxidative phosphorylation system components) may be adjusted to the environmental conditions. As long ago as in 1964, A. Lehninger [14] has indicated that in a cell mitochondria are usually located close to the substrate source and in the areas of cytoplasm, where ATP is intensively consumed and ADP is produced. Moreover, there are data showing that *implantation* of entire mitochondria into the cell induces their gradual degradation, and finally, the mitochondrial *homeostasis* is maintained [15]. Elimination of mitochondria from the cell by *digesting* in lysosomes is described in multiple publications (see, for example, [16]). It should also be noted that degradation of mitochondria, one of the stages of which is their swelling and the outer

membrane rupture, is accompanied by release of cytochrome *c* and some other proteins into the cytoplasm. Then these proteins induce the cell apoptosis. Such apoptosis mechanism is intensively discussed in the literature [17, 18].

SELECTION BY THE PERFORMANCE CRITERION AS A CONTROL MECHANISM FOR THE RESPIRATORY SYSTEM FORMATION

In 1976, we have observed [19] that injection of proteinase inhibitors into the *S. cerevisiae* growth medium reduced the contents of respiratory cytochromes without corresponding decrease of the rate of cell respiration (Figure 8). This unusual fact suggested that during formation of the cell respiratory apparatus, its components, which are not necessary for maintaining the cell respiration at a definite level, are initially accumulated. These extra components are eliminated during development of mitochondrial population. Later on, it has been cleared out [20] that *excessive* cytochromes are components of complete respiratory complexes which, being potentially active, make no contribution to integral activity of the respiratory chain and, consequently, are assumed excessive. Apparently, this is associated with inability of some complexes to contact with one another due to their irregular distribution in the membrane. Actually, there are experimental data indicating that the complexes may form homogenous clusters in the mitochondrial inner membrane [21].

As followed from the experiments with proteinase inhibitors, the contents of *excessive* respiratory complexes may be as high as 25 – 30% of the total content of complexes at the stationary phase of yeast growth [19]. Thirty years ago, such *profusion* of the yeast cell has seemed improbable. However, six or seven years after our publication, when studying dynamics of the respiratory apparatus in synchronously growing *Acanthamoeba castellanii* yeasts, D. Lloyd *et al.* (Cardiff University, UK) have found [22] that within the cell cycle the content of complex IV is doubled and then reduced to its basic level. This also holds for the ATPase complex of mitochondria [23].

Hence, a question arises: how the cell recognizes *excessive* components of the respiratory apparatus, and how it eliminates them. Since in our experiments this elimination was suppressed by vacuolar proteinase inhibitors, it may be suggested that the latter stage of this process is implemented in vacuoles. Meanwhile, the degradation may encompasses entire mitochondria

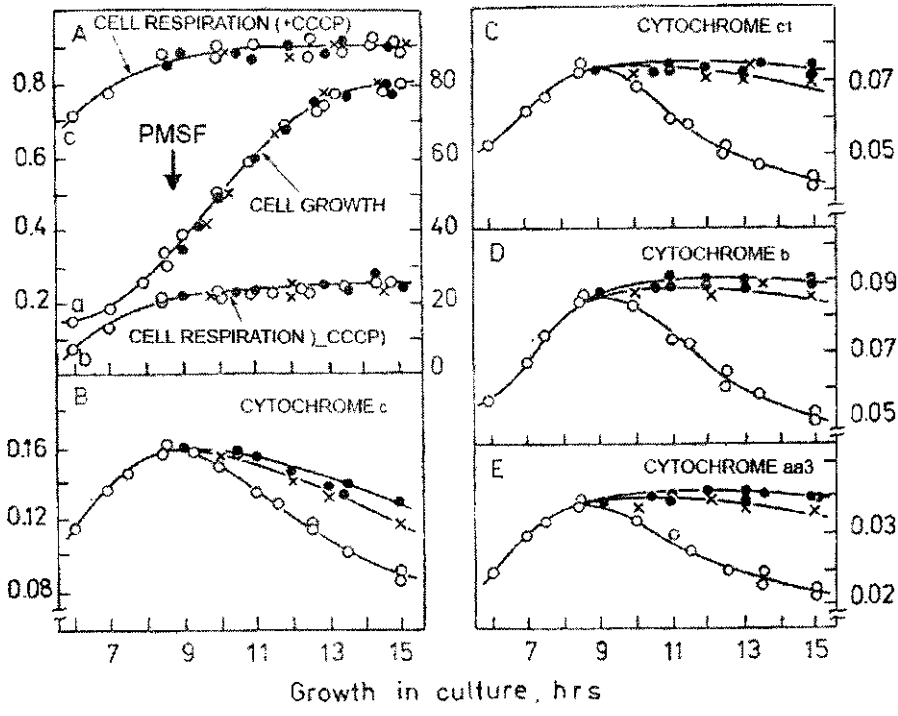

Figure 8. Development of the respiratory system in *S. cerevisiae* yeast growing on galactose and the effect of proteinase inhibitors.

o – control; • – in the presence of PMSF; × – in the presence of pepstatin.

A: yeast growth (a), cell respiration rate in the absence (b) and the presence (c) of uncoupler (CCCP); a – cell culture density in million cells per ml of suspension (right side); b and c – cell respiration on 1% ethanol, μg O atoms/min per mg dry weight of cells (left side)

B – E: cytochrome *c*, c_1, *b* and aa_3 content in whole cells, respectively, shown in optical density units from difference (reduced minus oxidized preparation) low-temperature spectra (22 g of dry-weight cells per mg of suspension).

B – cytochrome *c*, 548 - 533 nm; C – cytochrome c_1, 553 - 533 nm; D – cytochrome *b*, 559 - 570 nm; E – cytochrome aa_3, 603 - 630 nm. Data from [20]

and their fragments, that are ejected into the cytoplasm by virtue of local swelling of organelles and rupture of their membranes. These processes can be initiated by lowering activity of the oxidative phosphorylation system. Anyway, this concept requires special verifications, though there are some facts testifying to the involvement of lysosomes in the degradation of mitochondria induced by uncouplers of oxidative phosphorylation *in vivo* [24].

"QUALITY CONTROL" OF MITOCHONDRIAL PROTEINS

According to the above-said, 30 years ago the idea that during assembly of the respiratory apparatus about 25 – 30% of properly assembled (yet non-functioning) respiratory complexes are degraded seemed to be too progressive. However, multiple data accumulated by the present time indicate that even much simpler processes, such as folding and oligomerization of polypeptide chains, proceed with significant errors. The article by Schubert *et al.* [25], published in 2000, indicated that up to 30% of nascent polypeptide chains suffer ubiqitination and following degradation by proteasomes. Such polypeptide chains are accumulated in the cell, if proteasomal inhibitors are administrated. [Obviously, in 1976 we have used similar approach for detection of *excessive* components of the respiratory chain.] Studies of the fate of newly synthesized polypeptide chains led to a conclusion that in some cases only 15 – 20% of them get properly folded [26]. Concerning the assembly of heterooligomeric complexes, there is an example, when 90 – 95% of free subunits were degraded [27].

Recently, a new branch of cell molecular biology, named *protein quality control*, has come into prominance. This branch includes investigations on proteolytic systems, responsible for degradation of *defective* and *homeless* polypeptide chains, and mechanisms for delivery of these targets to the place of their elimination [28 – 31].

We have faced this problem 30 years ago in studies on the control over assembly of respiratory complexes. As is known, three of four complexes include subunits synthesized in both mitochondria and cytosol. The control mechanism for their stoichiometry was unclear. It was obvious that the control could hardly be implemented at transcriptional or translational level because two rather independent genetic systems are involved in encoding components of respiratory complexes. Moreover, since translocation of polypeptide chains from the cytosol into mitochondria is one of the stages of the complex

assembly, it might be assumed that the assembly is under post-translational control. In the late 1970ies we have published several works [32 – 35] indicating that, if the synthesis of mitochondrially encoded proteins is disturbed, their potential cytoplasmic partners imported to mitochondria are rapidly degraded. Accompanied by an article by Wheeldon *et al.* [36], this line of works anticipated intensive studies in the field of mitochondrial proteolysis, undertaken more recently in laboratories of V. Neupert (Munich), G. Schatz (Basel) and L. Grivell (Amsterdam). They have identified mitochondrial proteinases, deciphered the mechanisms of their action and the role in mitochondrial biogenesis [37 – 40]. Interestingly, that 15 years after the end of works on mitochondrial proteolysis in our group, we got an opportunity of cooperating with Neupert's laboratory, although no longer as the leaders [41].

CONCLUSION

In recent 10 – 15 years it became obvious that all stages of subcellular structure formation are controlled by various "*police*" systems, which detect and eliminate *defective* and *homeless* molecules of proteins, *jobless* protein complexes and even whole organelles. These ideas were forerun by the *principle of stabilization by functioning*, formulated in 1973 [2], and the principle of *selection by performance criterion*, suggested in 1981 [42]. According to the existing data (see above), the scope of action of "*police systems*" in the cell is rather impressive, and the fact of existence of such systems suggests mandatory and creative character of the controlling degradation processes in formation and maintenance of cellular architectonics.

At present, the "*quality control*" is the common notion, when we speak about proteins incapable of assuming native conformation by way of proper folding of polypeptide chains or their incorporation into oligomeric complexes. The formation of whole organelles is much more complicated process, as many additional processes must be controlled in this case, such as polypeptide chain targeting to particular subcompartments (as it is for mitochondria and chloroplasts), orientation of polypeptide chains in the membrane relative to its sides, definite lipid composition of the membrane, stoichiometry, structure and arrangement of supercomplexes (similar to the respirasome and the ATP-synthasome in mitochondria), etc. Apparently, for the whole organelle the *quality* criterion may be the ability to perform an integral, decisive function, which is oxidative ADP phosphorylation for mitochondria, fatty acid oxidation

(in mammals) or methanol (in yeasts) for peroxisomes, and light energy conversion to the energy of various chemical reactions for chloroplasts. The problem of rejecting organelles, which anyhow have lost the ability (or possibility) to carry out functions of this kind, is studied in detail on the example of peroxisomes, that suffer digestion in a cell when their substrates in the medium are exhausted [43]. To-date, the components of the cell apparatus responsible for delivery of *jobless* peroxisomes to vacuole are determined [44, 45]. However, the following questions have no answers yet:

(1) how this apparatus is switched on;
(2) how it distinguishes the targets;
(3) why it is selective for peroxisomes, but not other organelles, etc.

Thus, a break-through in these investigations demands another extraordinary step.

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PROTEIN AGGREGATION KINETICS

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Branched chain chemical reactions, discovered by N.N. Semenov [1], have given a strong impetus to investigations of free-radical processes proceeding in living organisms. N.M. Emanuel and his followers have made the essential contribution to understanding of kinetic regularities of pathological state development. The new aspect of application of the ideas about chain chemical reactions in biology concerns formation of protein aggregates. Nonnative forms of proteins which are intermediates in folding of newly synthesized polypeptide chains to native structures or formed from native proteins under the effect of adverse factors on the cells (increased temperature, for example), contain hydrophobic parts exposed to the aqueous medium and, therefore, are more active in intermolecular interactions forming functionally inactive aggregates [3]. The application of the chain chemical reactions allows for discussion of the protein aggregation question from new positions and description of the aggregation kinetics using the ideas about apical sites of the aggregates, which in the theory of chain reaction are active particles acting as the charge transmitters.

THE EFFECT OF CROWDING ON AGGREGATION OF NONNATIVE STATES OF PROTEINS

According to modern ideas, a great part of the living cell is filled with macromolecular components (proteins, nucleic acids, complexes of macromolecules, cytoskeleton, polysaccharides, etc.). In the literature, such conditions are mentioned as the crowding conditions (formed from the word *to crowd*). Figure 1 shows crowding conditions in cytoplasm of the intact cell [4, 5]. Crowding influences on biochemical processes proceeding with the volume change. Protein folding and unfolding, formation of complexes from

macromolecules, conformation transitions of macromolecules, formation of protein aggregates, etc. are crowding-sensitive processes [6 – 12].

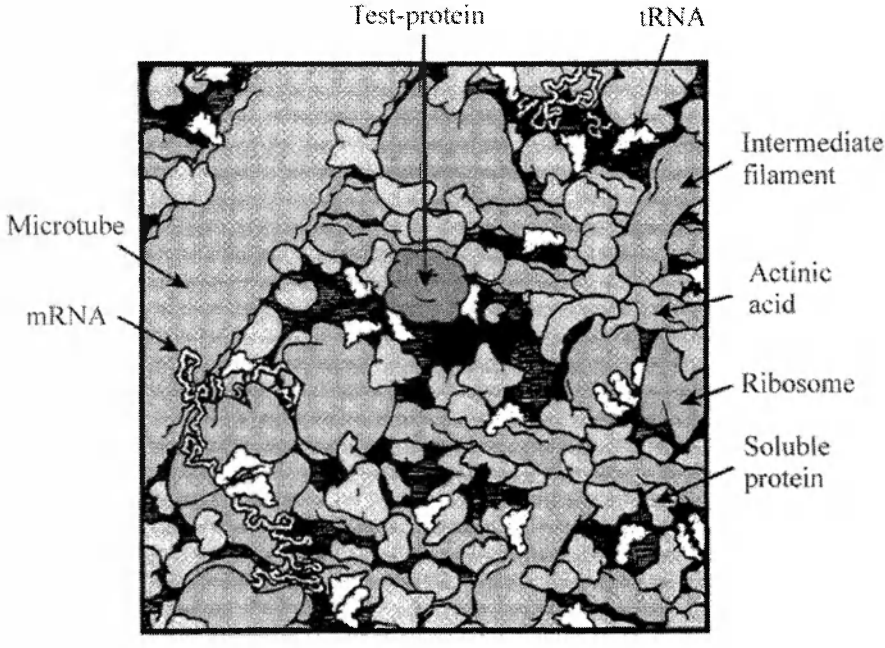

Figure 1. Schematic presentation of test-protein molecule in cytoplasm of a yeast cell, filled with macromolecular components creating crowding conditions: soluble proteins, RNA, ribosomes, and cytoskeleton [4, 5]

Folding of newly synthesized polypeptide chain produces a compact native structure. The crowding conditions promote formation of a native structure. However, due to the presence of hydrophobic parts exposed to aqueous medium, the folding intermediates easily aggregate [8, 13] (Figure 2). Crowding-stimulated aggregation of the intermediates reduces the native protein yield. The influence of thermodynamic imperfection, formed by crowding, on the aggregation rate of nonnative forms of protein can be explained using the theory of absolute reaction rates [7, 9]. For example, let us discuss the crowding influence on the rate of irreversible bimolecular reaction of unfolded protein molecules (U):

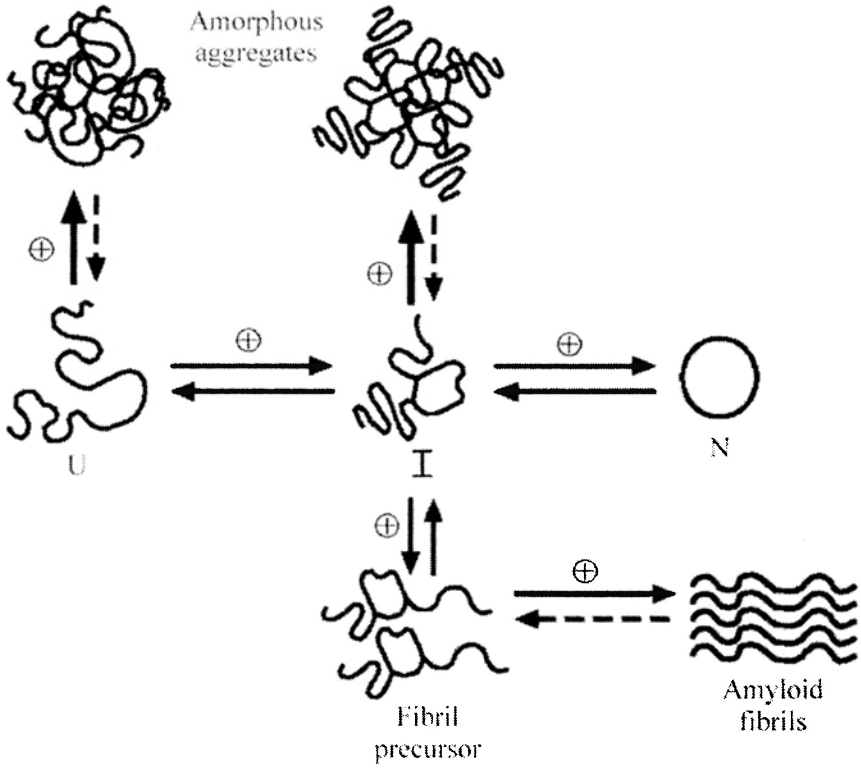

Figure 2. Aggregation of nonnative shapes of protein as a secondary folding reaction proceeding in the cell under crowding conditions [13]
U is the newly synthesized polypeptide chain; I is the folding intermediate; N is the native, completely folded protein. Sign ⊕ marks an increase of the mentioned stage under crowding conditions

$$U + U \xrightarrow{k} U_2. \tag{1}$$

The rate constant of the reaction k, measured in the presence of the crowding agent, is related to the reaction rate constant k_0, measured in the absence of the crowding agent, as follows:

$$k = \frac{k_0 \gamma_U^2}{\gamma_T}, \tag{2}$$

where γ_U and γ_T are thermodynamic activity coefficients for the shape U and the activated complex, respectively. Thermodynamic imperfection, induced by the crowding agent, is defined by the part of total volume of the solution, occupied by it. The activity coefficient for the substance of the *i*-th type is expressed as follows:

$$\gamma_i = \frac{v_{tot}}{v_i}, \tag{3}$$

where v_{tot} is the total volume; v_i is the volume accessible for substance of the *i*-th type. Activity coefficients of the shape U and the activated complex (γ_U and γ_T, respectively) are greater than one. It is obvious that the complex activated for the reaction (1) exceeds molecules U by size. Therefore, $v_T < v_U$ and, consequently, $\gamma_U > \gamma_T$. However, the numerator of the formula for the rate constant k contains γ_U^2, which exceeds γ_T; meanwhile, the crowding agent increases the protein aggregation rate. Note that in some cases coefficients of protein activity may be calculated with the help of simple structural models, in which globular proteins are represented by rigid spherical or spherocylindrical particles.

Besides the presence of highmolecular components, crowding conditions in the cell may be created by high concentrations of osmolytes, which are the compounds accumulated by living organisms in response to a stress (osmotic, chemical or thermal) [14 – 16]. According to chemical essence, osmolytes represent organic compounds, such as polyols, amino acids and their derivatives, and methylamines [14]. There are many experimental data indicating the protective action of osmolytes for proteins, affected by various denaturing factors [15 – 23].

Though the theory predicts intensification of protein nonnative form aggregation under crowding conditions [8, 24], there are no reliable experimental data confirming this fact. This is because the crowding effect is studied in the systems, where nonnative protein aggregation is accompanied by stages, which include origination of these forms. Let us illustrate this fact on the example of the crowding effect on the aggregation of glycogen

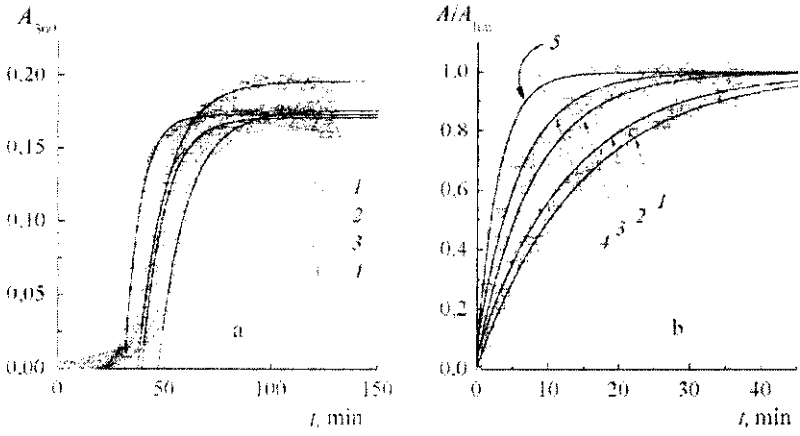

Figure 3. Aggregation kinetics for glycogen phosphorylase b (80 μg/ml), induced by 1 M GuHCl at 25°C (0.08 M Hepes; pH 6.8; 0.2 мM EDTA) [25]

a – TMAO is added to enzyme before GuHCl injection to the solution. Kinetic curves are presented in $\{A_{360}; t\}$ coordinates (A_{360} is the optical density at 360 nm). Points show experimental data. TMAO concentration: 0 (*1*), 0.1 (*2*), 0.2 (*3*), 0.4 M (*4*)

b – TMAO is added to the reaction mixture after the time period equal the lag-phase duration (38 min). Kinetic curves are presented in $\{A/A_{lim}; t\}$ coordinates (A_{lim} is the border value of A at $t \to \infty$). TMAO concentration: 0 (*1*), 0.05 (*2*), 0.1 (*3*), 0.2 (*4*), 0.3 M (*5*)

phosphorylase b from rabbit's skeletal muscles in the presence of 1 M guanidine hydrochloride (GuHCl). Similar to many other proteins, glycogen phosphorylase b has an affinity to aggregate in the presence of relatively low GuHCl concentrations [25]. Curve 1 in Figure 3a represents a kinetic dependence of glycogen phosphorylase b aggregation, tracked by the increase of seeming optical absorption at 360 nm. Protein aggregation is induced by GuHCl suppression (1 M is the final concentration). The presence of the lag-period on the kinetic curve is related to the presence of the protein denaturing stage. The kinetic curves of aggregation (2 – 4) were obtained in the presence of various concentrations of osmolyte – trimethylamine-N-oxide (TMAO). In these experiments TMAO was added to solution of glycogen phosphorylase b prior to injection of denaturing agent – GuHCl. Estimation of the crowding effect on the aggregation stage of denatured protein in this system is a difficult task, because the denaturation stage prior to the aggregation process is also sensitive to crowding. Actually, as shown in special experiments, TMAO reduces the rate of glycogen phosphorylase b denaturation rate. Glycogen phosphorylase b denaturation level was judged by the loss of enzymatic activity. In the presence of 0.4 M TMAO the inactivation rate constant is reduced by 110 times compared with the corresponding rate constant in the absence of TMAO. For the purpose of direct estimation of TMAO influence on glycogen phosphorylase b aggregation, once [25] osmolyte was added to solution containing glycogen phosphorylase b and GuHCl after the end of denaturation. In this experiment, the crowding-dependent effect of the aggregation intensification becomes clear (Figure 3b). As TMAO concentration is 0.3 M, the half-transformation time for the aggregation kinetic curve is reduced by 5.3 times compared with the corresponding value, obtained in the absence of TMAO.

PHYSIOLOGICAL FUNCTIONS OF CHAPERONS

Molecular chaperons take part in various intracellular processes, including co-translational folding of proteins, assembly and degradation of supermolecular protein structures, and protein transport through membranes [26 – 34]. Many molecular chaperons are known as heat-shock proteins (Hsp), synthesized in cells of all organisms in response to increased temperatures. Heat-shock proteins are required for cell protection from heat damage and

normalization of the cell functions after heat effect end [35 – 39]. Molecular chaperons have general properties characterizing the ability of these proteins to affect the conformation of protein substrates. Molecular chaperons selectively interact with definite intermediates of the protein folding processes, thus, affecting native-shaped protein output. Since some chaperons such as Hsp70 and Hsp60 (GroEL) promote polypeptide chain folding to a native structure, other chaperons, such as α-crystallines, heat-shock small proteins, immunophylins, cdc37 and Hsp90, are able to prevent aggregation of protein substrates, but are incapable of complete conversion of protein to the native shape. The following definition of chaperons was given: chaperons are proteins which interacting with folding polypeptide chains or aggregating supermolecular structures provide for a transition of spontaneous process to a stage, controlled by cell factors. The estimation of chaperon activity in tests, based on suppression of protein substrate aggregation, requires understanding of the protein aggregation mechanisms.

AGGREGATION TEST SYSTEMS

The tests of the influence of various factors (chaperons, crowding agents, etc.) on the protein aggregation are carried out using two types of test systems [45]. In the first type of systems, denatured proteins inclined to aggregation are produced from native protein by affecting it with denaturing agents. This type of test systems includes, for example, the following:

- systems including denaturation and aggregation of protein, affected by increased temperature; the experiments are carried out at temperature fixed or increasing at a constant rate;
- systems including denaturation and aggregation of proteins induced by the action of moderate concentrations of denaturing agents (about 1 M) of GuHCl or urea type;
- systems with aggregation, initiated by disulfide bond recovery in the protein molecule (for example, in insulin, lysozyme, β-lactalbumin, etc.);
- systems with protein denaturation and aggregation, induced by UV-light, ionizing radiation and other physical factors.

Test systems of the second types convert native protein to fully denatures state with the help of relatively high concentrations of denaturing agent (GuHCl or urea). As denaturing agent is diluted and its concentration decreased, proteins are renatured (e.g. convert to the native state). Refolding intermediates show affinity to aggregation.

KINETICS MODES OF PROTEIN AGGREGATION

Since particles possessing light scattering ability higher than initial protein molecules are formed at the protein aggregation, aggregation kinetics can be easily tracked by increase of seeming absorption in the visible spectrum. It is obvious that quantitative analysis of the aggregation curves obtained by turbidity method is possible in the only case, if the increment of optical absorption is strictly proportional to the quantity of aggregated protein. In some works the existence of such proportion is confirmed experimentally [41 – 43].

Formally, the process of protein aggregation may be considered as the n-th order reaction:

$$nP \xrightarrow{k_n} P_{agg}, \tag{4}$$

where P and P_{agg} are nonaggregated and aggregated protein forms, respectively; k_n is the reaction rate constant of the n-th order. If we accept that optical density (A) is proportional to the quantity of aggregated protein, the value A and the rate of its change are bound by the following relation [44, 45]:

$$\frac{dA}{dt} = \frac{nk_n}{\alpha^{n-1}}(A_{\lim} - A)^n, \tag{5}$$

where $A_{\lim}$ is the border value of optical absorption A, reached at $t \to \infty$ (t is time); $\alpha = \frac{A_{\lim}}{[P]_0}$ (here $[P]_0$ is the protein initial concentration). The use of equation (5) for analysis of the aggregation kinetic curves allows determination of parameters n, k_n and $A_{\lim}$. Determination of the protein aggregation mechanism requires the knowledge of parameters n, k_n and $A_{\lim}$, measured at various concentrations of protein. The linear dependence of $A_{\lim}$ on the initial

concentration of protein indicates probability of the assumption that the value A is proportional to the quantity of aggregated protein. The information about the aggregation rate order by protein is used for determining the kinetic mechanism of aggregation of the protein under study.

As frequently shown by the analysis of kinetic curves of protein aggregation obtained by the turbidity method, parameter n equals unit and, consequently, the aggregation process follows kinetics of the first order reaction [44, 45]. At $n = 1$, equation (5) is transformed as follows:

$$\frac{dA}{dt} = k_1(A_{\lim} - A), \tag{6}$$

where k_1 is the rate constant of the first order reaction. The integral shape of this equation is the following:

$$A = A_{\lim}\{1 - \exp(-k_1 t)\}. \tag{7}$$

As studying heat aggregation of proteins, it should be taken into account that the aggregation stages are preceded by the stage of protein molecule denaturation:

$$\mathrm{N} \xrightarrow{\text{denaturation}} \mathrm{D} \xrightarrow{\text{aggregation}} \mathrm{D}_i, \tag{8}$$

where N is the native protein; D is denatured protein; D_i is the aggregated shape of denatured protein. In accordance with the experimental conditions determined by many factors as initial protein concentration, temperature, pH, ionic strength of solution, etc., various kinetic aggregation modes can be realized.

As an example, let us discuss heat aggregation of creatine kinase from skeletal muscles of rabbit at $T = 50.6°C$ (30 mM Hepes, pH 8.0) [46]. Figure 4 shows kinetic curves of aggregation rated by the axis of ordinates by the value $A_{\lim}$. It is also observed that experimental sites, obtained at various protein concentrations, fit the unique general curve. This general aggregation curve is characterized by the lag-phase presence. After passing the lag phase, the kinetic curve is described by a varied shape of the equation (4):

$$A = A_{\lim}\{1 - \exp[-k_1(t - t_0)]\}, \tag{9}$$

where t_0 is the lag phase duration. For generalized curves, the corresponding values are the following: $k_1 = 0.066 \pm 0.001$ min^{-1} and $t_0 = 2.64 \pm 0.01$ min.

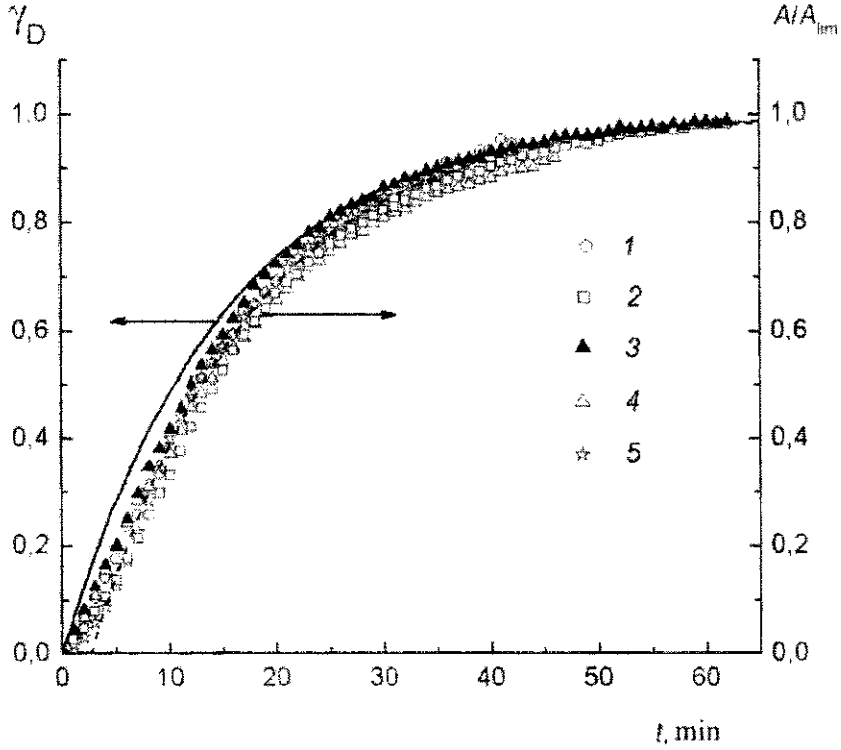

Figure 4. Kinetics of heat aggregation of creatine kinase from skeletal muscles of a rabbit at 50.6°C, registered by the absorption increase at 400 nm (A_{400}) in 30 mM Hepes buffer, pH 8.0, and the following protein concentrations: 0.05 (*1*), 0.1 (*2*), 0.125 (*3*), 0.15 (*4*), 0.2 mg/ml (*5*) [46].

Kinetic curves of aggregation are presented in $\{A/A_{lim}; t\}$ coordinates. Points represent experimental data; the dashed line is plotted with respect to all experimental points using the equation (9). Continuous line represents theoretical data on denatured protein part (γ_D) increase with time, calculated by the equation (11)

As shown by the method of differential scanning calorimetry, creatine kinase denaturation is describes by a single-stage model N → D. Parameters of the Arrhenius equation:

$$k_{\text{den}} = \exp\left\{E_{\text{a}}\left(\frac{1}{\text{T}^*} - \frac{1}{\text{T}}\right)/R\right\} \tag{10}$$

(where E_a is the activation energy; T is the absolute temperature; T^* is the absolute temperature, at which $k_{den} = 1\ \text{min}^{-1}$) are the following: $E_a = 461.0 \pm 0.7$ kJ/mol; $T^* = 329.01 \pm 0.1$ K. Using these values, one can calculate the denaturation rate constant for creatine kinase at $T = 50.6°C$:

$$k_{\text{den}} = 0.067\ \text{min}^{-1}.$$

Thus, the order one reaction rate constant calculated by the aggregation kinetic curve coincides with the denaturation rate constant. Besides aggregation kinetic curves, Figure 4 shows the change of denatured protein fractions (γ_D) with time, calculated by the formula:

$$\gamma_{\text{D}} = 1 - \exp(-k_{\text{den}}t). \tag{11}$$

The independence of the aggregation kinetic curve shape on the initial concentration of protein and similarity of the order one constant rate, calculated from the aggregation kinetic curve, and the rate constant of protein denaturation indicates that the aggregation proceeds in the kinetic mode, at which the stage limiting the general aggregation process rate is the protein molecule unfolding process.

The deviation between kinetic curves of denaturation and aggregation, observed at short times, is of special discussion. The reason for this deviation is the circumstance that the aggregate propagation stages precede thermodynamically unfavorable nucleation stage [48, 49]. Therefore, strictly speaking, for the experimental data under discussion protein molecule denaturation becomes the rate-limiting stage only after nucleation stage completion e.g. after the lag-phase end (to put it differently, the aggregate propagation stage proceeds at much higher rate rather than the stage of protein molecule unfolding).

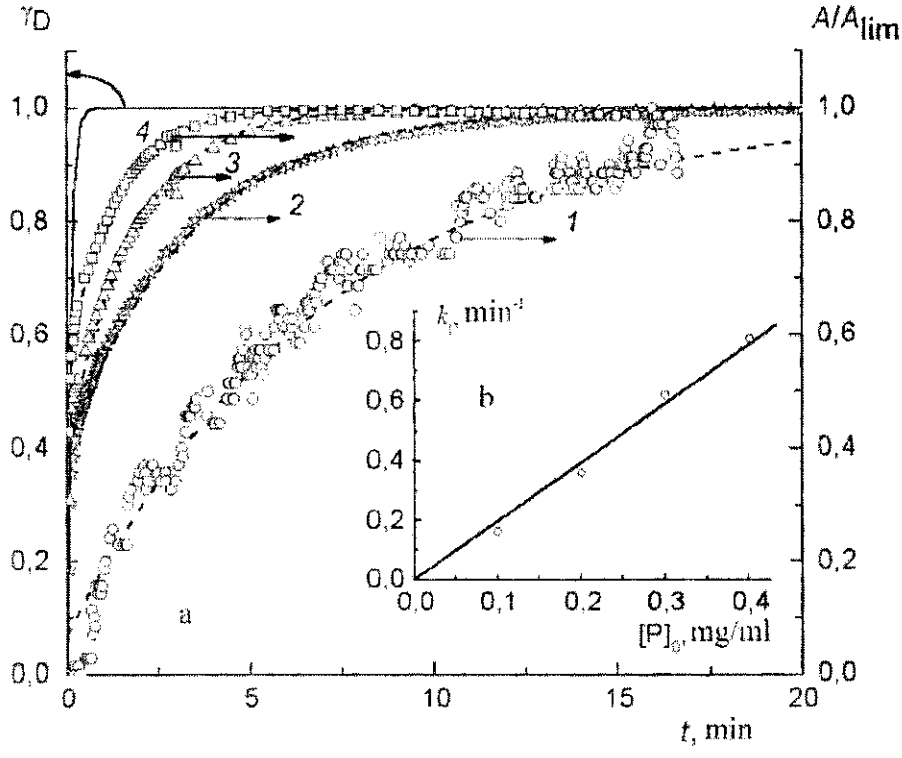

Figure 5. Kinetics of heat aggregation of creatine kinase from skeletal muscle of a rabbit at 60°C (30 mM Hepes buffer, pH 8.0) [46].
a – Kinetic aggregation curves registered by the absorption increase at 400 nm (A_{400}) and the following protein concentrations: 0.075 (*1*), 0.2 (*2*), 0.3 (*3*), 0.4 mg/ml (*4*). Kinetic curves are presented in $\{A/A_{lim};\ t\}$ coordinates. Points represent experimental data; dashed lines are plotted using the equation (12). Continuous line represents theoretical data on denatured protein part (γ_D) increase with time, calculated by the equation (11)
b – Dependence of the first order rate constant k_I on the initial concentration of creatine kinase $[P]_0$

Temperature increase causes a change of kinetic mode of aggregation process. Figure 5 shows kinetic curves of creatine kinase aggregation at T =

60°C in $\{A/A_{lim}; t\}$ coordinates. At this temperature denaturation rate constant k_{den} equals 9.35 min^{-1} (calculated from differential scanning calorimetry data). At this k_{den} value the part of denatured protein equal 0.99 is reached during 0.5 min. The aggregation rate decreases with the protein concentration (the half conversion time increases) and a situation can simply be realized, when during a short period of time protein completely transits into denatured state, and the investigator has an opportunity to study *purely* the denatured protein aggregation. If we process kinetic curves of aggregation, determined for various initial concentrations of the protein, by the equation:

$$A = A_0 + (A_{lim} - A_0)[1 - \exp(-k_I t)] \tag{12}$$

(where A_0 is the value at $t = 0$) equivalent to the equation (9), it is found that the rate constant of the first order reaction represents a linear function of the initial protein concentration (Figure 5b). Similar situation was also observed at heat denaturation of tobacco mosaic virus cover protein (TMV CP; 52°C; 0.05 M Na-phosphate buffer, pH 8.0) [50] and glowworm luciferase (42°; 0.025 M trypsin, pH 7.5) [51].

For the purpose of explaining kinetic regularities of aggregation observed [44, 45, 50, 52], protein aggregation is considered as a process including the nucleation stage

$$n\mathrm{D} \rightarrow \mathrm{R}, \tag{13}$$

where R is a nucleus consisting of n denatured protein molecules D; and the aggregate propagation stage

$$\mathrm{R} + \mathrm{D} \xrightarrow{k_{II}^{macro}} \mathrm{R}'. \tag{14}$$

Here R′ is a nucleus with additional molecule of denatured protein. The aggregate propagation stage represents a bimolecular reaction, which rate is expressed by the following formula:

$$v_{agg} = -\frac{d[\mathrm{D}]}{dt} = k_{II}^{macro}[\mathrm{R}][\mathrm{D}], \tag{15}$$

where k_{II}^{macro} is the macroscopic rate constant of the second order reaction. If we assume the concentration of nuclei R remaining constant during protein aggregation, the aggregate propagation rate represents the first order reaction with the rate constant of the pseudo-first order k_I equal the product k_{II}^{macro}[R]. In this case, k_I value changes with the protein concentration. The linear dependence of the pseudo-first order reaction rate constant on the protein concentration indicates that the concentration of nuclei, on which the aggregates propagate, is directly proportional to the initial protein concentration.

Therefore, temperature increase promotes alteration of kinetic mode of aggregation. At relatively low temperature, a kinetic mode in which the protein molecule unfolding is the rate-limiting stage is realized (strictly speaking, after the nucleation stage end). At relatively high temperature, a kinetic mode in which the rate-limiting stage of general aggregation process is the stage of the protein aggregate propagation.

For example, at TMV CP aggregation at 42°C (0.1 M Na-phosphate buffer, pH 8.0), the half transformation period ($t_{1/2}$) remains constant for kinetic aggregation curves in the range of initial TMV CP concentrations within 0.1 and 0.4 mg/ml. This means that under these conditions the rate-limiting stage of the aggregation process is protein denaturation. As the initial TMV CP concentration decreases, $t_{1/2}$ expands and, consequently, the propagation rate of the protein aggregate approaches the rate of protein molecule unfolding.

AGGREGATION OF PROTEINS AS CHAIN REACTION

In the general case, nucleus R contains several propagation sites (i.e. sites by which monomers D are attached). Let Ω be an aggregate propagation site and j – the number of propagation sites in the nucleus R. If [R] is molar concentration of nuclei, then molar concentration of propagation sites equals j[R]. The condition of nucleus concentration constancy is equivalent to the condition of the indestructibility of propagation sites and their constancy during aggregation:

$$\Omega + D \xrightarrow{k_{II}^{micro}} \Omega, \qquad (16)$$

where k_{II}^{micro} is a microscopic rate constant of the second order reaction. The following relation between microscopic and macroscopic rate constants of the second order reactions is true:

$$k_{II}^{macro} = j\, k_{II}^{micro}.$$

The aggregation rate is expressed as a decrease of monomers D concentration by the following equation:

$$v_{agg} = -\frac{d[D]}{dt} = k_{II}^{macro}[R][D] = k_{II}^{micro}[\Omega][D] = k_I[D]. \qquad (17)$$

Here k_I is the rate constant of the pseudo-first order:

$$k_I = k_{II}^{macro}[R] = k_{II}^{micro}[\Omega].$$

If experimentally determined rate constant of the first order reaction k_I shows linear increase with the initial protein concentration, this means that the propagation site concentration $[\Omega]$ is directly proportional to $[P]_0$.

Indestructibility of propagation rates of the aggregate nuclei means that the protein aggregation process is similar to chemical chain reactions, based on the principle of free valence indestructibility. In the early 1900 s, M. Bodenstein has discovered chain reactions. In 1958, N.N. Semenov was awarded the Nobel Prize for the discovery of branched chain reactions [1, 53]. Many important classes of chemical reactions such as oxidation with molecular oxygen, chlorination and bromination of many compounds, thermal degradation and many polymerization reactions proceed by the chain mechanism [54]. Free radicals participating in the chain reaction are called active sites of the chain reaction. As applied to the aggregation of proteins, active sites of the chain reactions represent aggregate propagation sites. The following stages are the main stages in chemical chain reactions: chain initiation, chain propagation and chain termination. The chain initiation is the stage of chain reaction, in which free radicals from valent-saturated molecules are formed. Concerning the aggregation of proteins, this stage is corresponded to unfolding of the protein molecule and nucleation. The chain propagation

reactions are elementary stages of the chain reaction proceeding with preservation of the free valence, consuming initial substances and forming reaction products. In the case of protein aggregation, this stage corresponds to the stage of aggregate propagation proceeding with preservation of the aggregate propagation site. The chain termination is the chain process stage (or stages), which cause elimination of the free valence. A chain may be terminated due to free radical capture by the reaction vessel wall or mutual saturation of free valences at the interaction of two free radicals. Concerning the aggregation of proteins, it should be mentioned, first of all, that in the case of use of relatively low protein concentrations possible participation of the reaction vessel surface during protein denaturation should be taken into account. It is known that in this case, the denaturation rate constant increases as the protein concentration decreases and in proportional to ration of the vessel surface to the solvent volume [55, 56]. The question about participation of protein, denatured on the vessel surface, in aggregation processes is unsolved yet. Concerning the interactions of aggregate nuclei carrying the propagation sites, but such interaction causes not only elimination of a definite number of propagation sites, but also, which is most important from the point of view of aggregation kinetics analysis, to break of a suggestion about proportionality between solution turbidity and the amount of aggregated protein. Interactions of this kind, which finally lead to precipitation of aggregated protein, become significant with increasing initial concentration of the protein.

Chain reactions proceeding without branching are called nonbranched chain reactions. In this case, at the stage of chain propagation one new active site is formed from already existing one. Drawing an analogy to protein aggregation and chemical chain reactions, we may suggest the protein aggregation to be a nonbranched chain reaction. Strictly speaking, this statement is correct only for the aggregate propagation stage. Concerning the nucleation stage, obviously, it is a branched chain reaction, because the essence of this stage consists in formation of a nucleus possessing several propagation sites (several active sites). Moreover, such approach to the nucleation stage provides for a strict definition of this stage duration. Propagation of the initial nucleus is accompanied with decrease of the number of propagation sites contained in it. Starting from a definite size of the nucleus, the number of propagation sites reaches its border value. This means that the nucleation process for this nucleus is finished. Further on, the nucleus formed propagates due to attachment of "*monomers*" (denatured protein molecules). Without changing the number of propagation sites in this nucleus.

Substances which injection to already proceeding chain reaction induces a replacement of free radicals, propagating the chain, by low-active ones, incapable of the chain propagation, are called chain reaction inhibitors. Therefore, chaperons which interlock aggregation of the protein substrate may be called inhibitors of the protein aggregation chain reactions. The function of a chaperon consists in full interlocking of the protein aggregate propagation site (i.e. active site of the chain reaction).

For luciferase aggregation induced by acidifying (25 mM Trypsin, pH 5.5; 30°C) [51], deviations from the first order kinetics are observed: $n = 1.29$. The reason for these deviations may be the change in propagation site concentration with the propagation of aggregates. If we take into account the fact that after the end of the nucleation stage concentration of denatured molecules D is proportional to $[P]_0\left(1-\frac{A}{A_{lim}}\right)$, then the rate of absorption change $\frac{dA}{dt}$ is expressed as follows:

$$\frac{dA}{dt} = k_{II}^{micro}[\Omega](A_{lim} - A). \tag{18}$$

Combining expressions (6) and (18), we deduce an expression for the concentration of the aggregate propagation sites:

$$[\Omega] = \frac{k}{k_{II}^{micro}}(A_{lim} - A)^{n-1}. \tag{19}$$

Thus, the multiplicand $(A_{lim} - A)^{n-1}$ defines the type of $[\Omega]$ value during aggregation.

QUANTITATIVE ESTIMATION OF CHAPERON-LIKE ACTIVITY

As mentioned above, for the extreme kinetic modes of aggregation, when the protein molecule unfolding or aggregate propagation is the rate-limiting stage, the aggregation obeys the first order kinetics. This explains the

experimental fact that kinetic curves of aggregation, obtained by turbidimetric method, are frequently satisfactorily described by the equation (9).

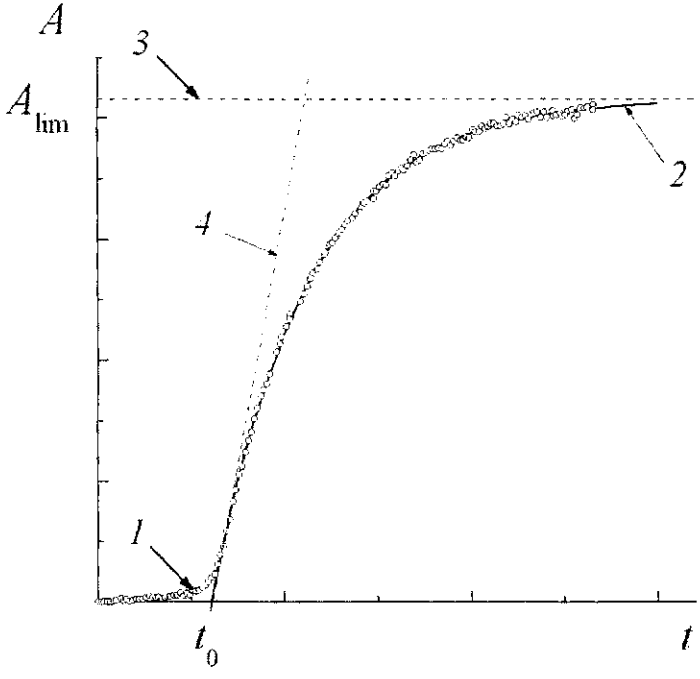

Figure 6. Quantitative estimation of chaperon-like activity [44]
1 – experimental time dependence of optical absorption (A); 2 – theoretical dependence corresponding to equation (9); 3 – the border value of A corresponding to A_{lim}; 4 – a tangent to the curve 2 passing through the point with coordinates $t = t_0$ and $A = 0$ with a slope equal the product $k_1 A_{lim}$

As the equation (9) is fulfilled, the protective action of chaperons can be characterized by a change in parameters t_0, A_{lim} and k_1. Chaperon present in the system binds denatured protein and prevents its aggregation. The action of chaperon is displayed in reduction of parameter A_{lim} with increasing chaperon concentration. Creation of A_{lim} dependence on the ratio [protein substrate]:[chaperon] allows for estimation of stoichiometry of a chaperon complex with denatured protein substrate. The complex stoichiometry is

determined from the cross point of the straight line and the abscissa axis in the above-mentioned coordinates [44, 45, 52].

In the range of low chaperon concentrations, the type of parameter k_I dependence on chaperon concentration is defined by the kinetic mode, in which protein aggregation proceeds. If protein denaturation is the rate-limiting stage, then parameter k_I remains constant at chaperon injection. For example, this picture is observed at heat aggregation of creatine kinase from skeletal muscles of rabbit in the presence of Hsp16.3 from *Mycobacterium tuberculosis* [46] and, as shown by the analysis [44], at heat aggregation of the catalytic subunit of protein kinase CK2 in the presence of glucose controllable protein grp94 [57]. In the case, when the protein aggregate propagation is the rate-limiting stage, addition of even small amounts of chaperon decreases parameter k_I. Similar case, for example, is observed at heat aggregation of malate dehydrogenase from pig heart mitochondria in the presence of *Pisum sativum* Hsp18.1 [58] (according to the data of analysis of the aggregation kinetics [45]).

The multiplication $k_I A_{lim}$ can be used as the measure of the initial aggregation rate (after the lag-phase). This expression equals the tangent to the aggregation kinetic curve passing through the point $t = t_0$ (Figure 6). The dependence of similarly expressed initial aggregation rate on the chaperon concentration is defined by the chaperon influence on parameters k_I and A_{lim}. It may be expected that the influence of chaperon on the initial aggregation rate will be stronger in the case, when aggregate propagation is the rate-limiting stage for the general aggregation process.

Since the lag-phase duration is defined by the nucleation stage, it can be expected that chaperon addition will increase parameter t_0.

THE MAIN WAYS OF PROTEIN AGGREGATE PROPAGATION

At present, the method of dynamic light scattering is widely used for obtaining full information on the dynamics of protein aggregate size change during aggregation process [59 – 62]. The method is based on registration of light scattering intensity fluctuations induced by the Brownian movement of particles in a small volume of the solution. Fluctuations are recorded in a microsecond time range. The diffusion coefficient (D) is calculated from autocorrelation function. For particles with the sphere-like shape, using the

Stokes–Einstein equation hydrodynamic radius of the particles R_h can be calculated:

$$R_h = kT/6\pi\eta D, \tag{20}$$

where k is the Boltzmann constant; η is the solvent viscosity. The advantage of dynamic light scattering method is, firstly, the ability to register initial stages of protein aggregation, when the part of aggregated protein equals tenth parts of percent of the total quantity of protein in the system; secondly, the possibility of detecting heterogeneity of the aggregate population; thirdly, the possibility od estimating the particle size (protein aggregates, chaperon complexes with denatured protein substrate, and initial protein substrate), not using other methods providing for particle separation by sizes. The latter circumstance is of special importance for the cases, when protein aggregates formed are unstable and affined to mutual adhesion. If in such a situation thermal aggregation is stopped by cooling the protein solution down to room temperature and of protein aggregates are distributed by sizes using gel-penetrating chromatography, sedimentation in analytical ultracentrifuge and other methods, a distorted idea about sizes of protein aggregates in the real system may be obtained.

Figure 7 shows the change of the protein aggregate distribution by size during TMV CP protein aggregation at 52°C (50 mM Na-phosphate buffer, pH 8.0) [63]. At relatively low recording times of the aggregation process single-type aggregates are synthesized in the system (Figure 7a) called the basic aggregates. As protein incubation time increases, larger size aggregates are synthesized in the system accompanying smaller ones (Figure 7b-d) called superaggregates. The change in light scattering intensity of TMV CP solution and dynamics of basic aggregate and superaggregate size change are presented in Figure 8. Hydrodynamic radius of basic aggregates increases with the aggregation time, at long times reaching the maximum at 130 nm. Interestingly, superaggregates are formed in the system after long time (over 20 min), at which the light scattering intensity approaches its maximum. The suggestion of superaggegate formation by adhesion of basic aggregates was confirmed by the following experiments. Times of incubation were selected, at which basic aggregates only were synthesized (t < 20 min). After cooling the solution down to room temperature the particle distribution by size was estimated by the dynamic light scattering method. It has been found that

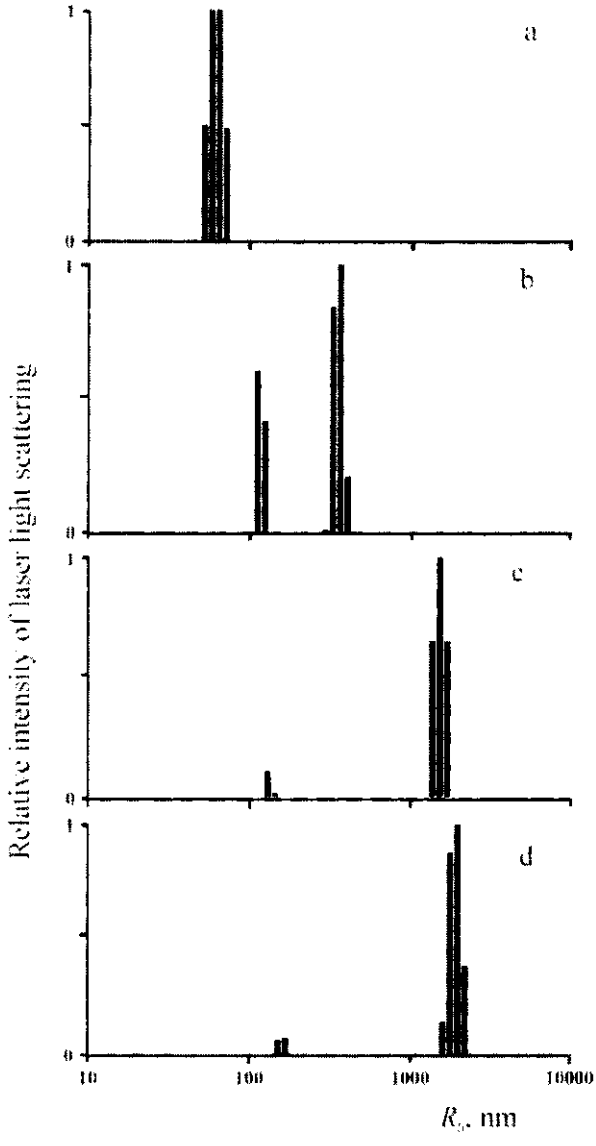

Figure 7. The aggregation of tobacco mosaic virus (0.1 mg/ml) at 52°C (50 mM Na-phosphate buffer, pH 8.0) [63]. Particle distribution by sizes recorded for the following incubation times (min): *a* – 10, *b* – 22, *c* – 45, *d* – 75

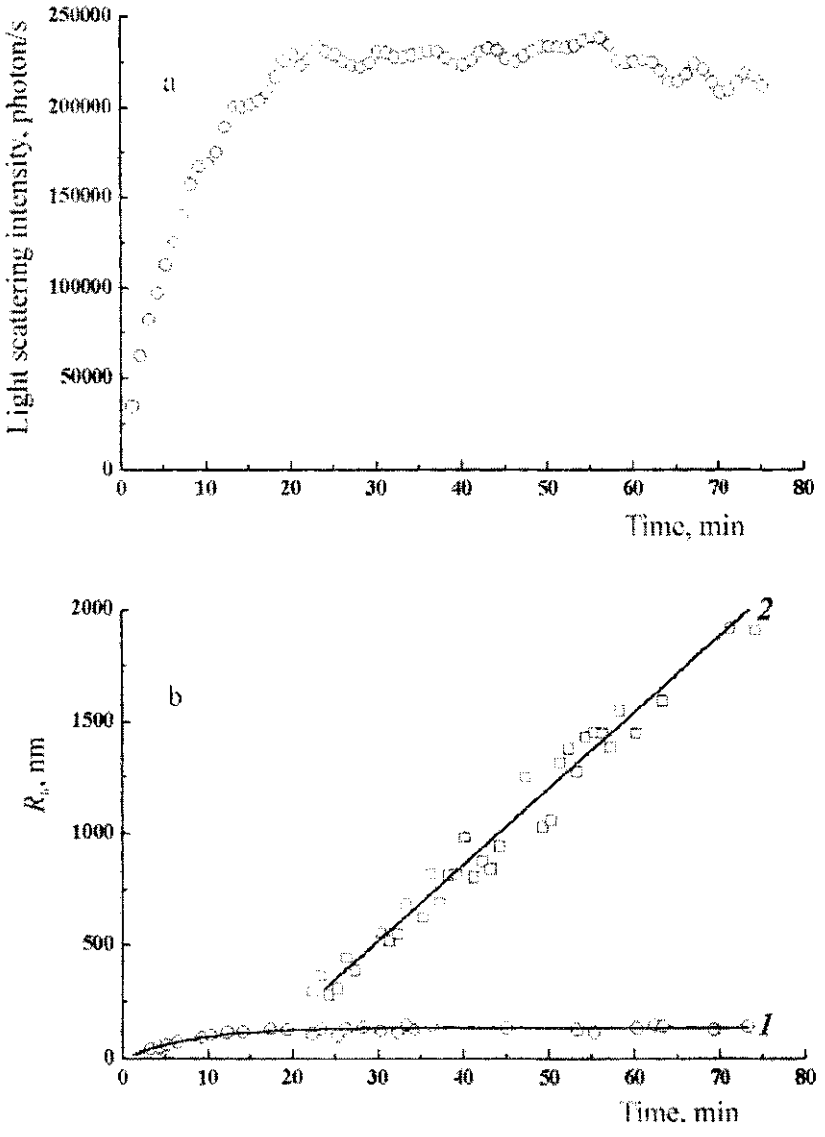

Figure 8. Kinetics of heat aggregation of tobacco mosaic virus (0.1 mg/ml) at 52°C (50 mM Na-phosphate buffer, pH 8.0) [63]. *a* – Time dependence of light scattering intensity; *b* – Time dependence of hydrodynamic radius (R_h) of basic aggregates (1) and superaggregates (2)

besides basic aggregates, cooled down solvent also shows superaggregates [63]. This means that basic aggregates are unstable and adhere to one another forming larger particles. The study of yeast alcohol dehydrogenase aggregation kinetics by dynamic light scattering method (56°C; 0.05 M Na-phosphate buffer, pH 7.4; 0.1 M NaCl) also showed formation of basic aggregates and occurrence of superaggregates after some time [64].

The formation of the aggregates of two types (basic aggregates and superaggregates) testifies about the existence of two main paths for protein aggregate propagation. For the aggregate system including heat aggregation, the *construction material* for growing aggregates is denatured protein. After formation of nuclei, the aggregate propagates by attaching denatured protein molecules (D) to the aggregate formed (A_i):

$$A_i + D \rightarrow A_{i+1}. \tag{21}$$

The aggregate propagates until full exhaustion of denatured (and, consequently, native) form of the protein. Obviously, such way of aggregation leads to formation of aggregates sized approaching some maximal value. At the given initial concentration of protein, the maximum size of basic aggregates is unambiguously defined by nucleus formation site concentration in the system.

The second path of the protein aggregate propagation is adhesion of previously formed aggregates:

$$A_i + A_j \rightarrow A_{i+j}\,. \tag{22}$$

Finally, this path of the aggregate propagation leads to formation of aggregates, sized within 1 – 2 μm. So big aggregates are inclined to precipitation. It should be noted that for definite proteins the aggregates formed adhere to one another already at early stages of the aggregation processes. Hence, under these conditions, no separation into basic aggregates and superaggregates are observed. The dynamic light scattering method registers aggregates of the single type (monomodal distribution). For example, this type of protein aggregation includes thermal aggregation of β-crystalline from bovine lens [65].

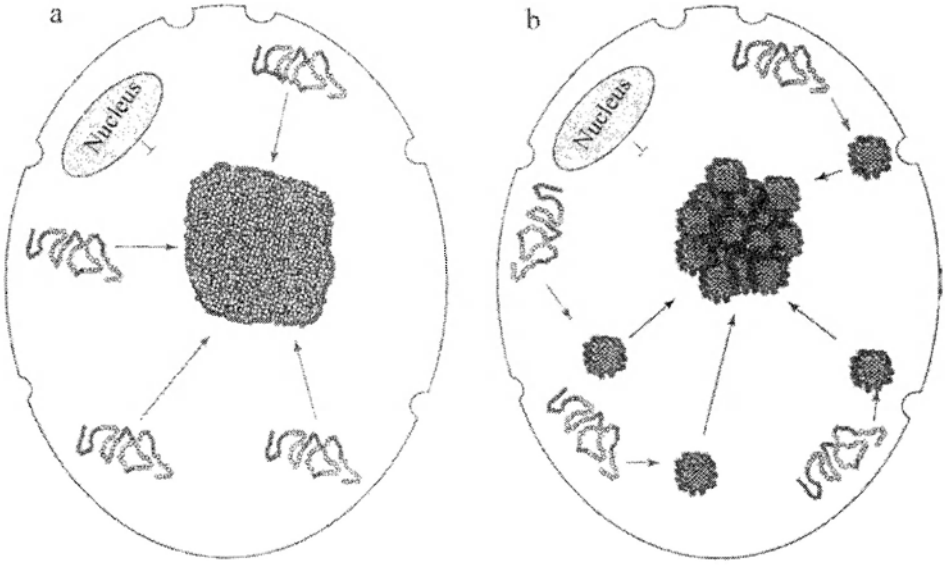

Figure 9. Two paths for protein aggregate propagation in a cell [66]

a – Nonnative protein shapes diffuse from the occurrence sites to precipitation points with inclusion body formation;

b – Nonnative protein shapes form aggregates of small size at the cell periphery. The formation of large aggregates (*aggregates of aggregates*) happens via adhesion of diffusing small-size aggregates or their directed transport with participation of microtubules

It is of interest that formation of big protein aggregates in the cell may be carried out by two different mechanisms [66], identical to those discussed above. Primarily, in prokaryotes improperly folded forms of protein create nucleation sites and, further on, aggregates propagate by adding nonnative forms of protein to separately propagating aggregates. In this manner, inclusion bodies are formed (Figure 9a). Primarily, relatively small-sized aggregates are formed in the living cells, which then adhere to one another forming larger aggregates – the "*aggregates of aggregates*" (Figure 9b). A microtubule system participates in the displacement of small-size aggregates.

CONCLUSION

Crowding conditions existing in the cell intensify aggregation of partly folded protein molecules, produced by folding of newly synthesized polypeptide chains or partly unfolded states of proteins converted from native states at stresses of different origins. The study of protein aggregation kinetics *in vitro* allows determination of the main regularities of the protein aggregate formation. Relatively low temperatures and relatively high protein concentrations promote for realization of the kinetic mode of aggregation, at which unfolding of protein molecule is the rate-limiting stage of the general aggregation process. Relatively high temperatures and relatively low protein concentrations promote for realization of the aggregation kinetic mode with the protein aggregate propagation representing the rate-limiting stage of the general propagation process. The results of investigation of protein aggregation kinetics by dynamic light scattering method allow for a conclusion about existence of two paths for propagation of protein aggregates. One of the paths is realized via addition of nonnative protein molecules to already formed nuclei, which may be described in the framework of the theory of chemical chain reactions invoking the notion of the aggregate propagation sites, equivalent to active sites in the chain reactions. As aggregates propagate according to this path, the size of aggregates approaches the maximum with exhaustion of nonnative protein shape. The second path of the protein aggregate growth is mutual adhesion of previously formed aggregates. This path leads to production of large aggregates inclined to precipitation.

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NIKOLAI M. EMANUEL – THE ORIGINATOR OF BIOCHEMICAL INVESTIGATIONS IN THE FIELD OF CHEMOTHERAPY IN N.N. SEMENOV INSTITUTE OF CHEMICAL PHYSICS, RAS

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In 1957, after PhD courses in A.N. Bach Institute of Biochemistry, AS USSR, Academician A.N. Belozersky introduced me to N.M. Emanuel and recommended for working in the field of oncology.

When Academician D.G. Knorre had left to Novosibirsk, N.M. Emanuel suggested me to head a small group of scientists and arrange investigations of biochemical mechanisms of new potentially active antitumor compounds among antioxidants.

In 1960, the first team work of our group was published [1]. During next six years we studied the effect of some antioxidants (propyl gallate, dibunol, etc.) on biosynthesis of cellular proteins, DNA and RNA in tumor cells *in vivo* (in rats and mice with grafted experimental tumors) and *in vitro* (suspensions of tumor cell cultures). It has been positively determined that antioxidants strictly suppress RNA and protein synthesis in tumor and actively proliferating cells in bone marrow and spleen. Messenger RNA (mRNA) fraction was found the most sensitive to inhibiting effect of intermediate ionol oxidation forms in Ehrlich's ascetic tumor cells [2, 3].

As shown in the model RNA-polymerase system, propyl gallate reacts with SH-groups of RNA-polymerase and suppresses RNA synthesis the transcription elongation stage [4].

In 1965, N.M. Emanuel suggested investigations of the mechanisms of a new class of N-alkyl-N-nitrosourea (ANU) compounds. This suggestion was based on a broad experimental material of animal tumors showing high antitumor activity of one of the compounds of this class – N-methyl-N-nitrosourea (MNU) [5].

Later on, the results of these investigations were generalized in our joint monograph [6] and some other publications.

It has been found that ANU antitumor activity is stipulated by a combination of their alkylating and carbamyling activities. Under conditions

approaching physiological ones, these compounds easily degrade to alkylcarbonium ions and isocyanates. The tests *in vivo* in mice having solid hepatoma 22a and *in vitro* in lymphatic leucosis L1210 cells indicated the highest sensitivity of DNA synthesis, partly stipulated by DNA matrix property variation, incubated *in vitro* with N-propyl-N-nitrosourea (PNU), to the damaging action of PNU.

Most probably, suppression of protein synthesis is mediated, because it appears only at high PNU doses and long-term effects [7, 8]. PNU in 10^{-4} M concentrations *in vitro* inhibits synthesis of arginine-saturated histones and activates synthesis of lysine-saturated histones; meanwhile, synthesis of salt-soluble proteins does not change [9].

Further on, the attention of our group was aimed at the studies of properties of RNA-polymerase activity in nuclei, extracted from tumor cells after ANU injection in therapeutic doses to mice–tumor carriers. It has been shown that ANU activates pRNA synthesis and suppresses iRNA synthesis [10]. MNU and PNU induce 40% suppression of soluble RNA-polymerase activity *in vitro*. It has been suggested that these ANU affect initial stages of transcription (DNA-enzyme complex formation and initiation). Distortion of DNA matrix activity was observed only in case of long-term DNA incubation with ANU [11].

Since the middle 1970 s, a plentiful intensification of biochemical studies in the field of chemotherapy of tumors was observed and new experimental models and investigation methods were developed.

The study of MNU effect on the protein-synthesizing system indicates that momentary therapeutic dose of this compound, injected to mice with hepatoma 22a, cause changes in polyribosomal complex and cytosol. These disturbances are observed 5 – 15 hours after injection of the compound to livers of mice-tumor carriers. Then these damages are completely reduced, whereas in hepatoma 22a cells they are preserved for, at least, 48 hours that testifies about definite selectivity of MNU effect on the protein-synthesizing apparatus [12]. The results of these investigations are confirmed by quick elimination of radioactivity from DNA, RNA and proteins of liver and spleen, and preservation of radioactive label in hepatoma cells after MNU–^{14}CO injection to mice – tumor carriers [13].

It has been suggested that renewal processes of macromolecules and lipids are very important for explanation of different sensitivities of normal and tumor cells to ANU effect. It is also suggested that the difference in elimination

rates of damaged macromolecules and lipids is associated with their turnover, quick in normal and slow in tumor cells.

Henceforth, a complex of fundamental studies of ANU effect on RNA synthesis has been implemented. It has been found that contrary to other ANU, MNU *in vivo* inhibits pro–mRNA synthesis and pRNA processing. For model RNA-polymerase systems, it is indicated that MNU directly affects RNA-polymerase enzyme and inhibits DNA-enzyme complex formation and transcription initiation. This confirms the above suggestion [14].

For the first time, multiple forms of DNA-dependent RNA –polymerase (I, II, III) were extracted from leucosis L1210 cells, purified and characterized. A complex study of chromatographic and catalytic properties of RNA-polymerase from leucosis L1210 cells allowed for unambiguous confirmation of their full conformity to three forms of this enzyme, detected in other tumor and normal eukaryote cells.

Most interestingly, leucosis cells shows RNA-polymerase I activity 10 times higher, approximately, than liver and spleen cells of healthy animals [15]. Later on, this observation was confirmed by S.T. Jacob and was included in all reviews and monographs on transcription.

Injection of momentary therapeutic ANU doses to mice with leucosis L1210 induces typical two-phase disturbance of transcription processes as a whole (nuclei, permeable cells). Primary inhibition of pre-pRNA and gyRNA synthesis in nuclei and permeable Leucosis L1210 cells proceeds during 2 – 4 h as a result of damaging ANU chromatin, RNA-polymerase molecules and regulatory transcription factors. Generally, secondary inhibition of RNA synthesis is a result of chromatin damage and proceeds until the observations end (48 h).

The depth and duration of the second phase of pre-pRNA and gyRNA synthesis in the system of nuclei and permeable cells correlates with antitumor activity of ANU increasing in the sequence:

$$\text{streptosotocine} \rightarrow \text{MNU} \rightarrow \text{BCNU} \rightarrow \text{chlorosotocine.}$$

Therefore, the damage of tumor cell transcription apparatus is directly related to molecular mechanisms of ANU antitumor action [16, 17].

Comparative study of the type of some ANU (MNU, streptosotocine, chlorosotocine, BCNU) effects on DNA synthesis in tumor and normal dividing cells indicated direct correlation between antitumor activity of some

ANU and the depth and duration of DNA synthesis inhibition in melanoma B16 cells [17].

By the early 1980 s, it became obvious that experimentally determined extremely high antitumor ANU activity is not fully realized in cancer treatment. One of the reasons of this event is the existence of resistance to ANU, inborn or acquired during treatment. Meanwhile, contrary to the majority of antitumor compounds, multiple drug resistance is not typical of ANU. Moreover, there is not full cross resistance among close ANU analogues [18]. In tests with leucosis L1210/0 (sensitive strain) and L1210/MNU (strain with acquired resistance to MNU), it has been shown that lysomustine, a compound from the ANU group, is equally effective for both strains [19]. Full cross resistance between MNU and BCNU was not also observed that allowed for replacing one compound by another during treatment in order to increase their therapeutic efficiency [20].

Centrifugation of leucosis cells indicated in neutral and alkaline saccharose velocity sedimentation gradient some differences in the rate of DNA damage occurrence and repair (single-stranded breaks, alkali-labile zones, intermolecular crosslinks), induced by MNU and BCNU [21].

A new stage of investigations in the field of drug resistance mechanisms in relation to ANU was initiated by discovery of a new enzyme (protein-transmitter), O^6–alkylguanine-DNA transferase (O^6–AGT). At present, it is found that ANU cytotoxicity is generally defined by the ability of alkylcarbonium ions, the products of ANU degradation under almost physiological conditions, to alkylate DNA and produce a minor adduct O^6–alkylguanine (O^6–AG). The lifetime of this modification is of great importance. In turn, it depends on O^6–AGT concentration in tumor cells [22, 23]. High concentration promotes occurrence of resistance to ANU. The uniqueness of the mechanism of this enzyme is in its role of alkyl group transmitter from O^6–alkylguanine in DNA structure to self cysteic residue in eukaryote cells. As a result, N-alkyl cysteine is formed and unmodified guanine is reduced in DNA.

The majority of test tumor strains show very low concentration of this enzyme, therefore, they are highly sensitive to ANU. Among human tumor cell cultures (120 tumor lines have been analyzed) only 20% possess low O^6–AGT concentration. It is apparent that these differences induce different antitumor activity of ANU in experiments and clinical tests [23].

We have found that in leucosis L1210 cells, resistant to aranose and ACNU, concentration of this protein–transmitter is 8 times higher than in the

initial sensitive strain L1210/0 [24, 25]. After injection to mice with leucosis L1210 and L1210/A, O^6–AGT activity was determined in lysates of extracted leucosis cells. Moreover, O^6–AG adducts in DNA samples were determined using HPLC method. It is shown that already two hours after injection to mice with leucosis L1210/A O^6–AGT activity is abruptly lost and then restored only 48 h later. Exhaustion of this enzyme pool in leucosis cells resistant to aranose proceeds with O^6–AG adduct concentration in DNA [25].

As a consequence, initially high O^6–AGT concentration in cells resistant to aranose is responsible for quick repair of O^6–AG adducts in DNA and the loss of aranose cytotoxicity. In cells sensitive to aranose O^6–AGT concentration is insufficient for repair of a great quantity of O^6–AG, induced by aranose. Under these conditions, aranose cytotoxicity is conserved.

However, explanation of the reasons for drug resistance to ANU must not be only limited with O^6–AGT concentration. It has also been shown that ANU resistant cells display DNA-polymerase repair enzyme activation and increase of reduced glutathione concentrations [25].

The studies discussed in the present review were initiated and headed by Nikolai M. Emanuel.

Recently, we were studying biochemical mechanisms of many other active antitumor compounds as, for example, cis-dichlorodiamino platinum derivatives (cisplatin, cycloplatam, etc.)

I am greatly thankful to Nikolai Markovich Emanuel for permanent interest in our researches and suggested direction in the studies, which are developing still.

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ANTITUMOR EFFECTS OF NITRIC OXIDES AND NO-DONORS

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In the development of ideas by N.M. Emanuel about desirability of antioxidant use in chemotherapy of tumors stable nitroxyl radicals and compounds representing NO donors were studied in the experiment.

Their ability to increase sensitivity of malignant tumors to antitumor compounds, inhibit growth of metastases and suppress development of drug resistance is shown.

Keywords: experimental chemotherapy, malignant tumors, nitroxyl radicals, NO donors

In the late 1950ies – early 1960ies in the Institute of Chemical Physics N.M. Emanuel formulated the concept of free-radical process role in various pathologies, including development of malignant tumors [1, 2].

In the same period, the possibility of using antioxidants as antitumor agents was shown [3, 4].

Later on, the application of nitroxyl radicals as powerful antioxidants for modification of antitumor cytostatics was suggested.

For the first time, the antitumor effect of stable free radicals was observed in 1964 for nitroxyls and triarylhydrazyls. This initiated systematic investigations of their pharmacological activity, carried out in Institute of Chemical Physics and, later on, in Institute of Problems of Chemical Physics, Russian Academy of Sciences.

Nitroxyl derivatives of antitumor compounds, related to the class of antibiotics, alkylating substances, antimetablites, and nitrosoalkylureas were synthesized and tested [5 – 8].

Experimental data show that in the majority of cases nitroxyl modification leads to a significant decrease of total toxicity and increase of

selectivity of antitumor compounds. In these investigations ruboxyl (emoxyl) compound, which is nitroxyl derivative of daunorubycin – antitumor anthracycline antibiotic – was synthesized (Figure 1). It is the most perspective for practical application.

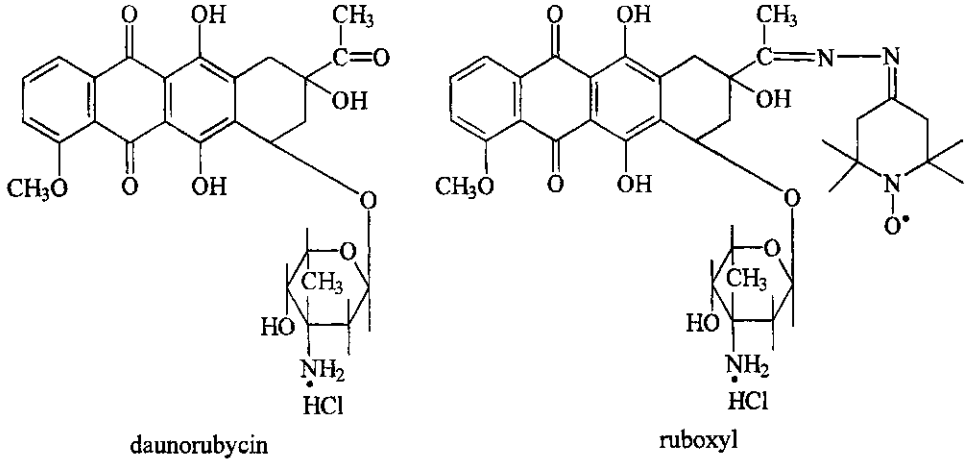


Figure 1. Structural formulae of daunorubycin and its nitroxyl analogue ruboxyl

As a rule, total toxicity of drugs correlates with manifestation of specific secondary effects. High cardiotoxicity is typical of anthracycline antibiotics. Comparative analysis of electrocardiograms of animals treated by equivalent doses of daunorubycin and ruboxyl indicated the absence of cardiotoxic action of the latter compound [9, 10].

It is known that cardiotoxicity of anthracycline antibiotics is related to the induction of reactive oxygen species in myocardium. Possessing antioxidant properties, nitroxyl acts as free radical trap [11]. Taking into account that in the experiments searching for new antitumor agents of special interest is determination of human tumor sensitivity to these compounds, numerous studies using the method of human tumors transplantation beneath kidney capsule of normal immunocompetent animals were carried out [12].

As an example, Figure 2 shows data on effectiveness of ruboxyl and other anthracyclines at large intestine cancer. Clearly by activity ruboxyl exceeds daunorubycin and adriamycin. Analogous data were obtained for cellular cultures of human large intestine cancer [13].

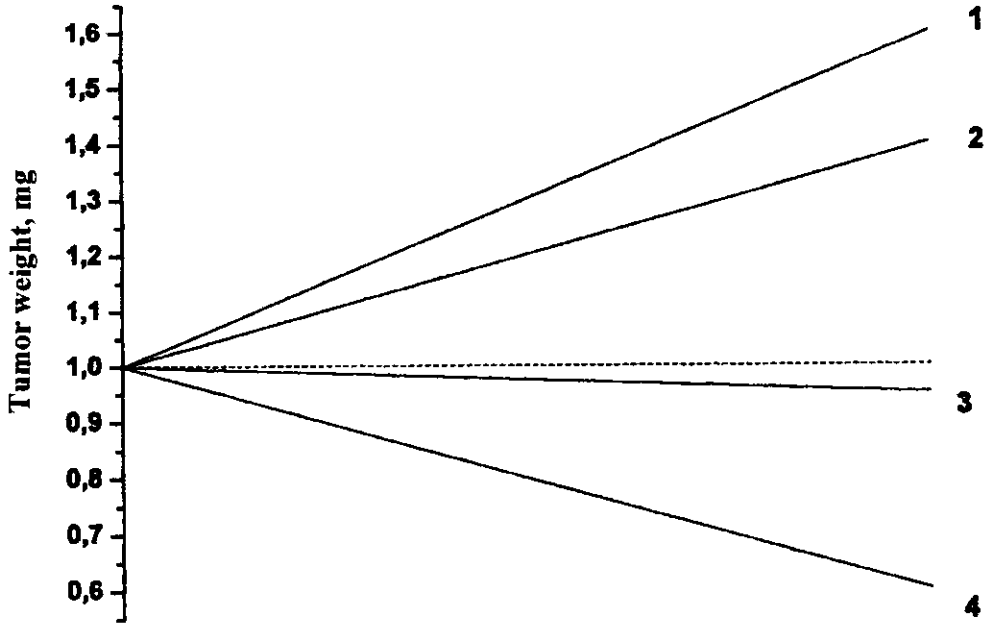


Figure 2. Sensitivity of human large intestine tumor heterotransplant to anthracycline antibiotics
1 – tumor growth in control; 2 – tumor growth after daunorubycin injection; 3 – tumor regression after andriamycin injection; 4 – tumor regression after ruboxyl regression

As is known, therapeutic and toxic effects of drugs mostly depend on their concentration and impact duration. Therefore, pharmacokinetic aspects represent the required unit in synthesis of new compounds. In this connection, nitroxyl fragment of the drug molecule may be the marker for studying pharmacokinetic regularities of its behavior in the organism using ESR-spectroscopy method [14].

The study by ESR method indicated differences in pharmacokinetics of ruboxyl in intact and tumor-carrying animals [15]. Analysis of the results shows that the marker content in some organs of animals is much higher.

The ESR method is used for detection of all metabolites containing the radical. Enzymatic hydrolysis of ruboxyl by N=C with formation of nitroxyl component is also possible. Parallel study of ruboxyl and nitroxyl radical indicated their pharmacokinetic differences, which allow a suggestion that in

continued experiment ruboxyl is not subject to hydrolytic decomposition (Figure 3).

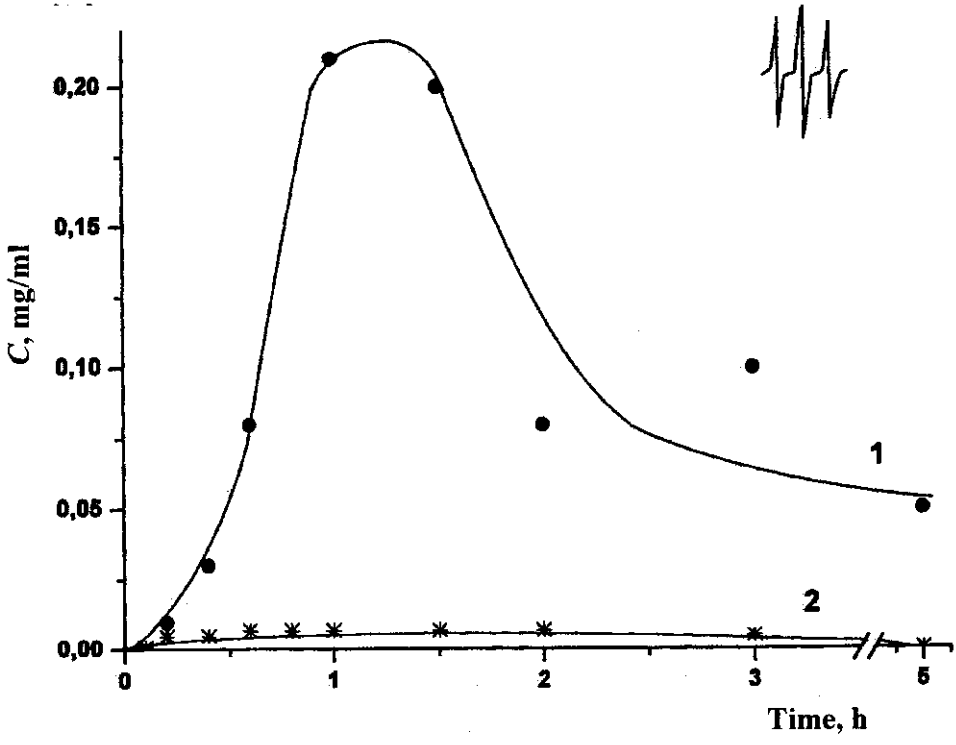


Figure 3. The change in marker concentration in bile of normal animals after injection of ruboxyl (1) and nitroxyl radical (2)

The results of detailed preclinical study of ruboxyl allowed for its recommendation for study in oncological clinics.

Data on phases I and II of clinical tests of the compound show that ruboxyl possesses antitumor activity; moreover, the spectrum of tumors, for which the effect from ruboxyl application was observed, differs from the spectrum of tumors sensitive to rubomycin.

There are some cases of full and partial remissions at breast cancer, small-cell lung cancer, Kaposi's sarcoma, and non-Hodgkin's lymphoma. Ruboxyl application is not accompanied by significant secondry effects [16, 17].

Also, data on the ability of nitroxyl radicals to act as detoxicants is on a definite interest. For example, injection of absolutely lethal dose of antitumor cytostatics (6-mercaptopurine, cyclophosphane, sarcolysine) together with nitroxyl radical decreases toxicity of compounds, which is manifested by occurrence of survived animals [18].

As an example, Figure 4 shows data characterizing death kinetics of animals after injection of cyclophosphane individually and in combination with nitroxyl radical. Similar data were also obtained for other compounds.

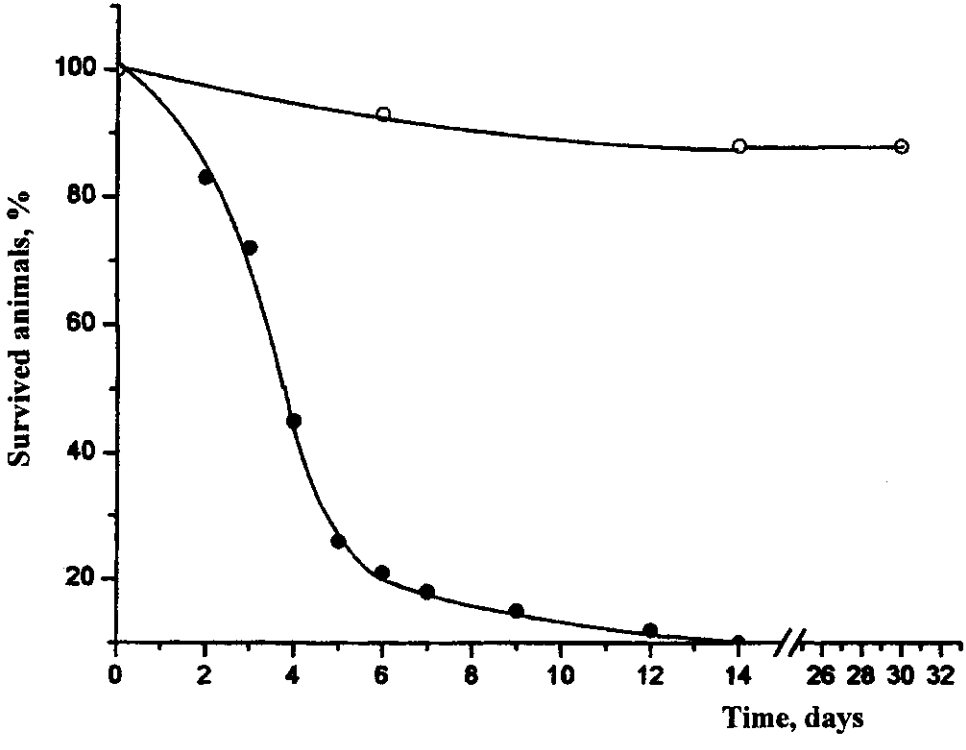


Figure 4. Kinetics of animal death after injection of absolutely lethal doses of cyclophosphane (1) and a combination of cyclophosphane and nitroxyl radical (2)

For the study of this effect by ESR method, the change in signal intensity of cytochrome P450 enzyme in liver after injection of cytostatic in toxic dose individually or in combination with nitroxyl. As is known, the

functional role of cytochrome P450 is associated with participation in metabolic processes providing for detoxication of antibiotics. Injection of cytostatics leads to an abrupt decrease of oxidized form of cytochrome P450. Addition of nitroxyl radical normalizes the enzyme level (Figure 5). These results confirm the suggestion that detoxicating action of nitroxyls is associated with activation of anabolic processes in liver [19].

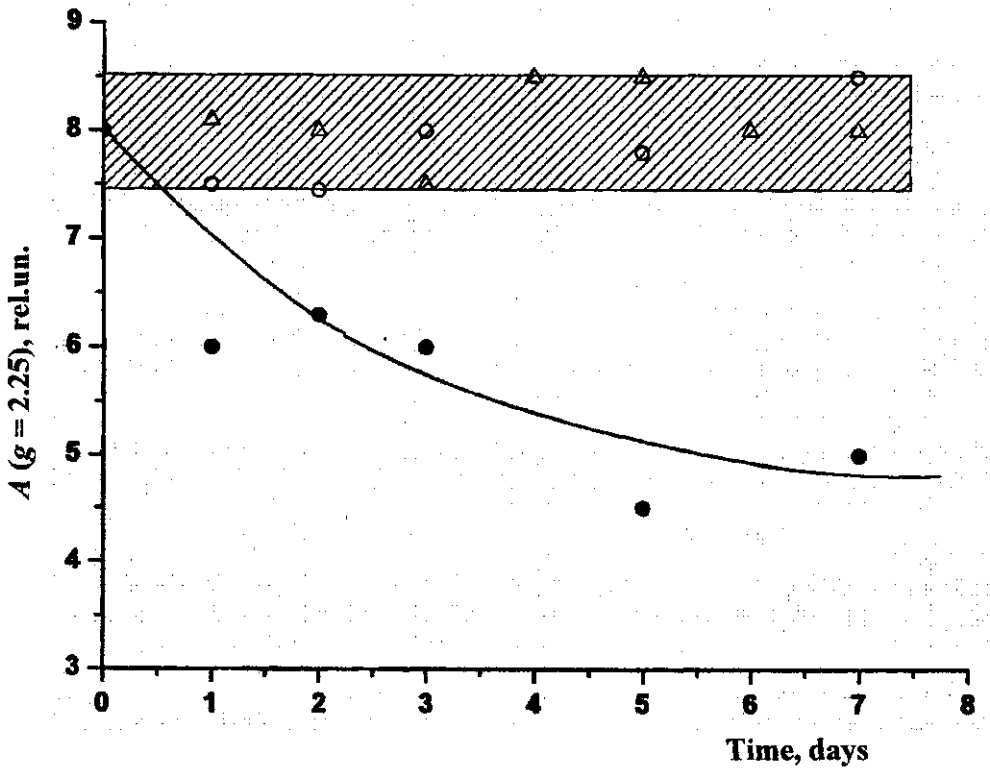


Figure 5. Dependence of ESR signal intensity for oxidized form of cytochrome P450 in liver of experimental animals on time after injection:
• – cyclophosphane; o – nitroxyl radical; Δ – cyclophosphane combined with nitroxyl radical

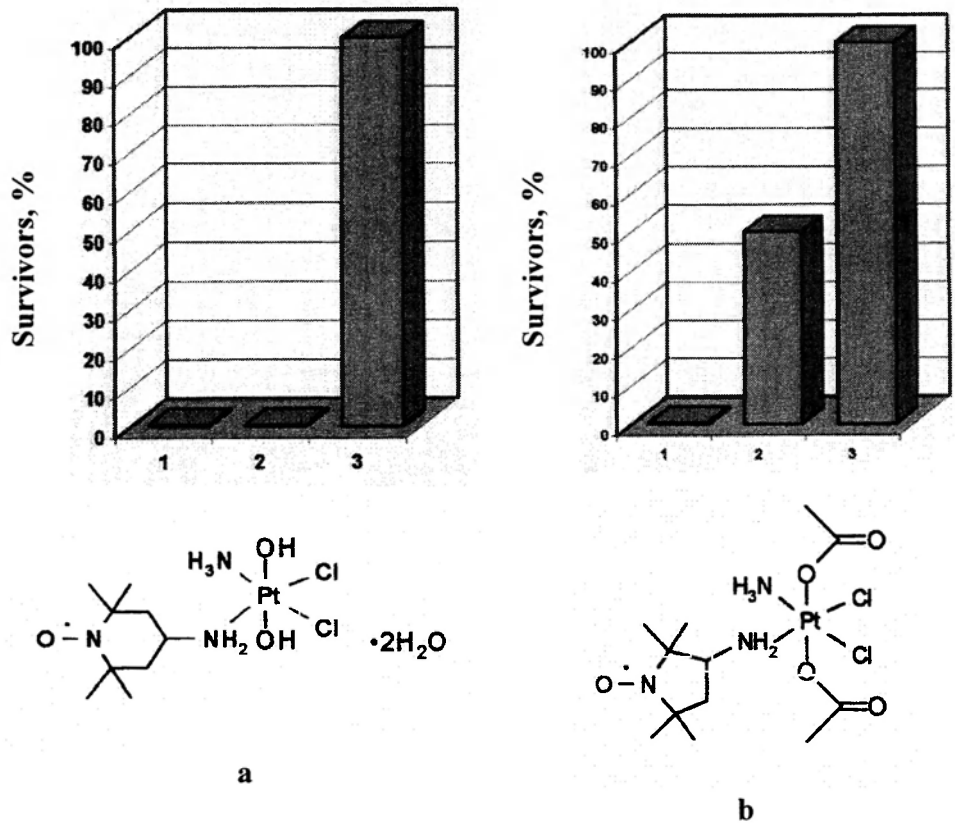


Figure 6. Synergic interaction between antitumor drugs of platinum group and platinum-nitroxyl complexes (leukemia P388)

a. 1 – platinum-nitroxyl complex, dose 1.35 mg/kg, day 1 – 7; 2 – cysplatin, dose 0.6 mg/kg, day 1 – 7; 3 – combined injection of cysplatin and the complex in the same doses simultaneously, day 1 – 7

b. 1 – carboplatin, dose 1.8 mg/kg, day 1 – 5; 2 – platinum-nitroxyl complex, dose 2.3 mg/kg, day 1 – 7; 3 – carboplatin (1.8 mg/kg), day 1 – 5, combined with platinum-nitroxyl complex (2.3 mg/kg), day 1 – 7

Thus, differences in biological properties of antitumor drugs and their nitroxyl analogues are clearly observed. Nitroxyl modification of cytostatics leads to creation of principally new antitumor drugs of the combined type of

action, the activity of which is implemented due to various functional groups in the compound structure. Acting in a manner similar to alkylating agents, intercalators, and antimetabolites, these compounds may also represent active antioxidants. Apparently, antioxidant activity stipulates their modifying effects. In these investigations, a sequence of platinum (II) and (IV) complexes with aminonitroxyl radicals was synthesized [20, 21]. Platinum compounds (cysplatin, carboplatin) are widely used in chemotherapy of tumors. However, their great disadvantage is high toxicity [22].

Experimental studies of aminonitroxyl platinum complexes possess several advantages: total toxicity decrease and, in some cases, increase of antitumor activity compared with cysplatin. The most interesting results were obtained in the case of leukemia P388 combined treatment with cysplatin in combination with aminonitroxyl derivative of Pt (IV) complexes. The efficiency criterion was the number of recovered animals (lived up 60 days or more after tumor transplantation). Each compound was used in very low, ineffective doses. Figure 6a shows that individual injection of cysplatin and its nitroxyl analogue did not saved animals with leukemia P388. At combined injection of these compounds, 100% of animals were recovered.

To put it differently, a strong synergic interaction of cysplatin and platinum-nitroxyl complexes was observed. Such effect, though lesser expressed, was observed at cysplatin replacement by carboplatin. In this case, there were no survived animals and the efficiency was estimated by an increase of the average life duration of treated animals compared with the control group (Figure 6b).

The investigations in the field of experimental oncology, initiated by N.M. Emanuel, are currently developed in IPCP RAS. In particular, they include studies of nitric monooxide donors as potential antitumor drugs.

Multiple types of biological activity of nitric oxide are known well [23]. Some of them directly relate to tumor growth processes. For example, NO participates in the antitumor effect, mediated by macrophages. It is a strong antioxidant and decreases hypoxia in tumor tissues [24, 25].

These data allowed a suggestion that potential nitric monooxide donors may be used at oncological diseases and initiated experimental studies of these compounds [26].

In the Institute of Problems of Chemical Physics, RAS, nitric monooxide donors of various chemical structures were synthesized and tested. The results of their study on metastasizing tumor models show that they all, to a different extent, manifest antimetastatic activity. For the sake of examples,

Figure 7 and Table 1 show data obtained for thiosulfate iron complex [27, 28], platinum and palladium complexes with ligands – NO donors [29].

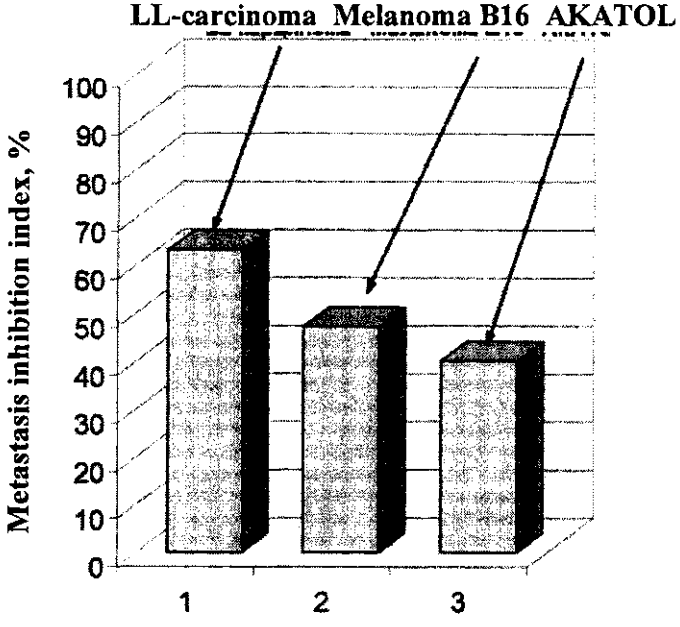


Figure 7. Antimetastatic activity of thiosulfate iron complex

It is indicated that all studied compounds, to a different extent, inhibit metastasis of various experimental tumors. Melanoma B16 was found the most sensitive to the action of nitric oxide donors. Also, it is of interest that the metal complexes used preserved activity at both intraperitoneal and peroral injection.

Recently, antimetastatic effect was also detected for nitrotriazole (sanazole), provided by the University of Kyoto (Japan) (Figure 8) [30].

It is known that endogenic NO participates in homeostasis of Ca^{+} ions [31], the break of which prevents thrombus formation and adhesion of metastatic cells to capillary walls. In this case, at the current stage metastasis is terminated [32].

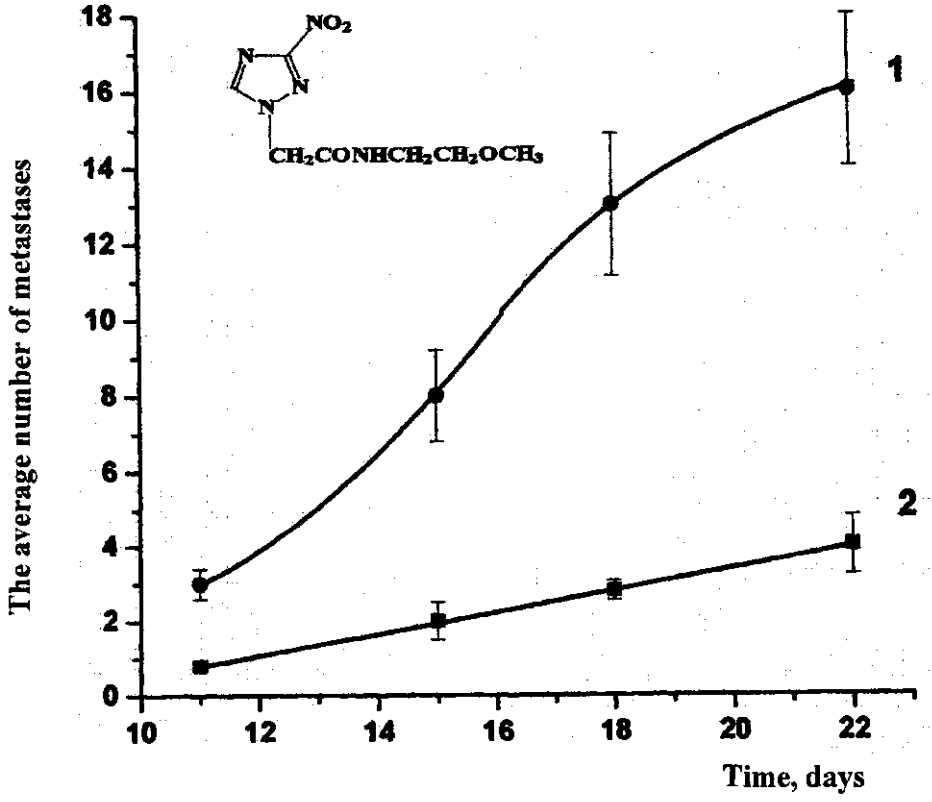
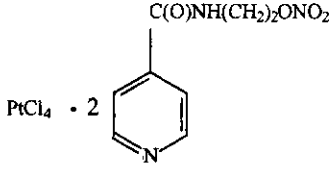
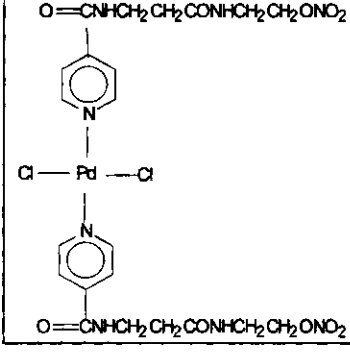


Figure 8. Kinetic curves of increase of the number of metastases in liver with AKATOL tumor:
1 – in control; 2 – after sanazole injection

Table 1

Antimetastatic activity of platinum group metal complexes with ligands – NO donors

Compound formula	Single dose / Total dose (mg/kg)	IIM%		
		Melanoma B-16	LL-carcinoma	Acatol adeno-carcinoma
	116/928 c/b	95		
	300/1500 p/os	99		
	120/840 c/b		62	
	200/1000 p/os			62
	200/1000 c/b			61
	200/1400 c/b	96		
	200/1400 p/os	98		
	150/1050 c/b		60	

The study of NO donor sanazole influence on active transport of Ca^{2+} ions, implemented by Ca^{2+}–dependent Mg^{2+}–activated ATPase of sarcoplasmic reticulum, shows that the compound inhibits the transport function of the enzyme even in low concentrations (Figure 9). In higher concentrations the inhibition reaches 100% [33]. Apparently, antimetastatic effect of NO donors is related with the balance break between intra- and extracellular Ca^{2+}.

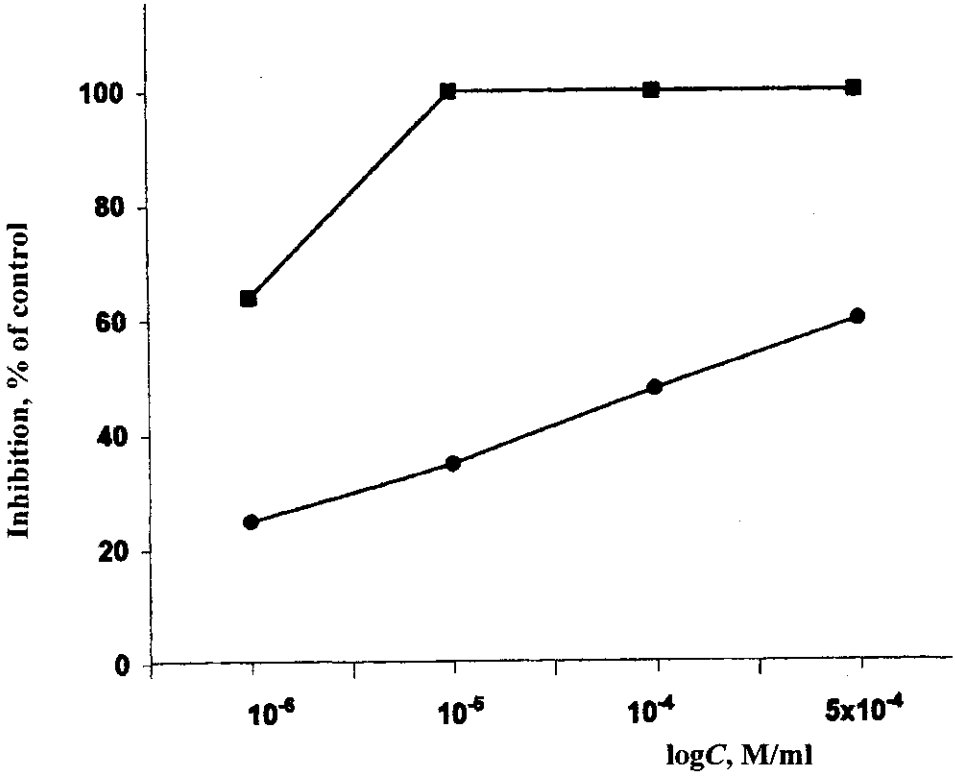


Figure 9. The change of hydrolytic and transport activity of Ca^{2+}–ATPase in dependence on sanazole concentration

Neoangiogenesis may be another target of nitric oxide action in realization of antimetastatic effect. It is shown that some NO donors inhibit cytokine – the induced neovascularization [34].

The participation of NO in inhibition of development of metastases is indirectly confirmed by data on reverse correlation between NO-synthase activity and metastatic potential [35].

The urgent task of studied in the field of tumor chemotherapy is development of methods increasing effectiveness, which are used in drug clinics.

When studying activity of the above NO donors combined with antitumor cytostatics, a hemosensibilizing effect of nitric monooxide was clearly observed.

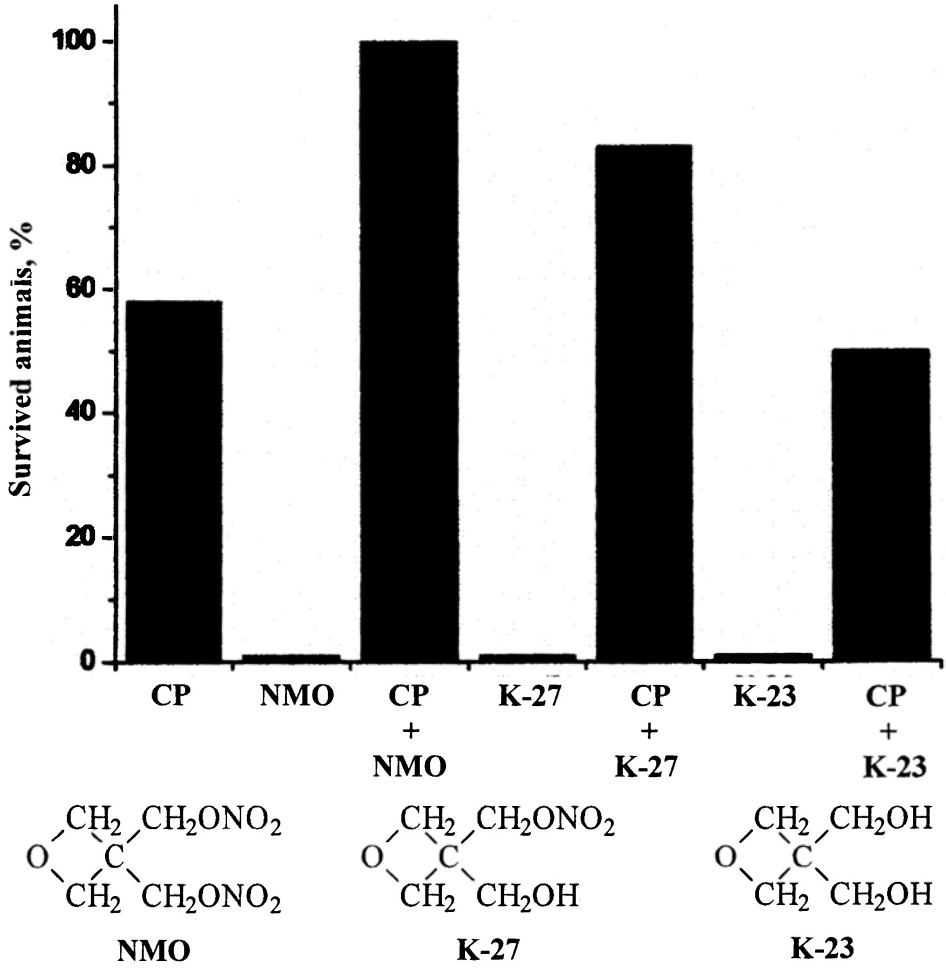


Figure 10. Dependence of hemosensibilizing effect of nitric oxide donors on the presence of nitrate groups in the compound structure (leukemia P388)

The combined injection of cytostatics in low, ineffective doses leads to a significant increase of the antitumor effect [36].

Nitric monooxide participation in sensibilization of tumors under chemotherapeutic influences may be proved in comparative study of this effect dependence on the presence of nitrate groups in the structure of chemical compound (Figure 10). At the therapy of animals with leukemia P388 by

cyclophosphane, an addition of compound possessing two nitrate groups (NMO) in the structure increases activity of cyclophosphane to 100%. The addition of preparation with a single nitrate group (K-27) decreases sensibilizing effect of NO donor. As nitrate groups are replaced by hydroxyl ones (K-23), this effect is absent. In particular, the sensibilizing action of nitric monooxide donors is associated with the ability of NO to inhibit DNA repair enzymes in the tumor cell [37]. Moreover, owing to dilatation of vessels induced by nitric oxide, a large quantity of drug may be delivered to the tumor.

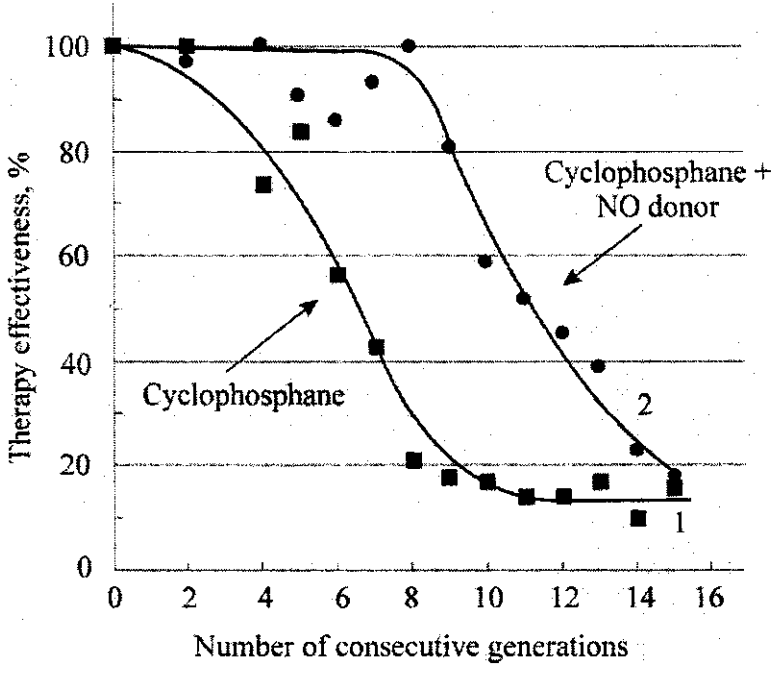


Figure 11. Delay in development of drug resistance of leukemia P388 to cyclophosphane at combined injection with a nitric oxide donor

It is known that nitric oxide induces apoptosis in tumor cells, which may also increase the effect of antitumor drugs [38, 39].

One of the most urgent problems in oncology is drug resistance of malignant tumors, which is formed during the treatment. Therefore, of special interest are data on nitric oxide donor effect on development of this process. At monotherapy of animals with leukemia P388 by cyclophosphane, total resistance to is developed in 8th transplantation generation (Figure 11). As

cyclophosphane is combined with NO donor, 100% sensitivity at 8th generation is still preserved, and full resistance is observed for 14th transplantation generation only.

Also, nitric oxide donors increase sensitivity of tumors with already formed phenotype of multiple drug resistance. This effect depends on the number of ONO_2 groups in the compound structure that indicates NO participation in this effect.

Thus the data obtained allow for considering NO donors as tumor drug resistance modulators. It is possible that these effects are implemented via the system of glutathione and associated enzymes (generally, glutathione-S-transferase). As is known, besides other factors, such system contributes into development of resistance to the drugs with alkylating type action [40]. Also, there are data indicating that glutathione-S-transferases are inactivated by reactive nitric oxide species [41, 42]. Moreover, it has been shown that replacement of endogenic NO by exogenic nitric oxide increases accumulation of protein p53 – the tumor suppressor – and activates apoptosis, which may also contribute into NO modulating activity [43].

Thus three types of biological activity typical of nitric monooxide donors, related to the influence on the tumor process, were determined in the experiment:

1) antimetastatic effect;
2) ability to increase tumor sensitivity to antitumor cytostatics;
3) ability to modulate drug resistance of tumors.

Taking into account the above facts, one may unambiguously indicate the ability of NO donors to increase effectiveness of chemotherapeutic agents of clinical use. It is also probable that introduction of NO donors to combined chemotherapy protocols may promote suppression of drug resistance development in tumors.

To conclude the article, the results of investigations performed indicate the desirability of clinical tests of nitroxyls and nitric monooxide donors and open broad prospects in creation of a new direction in the field of chemotherapy of tumors.

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NITROSOALKYLUREAS IN MODERN ANTITUMOR CHEMOTHERAPY

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In this country, the intensive development of antitumor chemotherapy as the independent scientific and medical branch began in the 1960ies. The significant contribution into development of this field was made by investigations, carried out in N.N. Semenov Institute of Chemical Physics (Moscow) and supervised by N.M. Emanuel since the late 1950ies.

In 1957, after reading the book "The progress in cancer studies", N.M. Emanuel became interested in the study of the tumor growth regularities and the problems of antitumor chemotherapy. He noticed that the tumor process proceeding bears a striking resemblance to the chain reactions. This induced a firm belief that the application of physicochemical and kinetic methods to investigations of tumor growth and influences on it discloses new prospects and possibilities for practical application of the results obtained.

To the end of his life N.M. Emanuel has not stopped working on these problems. As a result of this work, large-scale and remarkably new in approach, a new direction was founded, called physicochemical oncology [44]. It should be noted that primarily, in 1960 – 1970ies, these works were actively non-accepted by many scientists, including the leaders in traditional biology and medicine. However, usually, time puts everything in its rightful place – a plenty of new and unusual that has been discovered, studied and developed by N.M. Emanuel and his followers is now obvious.

Among these investigations, a special place is devoted to the works on creation and study of new effective antitumor drugs, which were always of special attention for N.M. Emanuel, which he was greatly satisfied with. It was clear that he deemed it duty to help medicine, and the drugs designed for routine treatment of patients – his greatest achievement.

One of the important results of these studies was creation of a highly effective antitumor drug – nitrosomethylurea (NMU), which is still included in the list of the most active anticancer means [45].

Nitrosomethylurea may be considered as the ancestor of a large group of antitumor drugs – nitrosoalkylureas (NAU), which currently form one of the main classes of chemotherapeutic medicines for treatment of tumors. All biological effects typical of this group of antitumor drugs are clearly demonstrated by biological properties of NMU.

In 1963, in the Institute of Chemical Physics, Academy of Sciences of the USSR, NMU antitumor properties were discovered, which initiated systematic investigations of this class of compounds. Meanwhile, special attention was paid to the studies of kinetic regularities of tumor growth affected by these compounds. The kinetic approach to estimation of antitumor properties of cytostatics, which N.M. Emanuel had suggested and developed, allowed for determination of many principal action features for preparations of this group.

Theoretic premise for discovering antitumor activity of NAU compounds was the concept of high probability of detecting new antineoplastic substances among biologically active compounds, first of all, among chemical mutagens. This concept was based on supermutagenic properties of class NAU compounds, discovered by I.A. Rapport [6, 25, 37].

The works in this predominant direction led to creation of a new original antitumor preparation – nitrosomethylurea (NMU), and development of physicochemical grounds of the biological action of NAU [45].

A significant efficiency of NMU and its analogues, then suggested in the USA: BCNU (carmustine), CCNU (lomustine), MeCCNU (semustine) – stimulated development of experimental and clinical studies of preparations of this group, carried out in many scientific and medical centers all over the world at the treatment of patients with definite forms of neoplasms.

Among hundreds of newly synthesized nitrosourea derivatives, modified by fragments of sugars, natural amino acids, aliphatic, aromatic and heterocyclic compounds, seven preparations are accepted for medical application, about a dozen of various derivatives are passing different levels of pre-clinical and clinical tests [41, 45, 56].

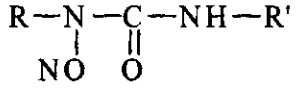
In modern chemotherapy of tumors, the NAU class is represented by 12 drugs, which comprehensively studied both experimentally and clinically (Table 1).

Molecular mechanisms of the biological action of NAU are stipulated by highly expressed reactivity of the products of their biodegradation to alkylation and carbamoylation of macromolecules. These reactions may cause modification of the DNA structure, damage of transcription and translation

apparatuses, interlocking of the repair system, and form the basis of carcenolytic and toxic effects of NAU [10].

Table 1

Antitumor preparations of nitrosoalkylurea class



Conditional preparation name	Chemical title of preparation
Nitrosomethylurea, NMU	1-nitroso-1-methyltylurea
Dimethynur	1,3-dimethyl-1-nitrosourea
Carmustine, BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
Locustine, CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
Semustine, MeCCNU	1-(2-chloroethyl)-3-(4-methyl)-cyclohexyl-1-nitrosourea
Nimustine, ACNU	1-(2-chloroethyl)-3-(4-amino-2-methylpyrimidin-5-yl)-methyl-1-nitrosourea
Photomustine, mustophorane	(2-chloroethyl-3-ureydol)-ethyldiethylphosphonate
Lysomustine, nitrullin	9-(2-chloroethyl)-7,9-nitroso-2-homocitrulline
Streptosotocine	2(3-methyl-3-nitrosoureido)-2-deoxy-D-glucopyranose
Chlorosotocine	2[3-(2-chloroethyl)-3-nitrosoureido]-2-deoxy-D-glucopyranose
ADECO	1-methyl-3-(B-D-xylosyl)-1-nitrosourea
Aranosa	1-methyl-3-(α-Larabinopyranosyl)-1-nitrosourea

Generalizing results of the experimental studies on antitumor properties and cellular mechanisms of NAU, let us note several fundamental features typical of this class of compounds and distinguishing it from the majority of the known preparations:

- the spectrum width of therapeutic action in relation to experimental tumor models;
- the ability to penetrate through hemato-encephalic barrier and associated activity at central nervous system tumors;

- cyclo-nonspecificity of action, expressed in the induction of irreversible lethal damages in both proliferating and resting cell;
- the absence of cross stability and possibility of combined application (simultaneous or consecutive) with drugs from other groups, including alkylating agents.

The wide action spectrum is typical of the majority of NAU and embraces all main histological types of neoplasts in animals, including spontaneous and induced tumors (Table 2).

Table 2

Characteristics of the action spectrum width for several nitrosoalkylureas in relation to experimental tumor models

Compound	Number of animal tumor strains sensitive to compound
NMU	30
BCNU	50
CCNU	17
MeCCNU	13

Kinetic approach to the estimation of NAU antitumor activity allows for classification of compounds by tumor growth inhibition rate and determine the differences in the action spectrum in relation to experimental models [27].

Among tumors most sensitive to simple alkyl-substituted nitrosoureas, first generations of spontaneous breast cancer should be mentioned [26]. As observed from dependencies depicted on Figure 1, NMU and dimethynur are most effective decelerators of the mentioned solid tumor development compared with other compounds. Meanwhile, some tumor strains, for example, leukosis L-1210 is highly sensitive to nitrosourea chloroethyl-substituted derivatives: carmustine, semustine and lomustine (Figure 2) [28].

It should also be mentioned that the group of tumors sensitive to chloroethyl-substituted derivatives includes strains with increased sensitivity to alkylating agents. On the other hand, tumors sensitive to NMU are famous with their resistance to various kinds of chemotherapy [45].

The results indicating high sensitivity of xenografts of some human tumors, melanomas, in particular, to NAU (Figure 3) are of special prognostic value for future clinical application of the drugs [29].

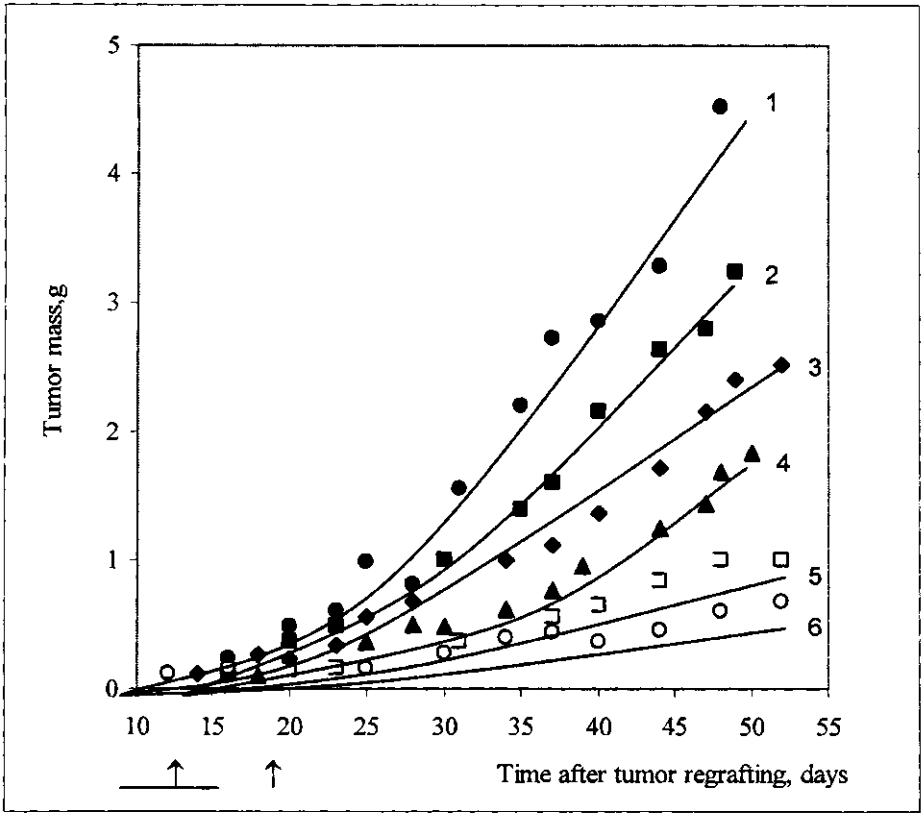


Figure 1. Tumor mass change at breast adenocarcinoma development in C_3H strain mice (the 1st generation of spontaneous tumor) in control and under the effect of nitrosoalkylureas:
1 – control; 2 – CCNU; 3 – BCNU; 4 – MeCCNU; 5 – dimethinur; 6 – NMU.
Drugs were injected intra-abdominally in equitoxic doses (0.67 LD_{10}) at 12th and 18th day after tumor regrafting

Searching for chemotherapeutic preparations effective in the treatment of cerebral tumors is still one of the most important tasks of experimental oncology. Preparations of this class are required for treatments of patients with primary CNS tumors and the therapy of cerebrum metastatic damages. Nitrosoureas represent the unique group of compounds, definitely effective in the treatment of patients with meningial tumors [45, 50, 54 – 56].

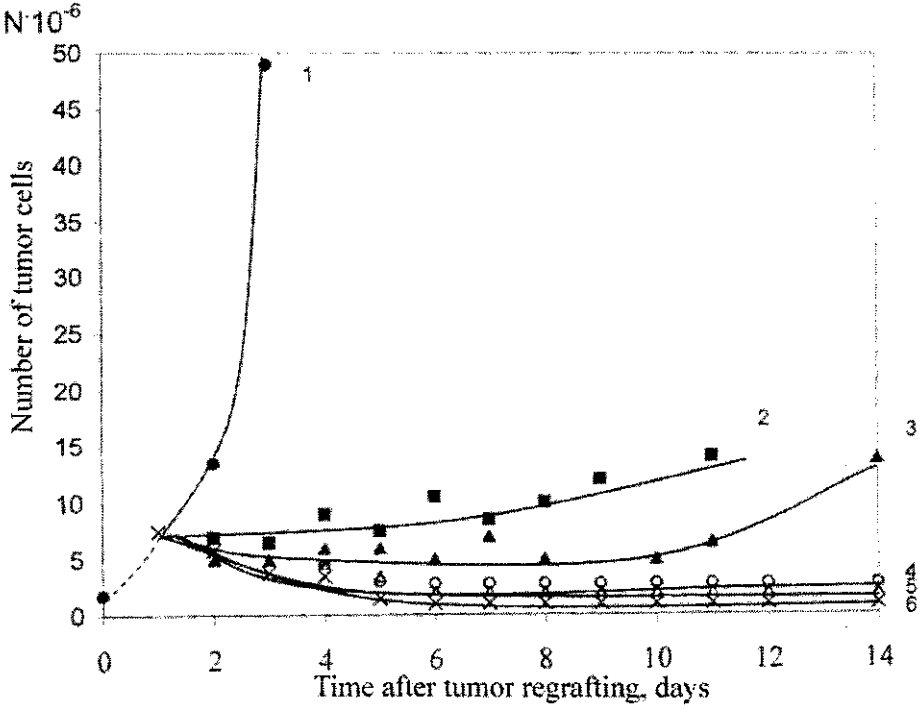


Figure 2. The influence of nitrosoalkylurea group of drugs on leucosis L-1210 development (BDF_1 mice).
1 – control; 2 – NMU; 3 – 1–methyl–1–nitrosobiurete; 4 – CCNU; 5 – MeCCNU; 6 – BCNU.
Drugs were injected intra-abdominally in equitoxic doses (0.67 LD_{10}) at 1st and 6th day after tumor regrafting

According to the existing ideas, NAU effectiveness in relation to cerebral tumors is stipulated by their ability to penetrate through the hemato-encephalic barrier owing to lipophilic properties of the compounds. The importance of relation between lipophilic characteristics of the compounds and tumor tissue saturation with the lipid components in realization of NAU antitumor effect is indicated by studies of the reasons for high sensitivity of Lewis lung carcinoma to MeCCNU [59] and intracerebral sarcoma 9L to PCNU [58].

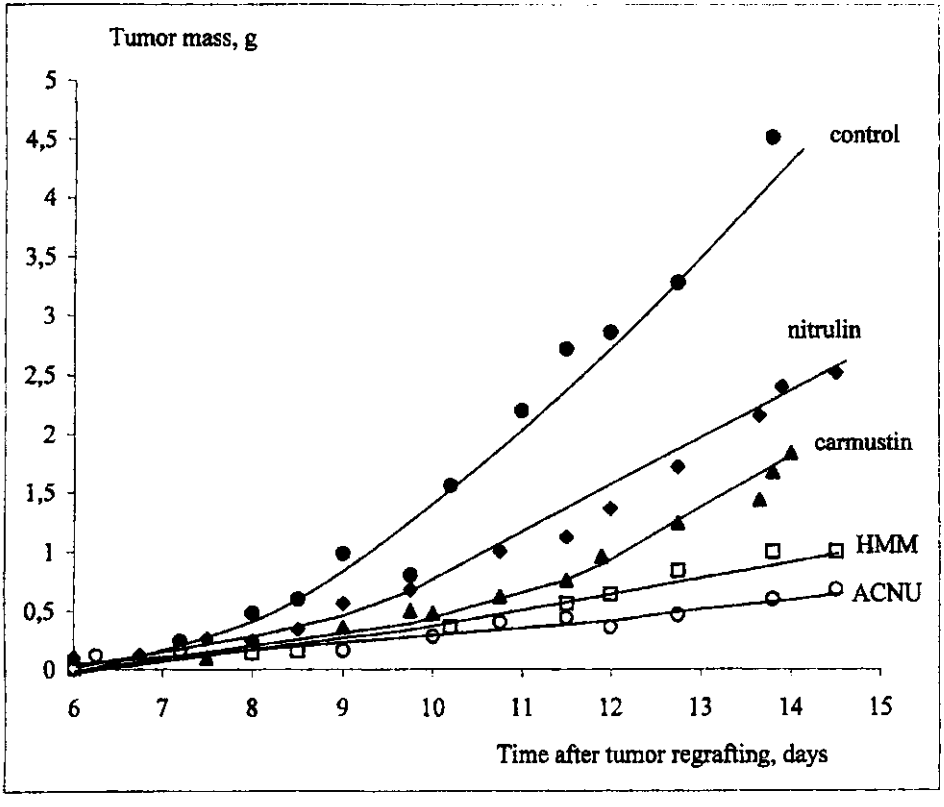


Figure 3. Kinetic curves of human Bro melanoma xenograft development in immune-suppressed CBA strain mice in control and affected by ACNU, NMU, carmustine, and nitrulin compounds
Drugs were injected intra-abdominally once on the 6th day after tumor regrafting

The efficiency of NAU in relation to 13 strains of neurogenic tumors, including virally and chemically induced ones, has been proved experimentally (Table 3).

Table 3

Intracerebral tumors in animals, sensitive to some nitrosoureas – NMU, dimethinur, ADECO, carmustine, semustine, and lomustine

Mice	Rats	Hamsters
Ependymoblastoma	Sarcoma 9L	Glioma induced by Rous sarcoma virus
Glioma 261	Gliosarcoma	–
Glioma 26	NMU–induced astrocytoma	SV-40 virus induced astrocytoma
Glioma induced by Rous sarcoma virus	ASV–induced astrocytoma Glioma 35 Glioma 2211	–

High activity in inhibition of intracerebral tumor development is displayed by compounds of the new generation: ADECO, dimethinur, mustophorane [17, 30].

Parmacokinetic studies of dimethinur showed that high sensitivity of intracerebral glioma to this compound is stipulated by its preferable accumulation on neoplast tissues (Figure 4). Simultaneous detection of dimethinur in blood, cerebral tissues and intracerebral tumor is the illustrative experimental fact, which confirms penetration of the compound through hemato–encephalic barrier [30].

It seems appropriate to mention that NAU demonstrates maximal therapeutic effectiveness at both parenteral and peroral intake in high doses, exceptional or injected with a long break between intakes [56]. The optimality of such regime for NAU was determined during kinetic study if the preparation action in the experiment and confirmed by its clinical application [45].

One of the typical features of nitrosourea pharmacological action, which distinguishes them from the majority of the known cytostatics, is cyclo-nonspecificity (Figure 5) e.g. the ability to lethally damage both proliferating cells (mostly at transition from pre-synthetic phase G_1 to the DNA phase S, and in the early phase S) and proliferation resting cells (phase G_0) [45, 46]. This property of the preparations is principally important for their use in therapy of solid tumors with low fraction of proliferating cells and a significant pool of resting cells resistant to the influence of usually applied chemotherapeutic preparations.

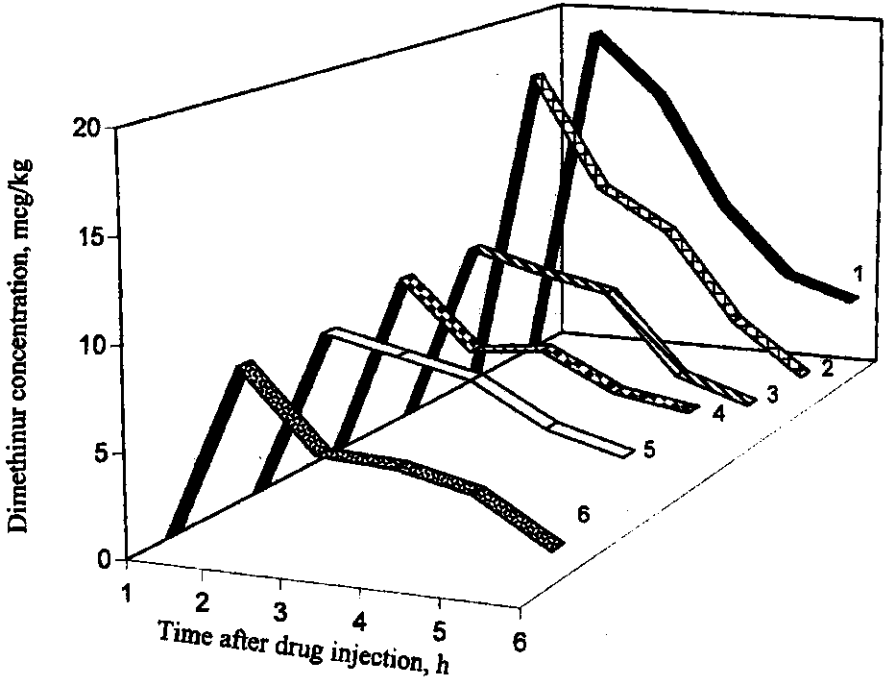


Figure 4. Pharmacokinetic curves of dimethinur concentration change in organs of rats with intracerebral glyoma 2211.
1 – intracranial tumor; 2 – cerebrum; 3 – blood; 4 – kidneys; 5 – liver; 6 – lungs.
The drug was injected once perorally in the dose of 100 mg/kg on the 7th day after tumor regrafting

It is apparent that high sensitivity of resting cells to NAU is the reason for preparation activity in case of slowly growing tumors with low proliferative pool, simulating in the experiment the development of human solid neoplasms (initial generations of spontaneous breast tumor). For the first time, the inverse correlation between the initial proliferative pool of the tumor, characterized by inclusion of labeled thymidine, and tumor sensitivity to NMU (Figure 6) has been determined *in vivo*. The highest activity of the preparation is observed, when tumor possesses initially low label index [47].

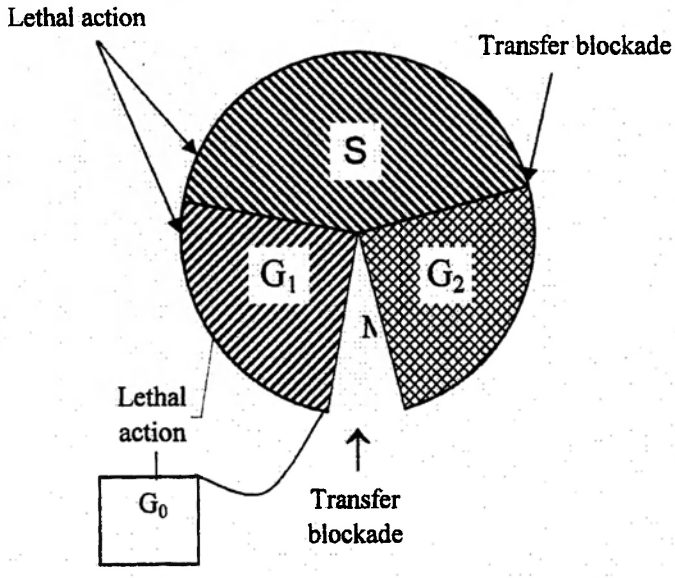


Figure 5. The influence of nitrosoalkylureas on the cellular cycle and viability of tumor cells (the scheme).
Distribution of sensitivity by cycle:
- cells in the period of transition from presynthetic phase G_1 to DNA synthesis phase S, in the early S phase and proliferative rest phase G_0 are most sensitive to lethal action of the drugs;
- cells in the DNA synthesis phase S and at transition from post-synthetic G_2 phase to mitosis M phase are most sensitive to interlocking "cytostatic" action

Apparently, the same factors provide for high effectiveness of NAU in relation to far gone tumors at injection of the preparations in later periods of the process development (Figure 7).

Not only cytolethal effect, which judging by values of survived fraction of tumor cells (Table 4) may differ by several orders of magnitude for some derivatives, is of importance for realization of NAU therapeutic action. The so-called "cytostatic" effect is all sufficient. It defines the rate of growth and repopulation of survived cell fraction, responsible for tumor relapse after the course of treatment. For some nitroso-derivatives, the contribution of these two factors into the final therapeutic effect may be much different (Table 4) [48].

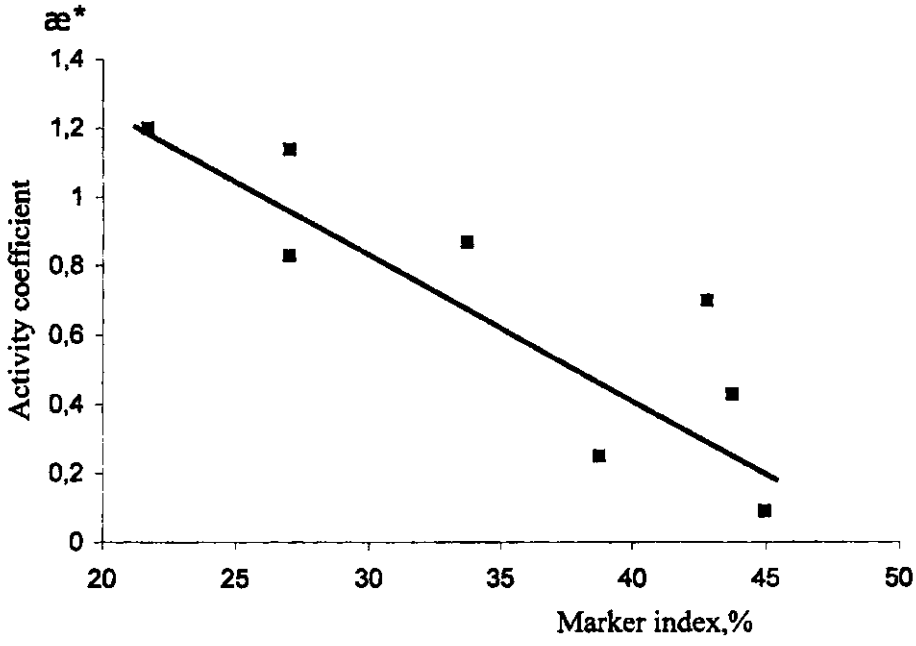


Figure 6. A correlation between NMU antitumor activity in relation to mammary gland tumors in CBA strain mice and the initial proliferative statute of tumors.
Antitumor activity of the drug is estimated with the help of the kinetic criterion – the activity index æ* [44]; the proliferating tumor statute was characterized by the label index, determined by inclusion of labeled thymidine into proliferating cells

For example, a possibility of combined application of various nitrosoureas [23] is based on the differences in these two cellular effects.

For example, combined application of dimethinur and lomustine increases with their growth-inhibiting effect at the background of better acceptability of the combined effect by animals compared with the individual use of each preparation (Table 5).

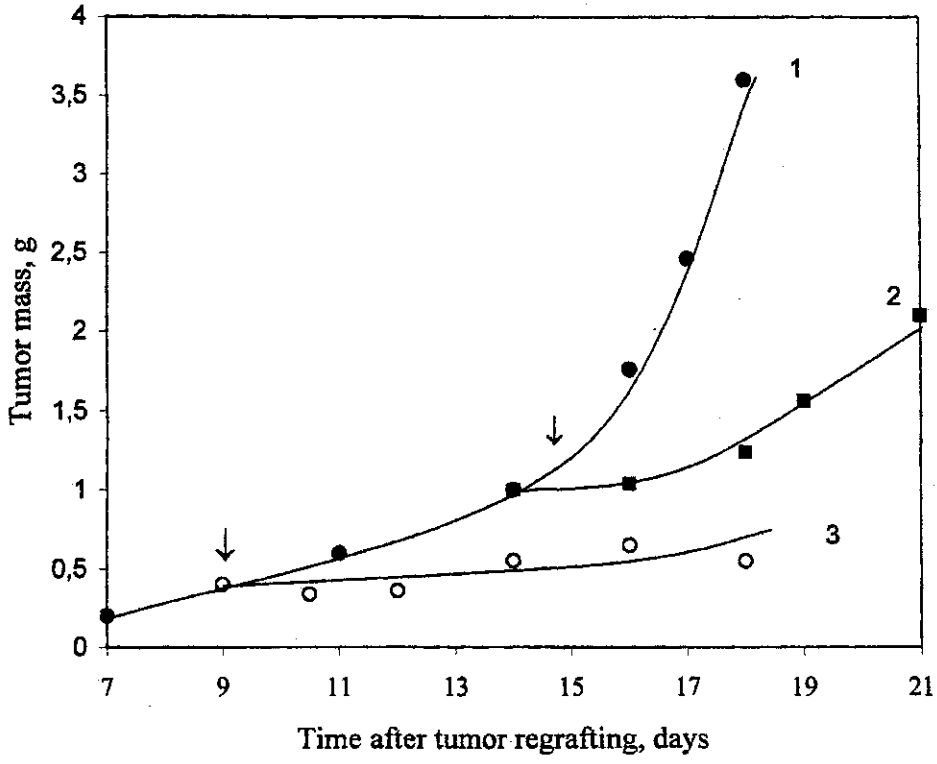


Figure 7. The influence of dimethinur compound on kinetics of solid leucosis P388 development in various application regimes in mice BDF_1.
1 – control; 2 – injection of dimethinur on the 14th day of tumor development (activity coefficient æ* = 0.68); 3 – injection of dimethinur on the 9th day of tumor development (activity coefficient æ* = 0.57).
The drug was injected perorally, once in the dose 100 mg/kg

Table 4

Characteristics of cytolethal and cytostatics effects of nitrosoalkylureas in experimental leucoses

Strain	Preparation	Survived cell fraction	Contribution into growth-inhibiting effect	
			Cytolethal action, %	Cytostatic action, %
Leucosis L-1210	HMU	$3.0 \cdot 10^{-3}$	60	40
	Dimethimur	$9.2 \cdot 10^{-2}$	60	40
	ADECO	$1.3 \cdot 10^{-5}$	95	5
	Semustine	$1.8 \cdot 10^{-4}$	95	5
	Lomustine	$6.2 \cdot 10^{-1}$	5	95
Leucosis La	NMU	$5.0 \cdot 10^{-7}$	100	0
	ADECO	$1.8 \cdot 10^{-7}$	100	0

Table 5

The effectiveness of dimethinur and lomustine combined application at peroral intake (Walker's carcinosarcoma in rats)

Preparations	Dose, mg/kg/day (injection scheme)	Survivability, % (25th day of tumor development)	Tumor growth suppression, % (TGS, %)*
Dimethinur	80 (2nd and 7th days)	70	70
Lomustine	38 (2nd and 7th days)	85	70
Dimethinur + Lomustine	80 (2nd day) + 38 (7th day)	100	85
Lomustine + Dimethinur	38 (7th day) + 80 (2nd day)	100	85

Note: * Index TGS (%) was calculated from the relation between tumor sizes in treated (T) and control (C) animals as follows:

$$\text{TGS, \%} = \frac{C-T}{T} \cdot 100\%.$$

In the control, all animals died by 25th day having tumors weighted, on average, 50 g.

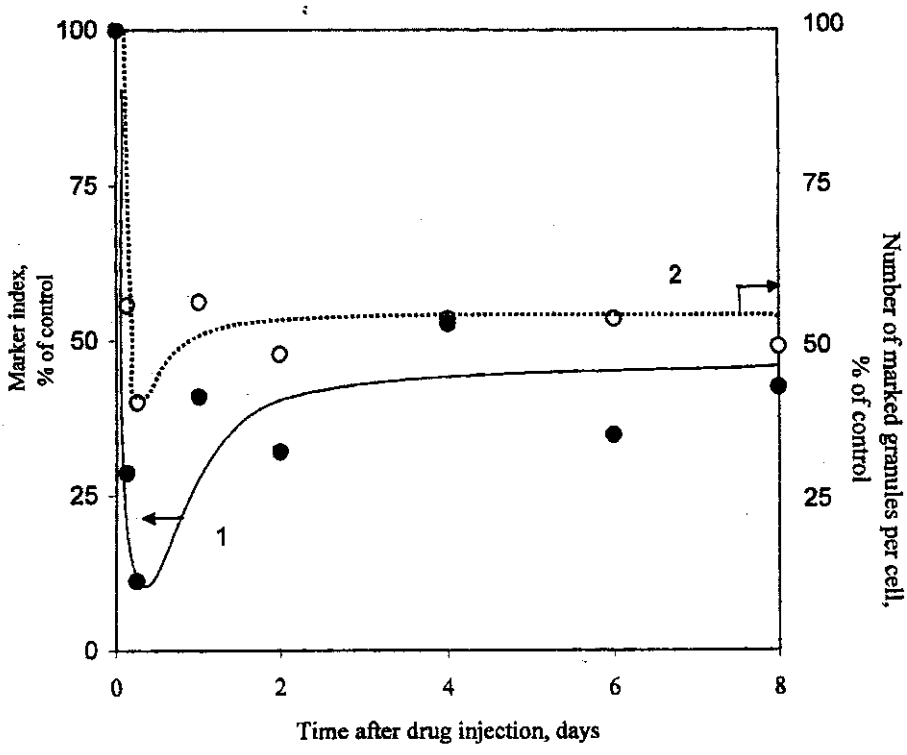


Figure 8. Inhibition of DNA synthesis in mammary gland tumor cells in C_3H strain mice by NMU

1 – kinetics of the label index change (in % to control); 2 – kinetics of changes in the number of labeled granules per cell (in % to control)

Radioautography method of DNA synthesis investigation on the inclusion of labeled thymidine. The drug was once injected intracranially in the dose 80 mg/kg

The cells of survived fraction usually possess much longer mitotic cycle rather than initial cells, which is stipulated by deep and long suppression of DNA synthesis (Figure 8) [31].

The fortune of survived tumor cell populations, which reproductive potency finally defines the substantiality of the treatment undertaken, is much stipulated by the damaging effect of NAU on the structure of tumor cell

chromosomes. Kinetic approach to the study of genotoxic effects of antitumor drugs allows determination of the type and duration of mutagenic effect and quantitatively estimation of their remote consequences for tumor cells and "critical tissues" of the organism (bone marrow).

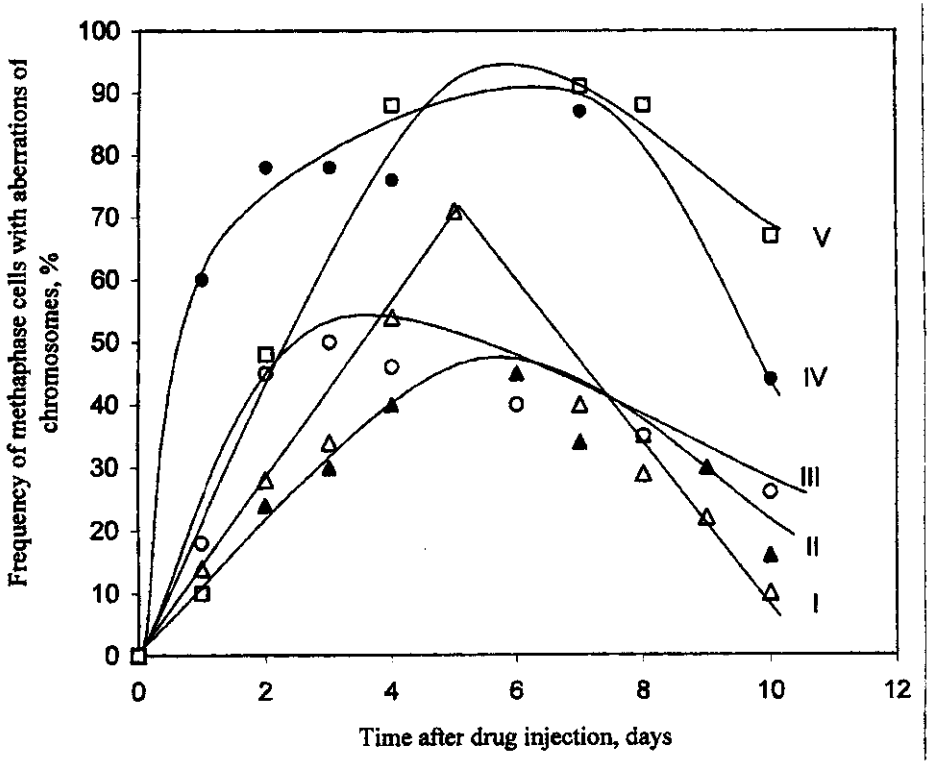


Figure 9. Kinetics of the change in the frequency of metaphase cells with chromosomal aberrations in cell populations of Ehrlich's tumor (SHK mice) under the effect of some nitrosoalkylureas
I – CCNU; II – dimethinur; III – NMU; IV – ADECO; V – BCNU
The drugs were injected once intracranially in equitoxic doses (0.67 LD_{10})

The studies of the "structure–effect" and "dose–effect" dependencies in the sequence of preparations forming the NAU class determined the induction effect of long existing damages of chromosomes, preserved in the tumor long

time after the impact in the absence of cells with chromosomal aberrations in bone marrow (Figure 9).

The level of tumor cells possessing residual chromosomal damages correlate well with the growth-inhibiting activity of preparations and is one of the factors defining tumor sensitivity to NAU (Figure 10) [32, 40 – 42].

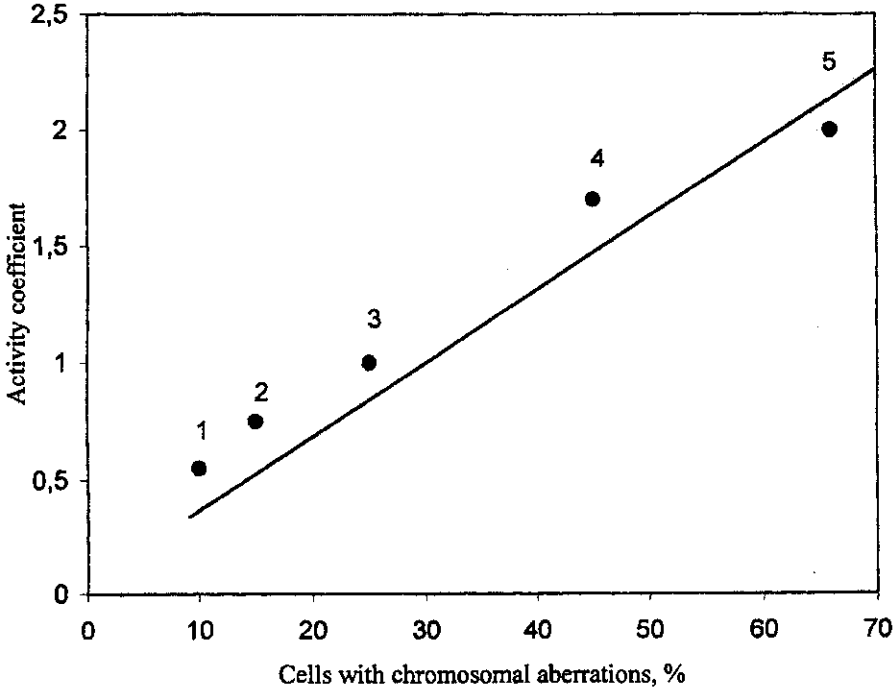


Figure 10. The correlation between antitumor activity and cytogenetic effect of nitrosoalkylureas (Ehrlich's tumor)
Changes in antitumor activity coefficient æ* with respect to the amount of cells having chromosomal aberrations on the 10th after single injection of drugs in equitoxic doses:
I – CCNU; II – dimethinur; III – NMU; IV – ADECO; V – BCNU

Table 6

Combinations of nitrosoalkylureas with other antitumor compounds, which possess therapeutic synergism in relation to experimental tumors

NMU	BCNU	CCNU	MeCCNU
Cyclophosphane Sacrolysine Methotrexate 6-Mercaptopurine Bleomycin	Cyclophosphane Yphosphamide BTIC Methotrexate Cytosar 6-Mercaptopurine 5-Fluorouracyl Vincrystine L-asparaginase	Cyclophosphane BTIC Cytosar 6-Mercaptopurine Vincrystine	Cyclophosphane Cytosar 6-Mercaptopurine 5-Fluorouracyl Adriamycin
Cyclophosphane + adriamycin		Cytosar + Vincrystine + Prednisolone	Cyclophosphane + methotrexate
Cyclophosphane + 5-fluorouracyl	Cytosar + DTIC		Cyclophosphane + adriamycin
Taxoter	Cytosar + DTIC + Methotrexate		Cyclophosphane + Cytosar
	Amphotericin Twin 80 Vitamin A		Cyclophosphane + Cytosar + 6-thioguanine
	Vitamin A + caffeine		Cytosar + Vincrystine + Prednisolone
	Chloropromasin + caffeine		Cyclophosphane + Vincrystine + Prednisolone
	Oxyurea + caffeine		

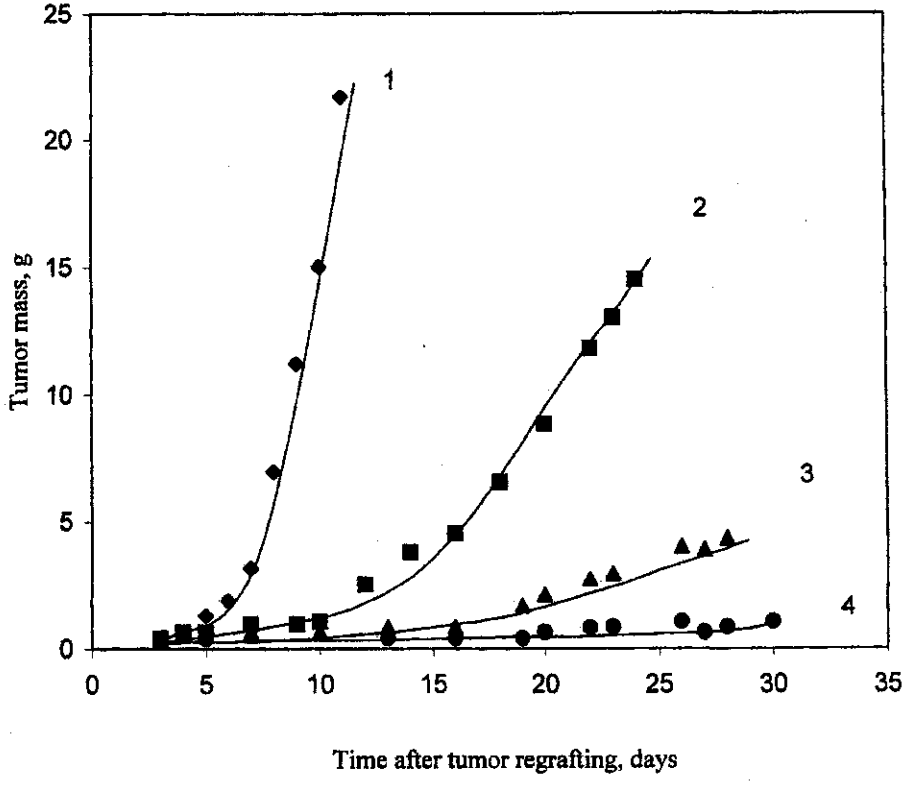


Figure 11. The effectiveness of inhibition of Walker's carcinosarcoma development in rats by combined influence f nitrosomethylurea and cyclophosphane:
1 – control; 2 – NMU, 20 mg/kg per day (the activity coefficient is æ* = 0.55); 3 – cyclophosphane, 20 mg/kg per day (æ* = 0.73); 4 – NMU, 10 mg/kg per day + cyclophosphane, 10 mg/kg per day (æ* = 0.90).
The drugs were injected ntracranially six times since the third day after tumor regrafting

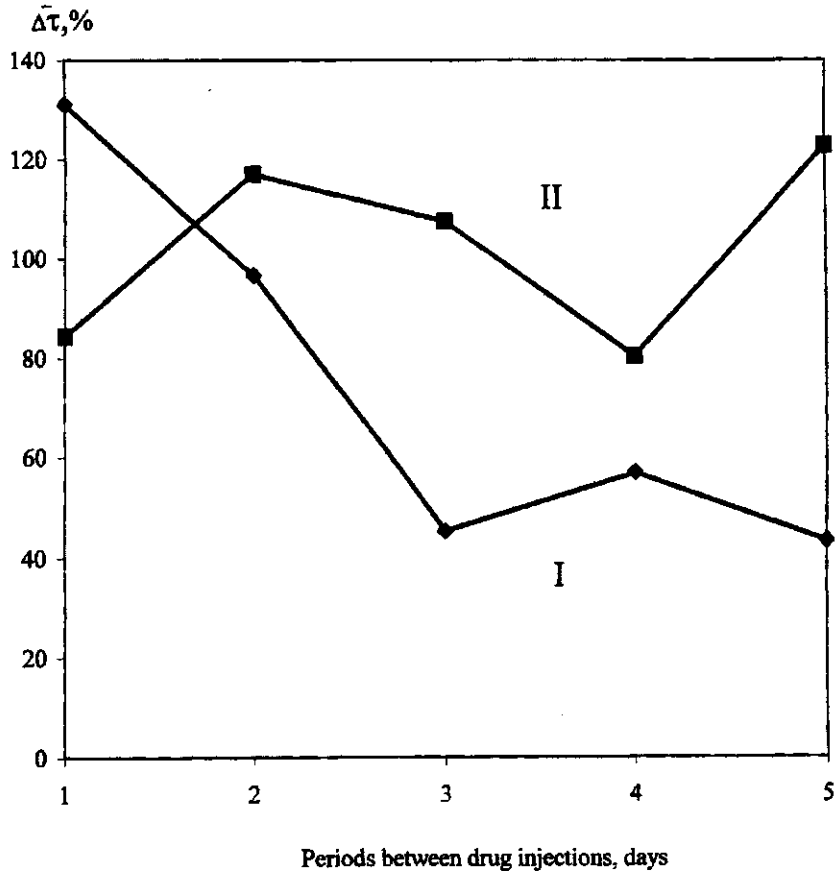


Figure 12. The effectiveness of taxoter and NMU combination with respect to the application mode of P388 leucosis model
I – Drug injection sequence: taxomer – NMU;
II – Drug injection sequence: NMU – taxomer
The single dose of each drug is 50 mg/kg
Abscissa axis: the interval between injections in days;
Ordinate axis: the average life duration increse for treated animals compared with the control, %

According to the rational selection of drugs for combined application, appearance of the class of compounds similar to NAU, possessing the above-mentioned features of pharmacological action, discloses new abilities for

combined treatment of patients with the far gone, widespread tumor process and refractoriness to the majority of chemotherapeutic agents. In this connection, it seems natural that NAU have occupied one of the leading places in modern polychemotherapy and are actively studied as the components of many therapeutic schemes.

Table 6 presenting quite broad list of compounds, which includes practically all most widespread cytostatics used, show the NAU, obviously, owing to the features of their mechanism, are capable of potentiating the effect of the majority of antitumor preparations – the alkylating agents, antimetabolites, and antibiotics [23, 45, 49, 50, 57].

Some of combined therapy schemes, experimentally confirmed, which included NMU, for example, NMU combinations with cyclophosphane (Figure 11), taxoter (Figure 12), bleomycin or cyclophosphane and adriamycin are used in clinical practice [33, 45, 49, 51, 52, 57].

Modern ideas about NAU role in clinical chemotherapy of tumors are based on the experience of wide medical application of preparations of this class during almost thirty years [50]. Hence, it should be noted that clinical investigations confirm many of experimentally forecasted features of nitrosoureas' action.

At the present time, one may accept strictly stated high effectiveness of NAU at lymphomatoid granulomatosis, lung cancer, lymphosarcoma and melanoma – these tumors are the main indications for application of these preparations. Antibiotic streptosotocin representing natural derivative of nitrosomethylurea, in which amino group is replaced by sugar, is effective in cases of insulinomas and malignant carcinoid.

As an example, Table 7 shows generalized data of various investigations, which illustrate effectiveness of NMU application to these tumoral diseases.

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As an example, Table 7 shows generalized data of various investigations, which illustrate effectiveness of NMU application to these tumoral diseases.

Table 7

The effectiveness of combined chemotherapy including NMU in relation to various tumors

Tumor	Number of patients	Treatment results			
		Full remission	Partial remission	Stabili-zation	No effect
Lymphomatoid granulomatosis	112	61 (54.5%)	21 (18.8%)	12 (10.7%)	18 (16.0%)
Non-Hodgkin's lymphoma	92	22 (23.9%)	40 (43.5%)	20 (21.7%)	10 (10.9%)
Small cell lung carcinoma	157	7 (4.4%)	34 (21.7%)	52 (33.1%)	64 (10.8%)
Skin melanoma	290	40 (13.8%)	55 (19.0%)	81 (27.9%)	114 (39.3%)

The results of NMU application to lymphomatoid granulomatosis (LGM) give grounds to relate a compound to the most effective means for medical treatment of this disease. With the help of NMU, monochemotherapy has induced the expressed objective improvement in 79% of patients, including 25% of complete remission [8].

Table 8

The effectiveness of monochemotherapy by nitrosomethylurea for previously treated and untreated patients with lymphomatoid granulomatosis

Group	Number of patients	NMU treatment with objective effect
Previously untreated patients	11	8 (73%)
Previously treated patients	18	13 (72%)

It should be emphasized that these results were obtained for patients with generalized process, damages of multiple groups of peripheral and visceral lymph nodes, and different organs with expressed intoxication symptoms. The important features of NMU action at LGM are quick positive effect occurring already after the first or second injection in previously untreated patients and the effectiveness at damaging of various organs, including liver, skin, bones, and CNS. Of special attention is identical NMU effectiveness for patients, previously untreated by chemotherapy, and patients

multiply treated by various drugs and irradiation, which made them resistant to these sorts of treatment (Table 8). Obviously, this can be considered as the indication of the absence of the cross resistance at LGM between NMU and other preparations [7, 21, 38].

Several schemes of combined chemotherapy of LGM, one of which is NMU, have been studied (Table 9) [9, 11, 21, 22, 38]. The objective effect from application of these combinations to various investigations equaled 50 – 85%, including 20 – 50% of full remissions durable up to 1.5 – 3 years, on average. For some patients, full remissions lasted over 5 – 10 years (Table 10).

As shown by survivability analysis of patients with LGM, the life duration after treatment by NMU may be very high. Among 101 patients, 38 persons (37.7%) lived up longer than 5 years, including 26 patients (25.7%) lived during 5 years or more without tumor signs [18].

The effectiveness of NMU for lymphosarcomas was observed already during the phase I of clinical study, when a single case of long-term full remission after NMU monotherapeutic treatment of patients with small intestine and testicle lymphosarcoma [5] was observed, respectively. In the following years, several schemes of combined therapy of lymphosarcomas (Table 9) were studied. As a rule, these schemes were used for line 2 – 3 therapy; nevertheless, about one third part of the patients displayed full remissions up to 2 years long (Table 7) [11].

NAU are effective at lung cancer, generally, at its small cell type (SCC). For example, the objective effect of NMU application, up to full regression of tumors, is observed at monotherapy of this tumor in 46 – 75% patients [3, 4, 15, 16, 19, 34]. The comparison of NMU and embichin and cyclophosphane activities show for this tumor equal activities of NMU and embichin (25% and 24% of full and partial regressions, respectively), which exceed cyclophosphase effectiveness (16%) [36].

The studies of combined chemotherapy for SCC, which includes NMU (Table 9), indicate quite high effectiveness of such combinations (Table 7). In case of SCC, the comparison of application results for cyclophosphane and NMU or CCNU combinations demonstrated definite advantages of the first combination: the objective effect was obtained in 30% of cases with the average life duration of patients about 7.5 months. Hence, a combination of cyclophosphane and CCNU caused objective improvement in 9% of patients at the average life duration about 5.2 months [1, 35, 36].

Table 9

NMU combinations with other antitumor drugs applied to various tumors

Tumor	Composition of drugs
Lymphomatoid granulomatosis	NMU + procarbazine hydrochloride + vincristine (vinblastine) + prednisolone
	NMU + cyclophosphane + vincristine + prednisolone
	NMU + bleomycin + vinblastine + prednisolone
	NMU + prospidinum + prednisolone
	NMU + cyclophosphane
	NMU + doxorubycin
Lymphosarcoma	NMU + cyclophosphane
	NMU + cyclophosphane + vincristine + prednisolone
	NMU + cyclophosphane + doxorubycin + prednisolone
	NMU + cyclophosphane + vincristine + methotrexate + prednisolone
Small cell lung cancer	NMU + cyclophosphane
	NMU + cyclophosphane + vincristine + prednisolone
	NMU + cyclophosphane + vincristine + CCNU
	NMU + cyclophosphane + doxorubycin
	NMU + cyclophosphane + methotrexate
Skin melanoma	MNU + dactinomycin + vincristine
	NMU + prospidinum
	NMU + prospidinum + vincristine
	NMU + bleomycin + vincristine
	NMU + CCNU

The specific feature of NAU is the effectiveness at skin melanoma, which is practically insensitive to cytostatics of other groups. According to results of systematic clinical investigations, the effectiveness at melanoma is higher for NAU rather than standard dacarbazine (DTIC) preparation [43, 45, 52, 53].

One of the early clinical investigations carried out on 30 patients with generalized melanoma applied NMU monochemotherapy, which gave 9 cases of objective effect, among which tumors were completely eliminated in 3 patients. In these patients, full remissions lasted 3 years, 12 months and 6 months; the average duration of partial remissions equaled 7 months. NMU was found effective for treating melanoma metastases in skin, hypoderm, lymph nodes, and lungs [8, 11, 12].

Table 10

Postponed results of combined chemotherapy with inclusion of NMU applied to treatment of patients with lymphomatoid granulomatosis

Direct treatment results	Number of patients	Survivability median (months)
Full regression	61	92
Partial regression	21	33
Without objective effect	30	6

In another investigation at monotherapy by NMU the objective effect was observed for 4 of 21 patients with disseminated melanoma [9]. For this tumor, similar effectiveness is shown by other NAU. Recently, of great attention is comparatively new preparation from this group (mustophoran). According to clinical test results, this compound is now considered as one of the most active drugs for treatment of patients with melanoma.

In the case of melanoma, a significant effectiveness is shown by various combinations of NAU and other cytostatics. One of the most effective schemes of combined chemotherapy of melanoma is NMU combination with vincristine and dactinomycin. The use of this scheme led to full remission in 10 (10.9%) and partial remission in 22 (24%) of 92 patients [2].

The multisite randomized investigation compared the efficiency of three schemes of combined chemotherapy for the treatment of disseminated melanoma:

1) a combination of HMU, vincristine and dactinomycin;
2) the first combination added by DTIC;
3) a combination with NMU replaced by CCNU.

It is found that DTIC addition to NMU combined with vincristine and dacarbazine does not increase the treatment efficiency. NMU replacement by CCNU reduced the frequency of full remissions (from 11.4 to 6.3%), partial remissions (from 18.2 to 12.5%), and significantly decreased the average life duration of patients (from 30.4 to 20.5 months) [13, 24].

Another multi-site investigation was carried out on 114 patients with disseminated melanoma. As is indicated, the combination of NMU, vincristine and dactinomycin gives twice more full remissions than single DTIC application (13.7 and 7.1%, respectively) [3, 24]. Full remissions, obtained

with the help of this scheme, may exist for quite a long time: 15 of 19 patients with full remissions lived out for 3 years, moreover, 12 of them lived out more than 5 years without any signs of the disease (Table 11) [3].

Similar effectiveness is shown by NMU combination with prospidinum – in 50 treated patients full remissions are observed in 16% and partial in 10% with duration up to 37 months [12 –14]. In the case of melanoma, approximately identical effectiveness is displayed by NMU combination with bleomycin [39].

Table 11

Remote results of combined chemotherapy treatment, including NMU, of patients with disseminated skin melanoma in case of full regression of the tumor

Number of treated patients	Number of patients with full regression of tumor	Number of patients living without tumor signs		
		3 years	5 years	6 years
177	9 (10.8%)	4	6	6

The investigations in the field of antitumor chemotherapy, carried out by N.M. Emanuel, which were initiated more than 40 years ago, led to important results, valuable both scientifically and for practical medicine. One of the demonstrative examples of this is the development, comprehensive study and introduction into clinical practice of nitrosomethylurea – the primary representative of the broad group of compounds. Scientific grounds of the antitumor action of nitrosoalkylureas founded in these investigations have played the important role in development of this direction in the drug treatment of cancer, which is still urgent [60].

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THE VALUE OF ANTIOXIDANT PROPERTIES OF LIPIDS IN RADIATION DAMAGE AND MEMBRANE REPAIR

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The investigation results on the role of antioxidant properties of lipids in determination of natural radioresistance of animals from different species and strains are generalized. It is shown that antioxidant activity of lipids in spleen is the main component in providing for animal stability under acute irradiation in sublethal and minimal lethal doses. The role of parameters of physicochemical regulation system for peroxide oxidation of lipids in membrane repair after the radiation impact on animals is analyzed. The idea about two levels of membrane repair after radiation impact is formulated.

Keywords: lipids, membranes, repair, antioxidant activity, peroxide oxidation of lipids (POL), composition, radioresistance, laboratory animals

In 1950ies, Academician N.M. Emanuel has proposed a hypothesis about urgency biophysical shifts, related to the change in free radical concentration in tissues, at radiation damage and cancer growth. It has been found [1 – 4] that antiradical properties of biochemical components of the cell are important for providing natural and modified radioresistance. The investigations of lipid oxidation processes were initiated in the middle 1950ies in works by Professor B.N. Tarusov (M.V. Lomonosov Moscow State University) and Academician N.M. Emanuel (Institute of Chemical Physics, Academy of Sciences of the USSR). The role of oxidative reactions in autooxidation of food, technical and cosmetic fats and oils is commonly known [5 – 8]. At the same time, the hypothesis about possible participation of free-radical reactions in the mechanism of radiation damage and cancer growth [1, 9, 10] was not accepted by the majority of biologists and medics for long. Nevertheless, during recent 10 – 15 years, the field of "*free-radical investigations*", as accepted by the authors of [11], became the main direction in biomedical science. Sing the early 1970ies, the vigorous development of

membranology allowed stipulation of the biological urgency of free-radical reactions proceeding in different compartments of cells in the regulation of cellular metabolism in norm [12 – 17]. It is believed to be proved that peroxide oxidation of lipids (POL) processes play the leading role in pathogenesis of radiation disease and burns, malignant neoplasms, atherosclerosis, diabetes, ischemic heart disease, Alzheimer's and Parkinson's diseases, etc. [13, 18 – 27]. Now determination of precise mechanisms of lipid physicochemical property influence on cellular metabolism regulation is one of the predominant directions in the field of physicochemical biology, established by Academician N.M. Emanuel and his followers. Moreover, the mechanism of liquid-phase oxidation of organic compounds, suggested by them, is considered as the basic mechanism of POL process for biological systems, too [12, 14, 16, 28, 29].

Natural and synthetic antioxidants (AO) are the effective regulators of oxidative processes. One of the most widespread model systems, used for analysis of antioxidant properties of both individual synthetic and natural AO, and biologically active substances, methyl oleate oxidative model, i.e. thermal autooxidation of methyl oleate. Being the chain reaction with clear branching, autooxidation represents an autoinitiated process with positive feedback, implemented via hydroperoxide [30]. Formation of more than 80% of the main oxidation products due to further transformations of peroxides [31] and linear dependence of the induction periods of inhibited autooxidation of methyl oleate on the concentration of added inhibitor [13, 32] formed grounds for suggesting the methyl oleate oxidative model for analysis of antioxidant properties of lipids involving their ability to decelerate peroxide synthesis at thermal autooxidation of methyl oleate [4]. Subsequently determined equality of recombination constants for peroxide radicals of methyl oleate and natural fats (pig fat, cacao oil) [33] became an additional confirmation of the justified selection of methyl oleate as the model oxidation substrate for analysis of antioxidant properties of biological system components.

It is shown [34] that there are different ways of death of complex biological organisms impacted by acute ionizing radiation in the dose range of 1.0 – 1,500 Gy. Therefore, estimation of the action of damaging factors on biological objects requires determination of metabolically urgent indices, which variations give an opportunity to judge about changes in the whole system of cellular metabolism in particular situations. The studies of the role of physicochemical properties of lipids in development of radiation damage, induced by acute radiation or radiomimetics [4, 13, 35, 36], allowed a conclusion that for laboratory rodents in the dose range of 3.5 – 7.0 Gy one of

such indices is the antioxidant activity (AOA) of lipids, determined with the help of methyl oleate oxidative model. It is found that changes in lipid AOA in liver homogenate – the integral characteristic reflecting physicochemical changes in membrane lipids and redistribution of antioxidants between organelles – are interrelated with intensity variations of oxidative processes proceeding in membranes [37]. The study of influence of radioprotectors from the class of free radical inhibitors, injected before and after acute radiation impact on animals in sublethal and lethal doses, on the animal survivability allowed suggestion of lipid AOA value as a test for selection of radioprotectors and optimal conditions for their injection, because the effects of both radioprotection and radiosensibilization may be reached in accordance with the influence of these agents of the lipid AOA level in animal liver [13].

The intensity of POL processes at a stationary level in norm is supported by physicochemical system of oxidative reaction regulation in lipids of membranes, which parameters are AOA and composition of lipids, their ability to be oxidized, and structural transitions in the components of membranes [25, 38]. At present, the existence of physicochemical system for POL regulation at the cellular and organ levels is experimentally shown. This system provides for proceeding of POL processes of the membrane systems in the cell and the organism as entire system [39, 40]. This requires consideration of the following factors in analysis of consequences of impacts of various damaging agents on complex biological objects:

1. the uniformity in changes of the state indices and functioning of membranes under different impacts on the organism;
2. impossibility, specially for *in vivo* conditions, of arbitrary changing of any parameter in the regulatory system with no effect on others [38];
3. the necessity of taking into account temporal factors in consideration of system parameter variations after damaging impact [13, 41, 42].

Moreover, the analysis of radiation damage consequences requires accounting for processes developing in organisms in norm and under impacts of damaging factors. First of all, the attention should be paid to the fact that the lipid AOA value in norm is the labile index.

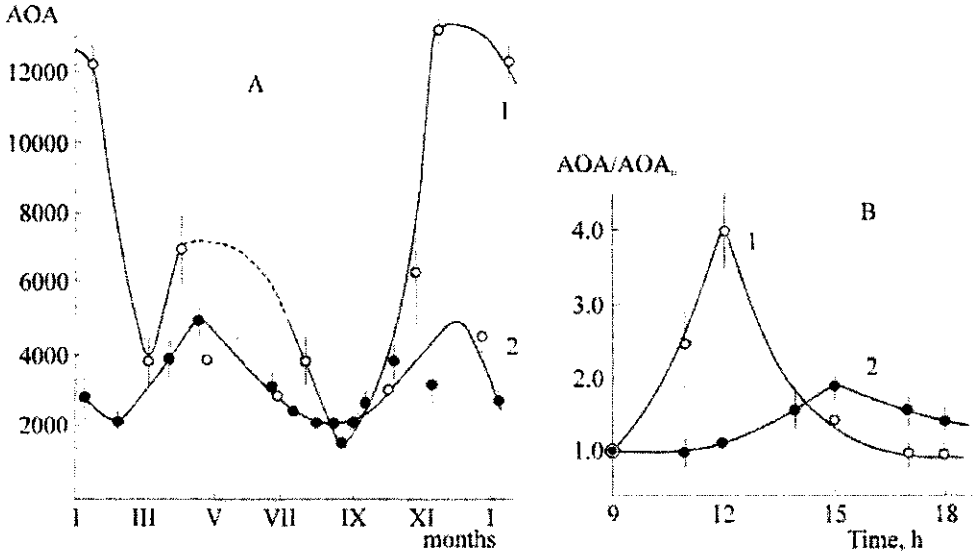

Figure 1. The change of antioxidant activity (AOA) of lipids in spleen (1) and liver (2) of Balb/c strain mice with respect to the observation season (A) and time of day (B)

In our experiments (Figures 1 and 2) and other works [43, 44], the stage changes in lipid AOA with respect to the season and daytime were observed at the level of organs and cellular organelles. As indicated, dynamics of changes in AOA of lipids extracted from cellular organelles or tissue homogenate of intact mice are highly different (Figures 1 and 2; [41]). The stage changes in lipid AOA and microviscosities of different zones of membranes in liver nuclei with time after acute radiation impact (6.5 Gy) were observed in both tests with radiosensitive Balb/c strain mice [13, 41] and experiments with higher radioresistant SHK mice. The lipid AOA value in liver nuclei of irradiated SHK mice 5 h after the impact is reliably equal to the control ($\Delta AOA = (190 \pm 190)$ h·ml/g), whereas a day after the impact the lipid AOA is found much lower than in intact animals ($\Delta AOA = (-1{,}400 \pm 190)$ h·ml/g). A decrease in the lipid AOA in nuclei is accompanied by a significant increase of viscosity of both lipid and near-protein zones of membranes in liver nuclei of mice one day after the radiation impact (Table 1). As a consequence, for different indices of the membrane state, sensitivity and ability to normalize it are also different. Apparently, this relates to the presence of unrepairable damages of membranes or disintegration of the membrane repair in time by separate indices.

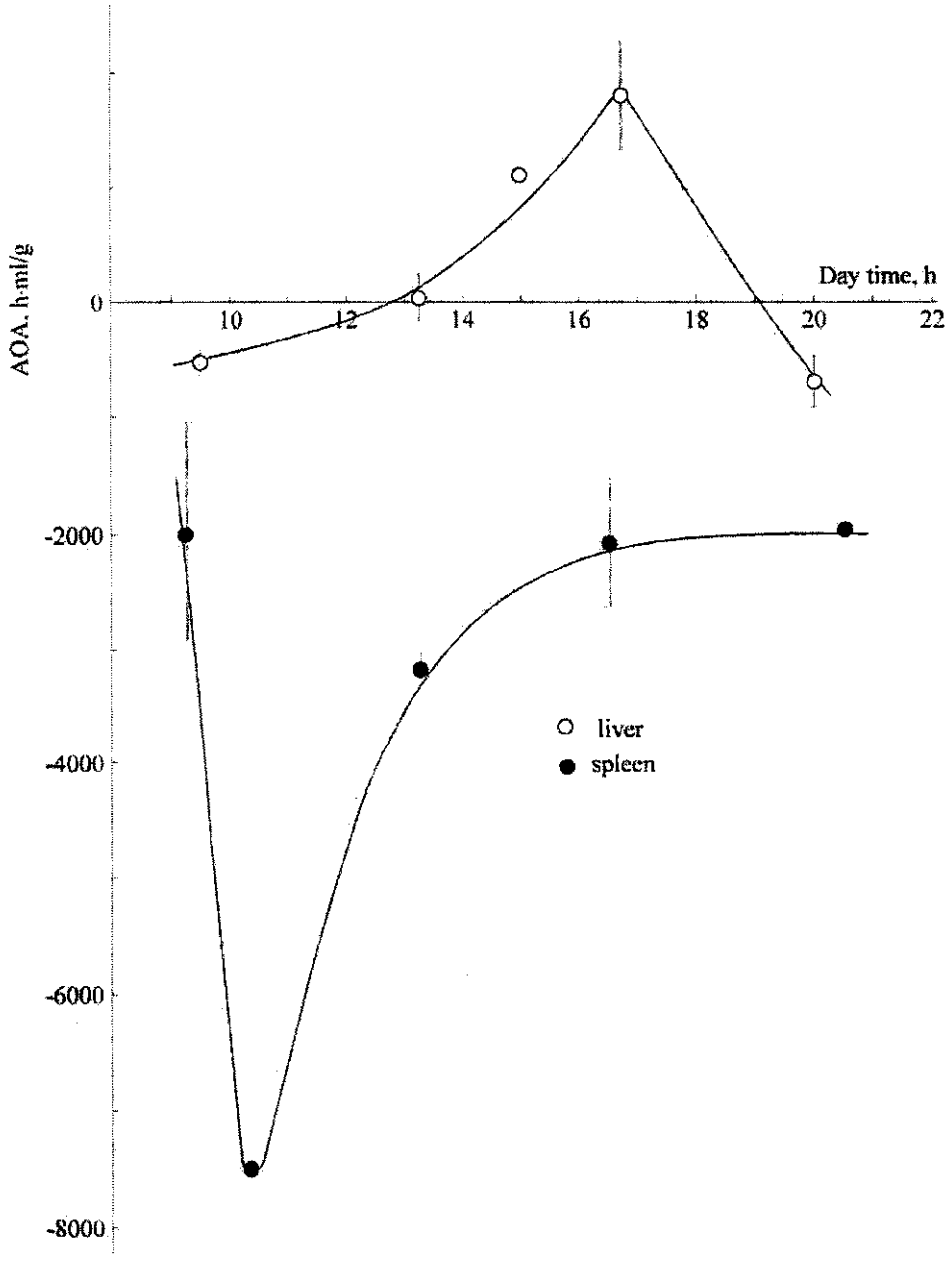

Figure 2. The change of lipid AOA in mitochondria of liver and spleen of Balb/c strain mice (females) during all day round

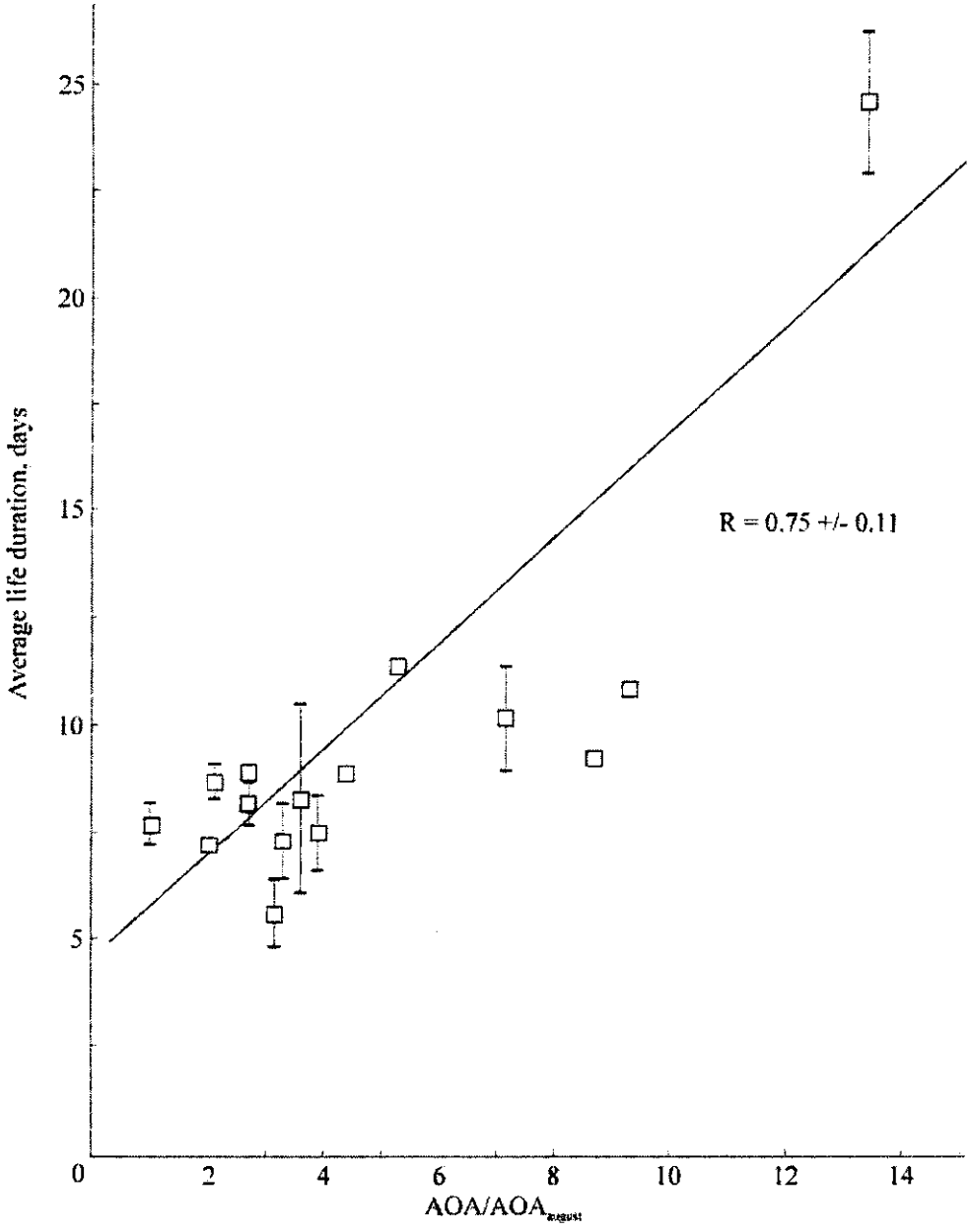

Figure 3. The relation between lipid AOA in spleen and the average life duration of Balb/c strain mice (males, females), impacted by 6.5 Gy dose of radiation in different seasons

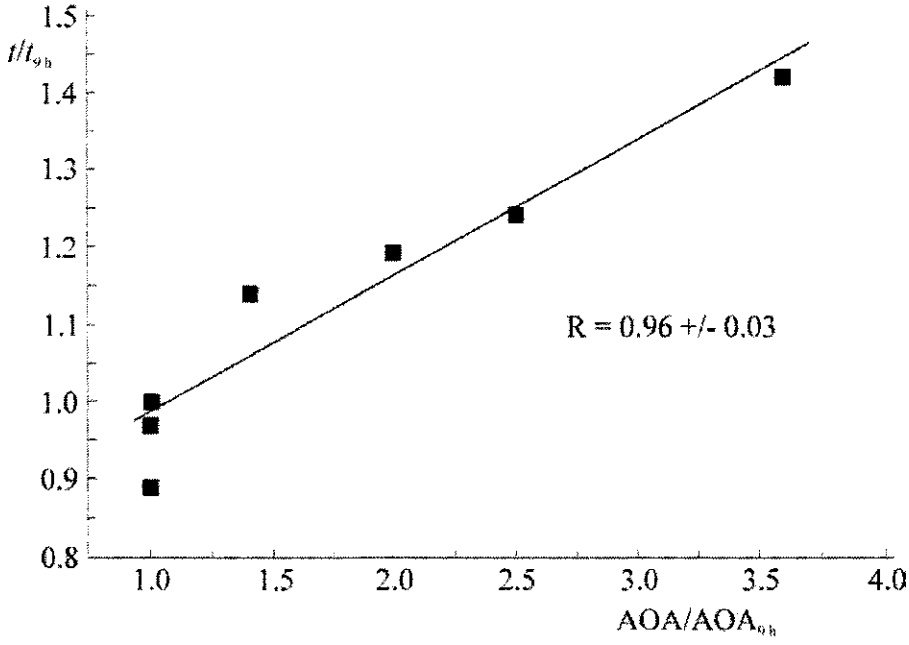

Figure 4. Dependence of relative increase of the average life duration for Balb/c strain mice impacted by 6.5 Gy dose of radiation in different time of day on the lipid AOA in spleen of intact mice currently at the impact time in relation to AOA level at 9 o'clock

The comprehensive study of radioprotection properties of synthetic AO shows that dose and time of agent injection should be selected basing on the changes of the organ lipid AOA, which is critical under the impact of a definite damaging factor, though the character of lipid AOA change in all organs is uniform. For example, the interrelation between targeted change of lipid AOA level in spleen by injection of an AO from the class of free-radical process inhibitors and the average life duration of Balb/c strain mice impacted by acute X-ray radiation in the minimal lethal dose (6.5 Gy) showed good correlation with the coefficient $r = 0.89 \pm 0.05$ ($n = 19$) [45].

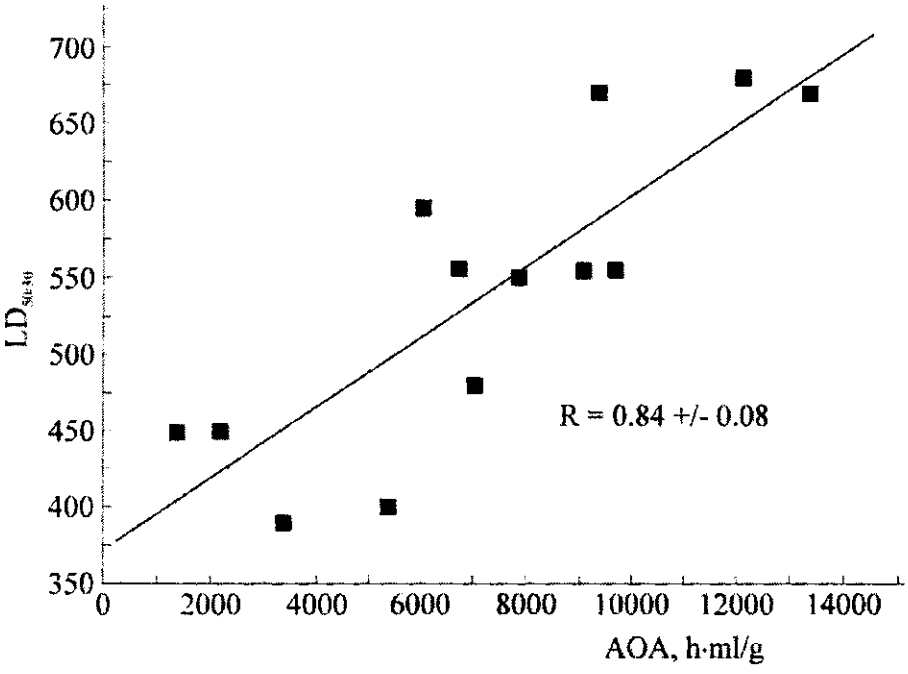

Figure 5. The relation between lipid AOA values in spleen of animals from different species and strains (Sybsa rats, Wistar strain; gold hamsters; SHK mice, Balb/c and CBA/J strains) and their $LD_{50/30}$ values under acute X-ray impact

The same was concluded about systematic study of the influence of various natural factors modifying the acute radiation impact (season, time of day, diets, various species and strains of animals) on changes in the level of lipid AOA in organs and radiosensitivity of animals. The results of experiments are shown in Figures 3 – 6. The analysis of interrelations between saturation of various tissues with AO and resistance of animals to acute radiation impact in sublethal and minimal lethal doses outlined positive correlation (with quite high coefficient) between radioresistance of animals with the lipid AOA level in spleen, which is one of critical organs at acute radiation of animals in the dose range of 4.0 – 8.0 Gy, which mostly induced the bone marrow way of death. The coefficient of correlation between the lipid AOA level in spleen (Figure 3 shows it in relation to the minimal value in august, Figure 1a) and the average life duration of Balb/c strain mice (males and females), impacted by 6.5 Gy in different seasons, is somewhat lower that the corresponding value at

irradiation of mice of this strain in different time of day (Figure 4). The study of radiation impacting of animals in different strains and species also indicates good correlation between the lipid AOA level in spleen and $LD_{50/30}$ values, determined simultaneously (Figure 5). Feeding of CBA/J and SHK strain mice by granulated concentrates with various fat concentrations and guinea pigs by granulated concentrates with various contents of proteins or raw cellular tissue increased radioresistance in mice of both strains at acute X-ray radiation impact in the dose range of 3.0 – 8.0 Gy and radiosensitivity of guinea pigs at acute γ-radiation impactin the dose range of 3.0 – 7.0 Gy [46] compared with control animals, bred and fed by general vivarium diets (Figure 6). Meanwhile, an increase of lipid AOA in spleen for mice of all test groups and decrease of this parameter in guinea pigs in test groups compared with the control were observed. High changes in lipid exchange indices at rat breeding on diets with increased fat content or deficient protein were observed [47 – 49], as well as modification of AO status and the functional state of clawed jirds at their moving from natural environment to a breeding farm, induced by the feeding change [50]. The interrelation between the lipid AOA values in spleen and $LD_{50/30}$ for the control and test animals are shown in Figure 6. As a consequence, in the case of variable lipid AOA level, which is caused by changes in diets, the radiaresistance of both species is also stipulated by AOA value.

Obviously, radiosensitivity of a cell, a tissue or an organism should be considered as a complex, multifactor phenomenon [51]. Since the limits of biochemical and physiological reactions in organs and tissues of the organism are rather narrow, this stipulates their high sensitivity to impacts of the damaging factors [52]. For example, it is found that radiosensitivity of complex biological objects correlates with the volume of chromosomes and nucleus in the cell [53], proteinase activity of peripheral blood [54, 55], the level of endogenic catecholamines and biogenic amines in tissues [56], the amount of phospholipids [57], the level of glycogen in white blood [58], etc. The role of sulfhydryl groups in natural and modified radiosensitivity is broadly discussed in the literature [59 – 64]. The detailed studies show that AOA levels of lipids and endogenic thiols reflect different aspects of cellular metabolism and provide for resistance of the organism to radiation impact by different metabolic mechanisms [60, 63]. For low doses of acute radiation impact, radioresistance of different species and strains is defined by high lipid and non-protein thiol AOA levels in spleen, and at lethal doses – by high level of total

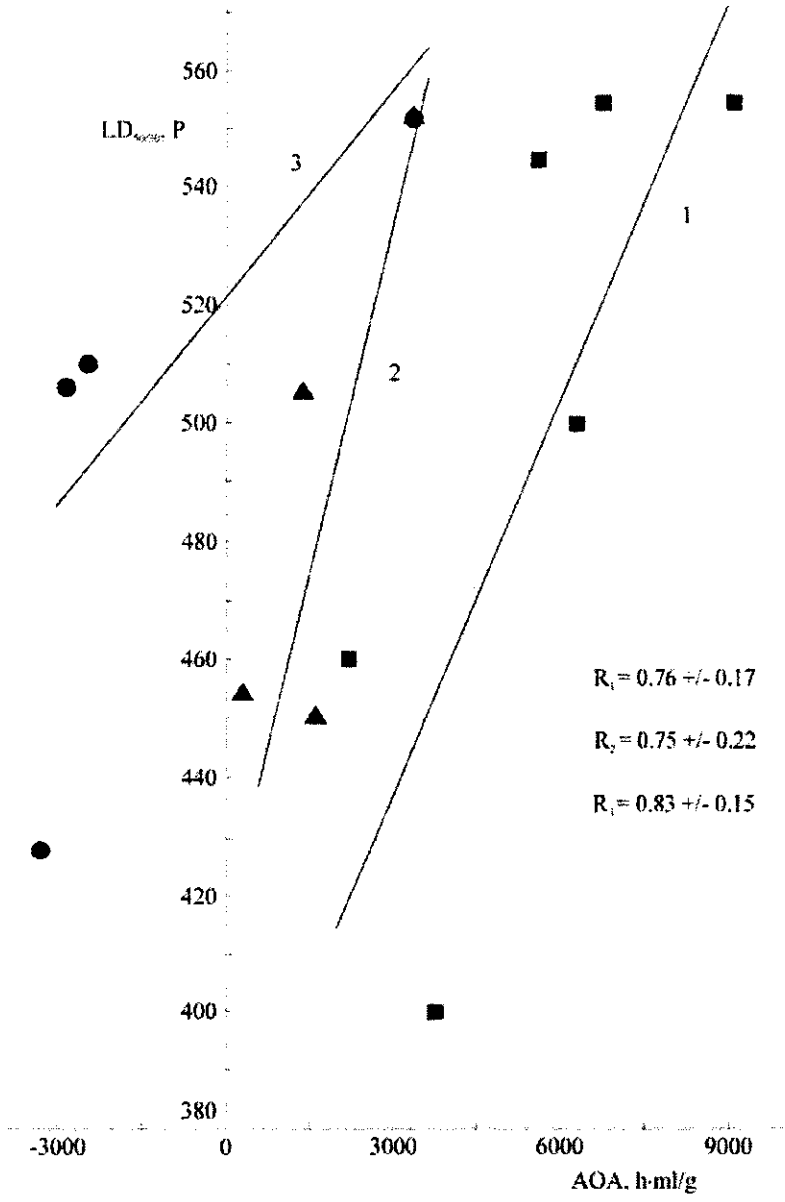

Figure 6. The relation between the lipid AOA in spleen and $LD_{50/30}$ values under X-ray imapcting of SHK and CBA/J strain mice, fed by diets with different fat content (1), at γ-radiation of guinea pigs fed by diets with different contents of protein (2) or cellular tissue (3)

(protein) thiols [63]. This corresponds to the idea of different main molecular mechanisms for high and low dose radiation impacts on the organism [39, 63 – 66].

The active study of the membrane role in regulation of biochemical processes in cells provided for understanding of the functional meaning of oxidative reactions proceeding in lipids in formation of biological consequences of ionizing radiation impacts. T. Alper has formulated an idea about the membrane, similar to DNA, as one of the targets for the radiation impact on the cell [67, 68]. Basing on the data about post-irradiation increase of death of bacterial cells at protein iodination, a model of "cooperative" DNA and membrane target was suggested [69]. According to this idea, damaging events may be observed in each of the components, and when being repaired they depend on one another.

Lipids represent one the basic components of biological membranes. They implement energetic, structural and barrier functions, and represent specific regulators of intracellular processes [38, 70 – 78]. Phospholipids are the most important components of the membrane system of cells. They generally determine active and passive transport of substances and pinocytosis, participate in signal transmission and regulation of immunological reactions, and other important metabolic processes [72 – 82]. Changes in the composition and oxidation degree of lipids, their orderliness and packing in the bilayer are very important for cell adaptation to the environmental conditions [71, 83 – 85] and development of degenerative changes and apoptosis [86 – 88]. The functional response of the cell to various external signals and phosphotidylinosite degradation are bound by phospholipid-dependent protein kinase C, activated by calcium ions [77, 89 – 94]. At present, phosphodiether hydrolysis of polyphosphoinositides is considered as the key reaction of the cascade amplification of signals received from receptors of calcium-mobilizing agents [93, 95] that revealed the interrelation of various regulatory mechanisms of the vital activity of cells [91, 95 – 98]. Also, POL process participating in the metabolism regulation in norm and under the impact of damaging factors, is closely associated with metabolism of the cell components, including renewal of the phospholipid composition [11 – 13, 17, 99 – 107].

The change of lipid metabolism in cells is of special value for gene activity regulation, which accompanies proliferation of both normal and tumor cells [13, 97, 108 – 110]. Already in 1970ies, the data were obtained about participation of lipids and products of their peroxide oxidation in DNA biosynthesis and functional genome activity [111 – 114]. It has been found that

DNA repair enzymes represent SH-containing, lipid-dependent and membrane-linked [113 – 119]. The existence of the DNA-membrane complex, playing the important role in DNA replication, was proved experimentally [112]. Hence, linkers binding DNA with nuclear structures include phospholipids, sphingomyelin (SM) and cardiolipin (CL), in particular [120 –123]. The role of DNA-lipid interactions in the cell functioning and intracellular structure formation, and the influence of lipids on activity of replication and transcription enzymes are discussed in many reviews [97, 122, 124, 125]. The influence of ionizing radiation and chemical toxicants on the relation between intensity of POL processes and the structural state, as well as completeness of lymphocyte DNA biosynthesis in blood of mice is shown in many experiments [40, 126, 127].

It should be emphasized that the ionizing radiation impact induces damages in both separate components of the membrane and structural organization of the entire membrane. Among the main damages in the membrane components, observed for various cells and cell structures, the following are most typical:

in proteins [128 – 133] – decrease of the number of SH-groups, oxidative deamidization, formation of peroxide groups and cross links of the Schiff base type, Degradation of cyclic amino acid residues; reactions with alkyl radicals of CH_3 type may be of high importance for damaging of membrane proteins [131, 134, 135];

in lipids [136 – 139] – formation of peroxides, oxides, aldehydes, and polymeric products (at the late stages of the damage), as well as increase of the amount of lisoforms, phosphodiether bond break and inorganic phosphate release, fragmentation of lipids due to dissociation of radicals, which occur in the hydrophilic part of lipids containing OH-group in β-position to phosphoether or amide bond [140 – 144];

in sugars [128] – formation of toxic α,β-unsaturated carbonyl compounds.

The following processes are typical of the radiated membrane as the cellular structure:

- the change of relative amount of various lipids of proteins [25, 145 – 147];

- lipid/protein ratio break and the change of structure and accessibility for lytic enzymes (phospholipases and proteases) [13, 128, 148 – 156];
- shifting of structural transition temperatures in various membrane zones [13, 157 – 162];
- formation of cross links of various types;
- formation of new phospholipids, untypical of this type of membranes [145, 163].

It is obvious that all the above-mentioned processes are interconnected and usually proceed at the background of POL intensification [13, 149, 151, 164] and lead to transformation of the membrane surface [153, 154]. For example, strong activity inhibition of biosynthesis and degradation of phosphatidyl choline (PC) enzymes, incorporated in membranes, extracted from microvessels of bovine cerebrum, bovine and rat cerebrum microsomes and rat liver microsomes, were obtained at high rate of POL. The authors suggest that the relation between generation of oxygen radicals and restructuring of the membrane structure of microvessels is stipulated by modification of SH-groups of proteins [165]. Actually, oxidative modification of SH-groups of proteins in erythrocyte membranes promotes a change of microviscosity of their lipid bilayer that increases vesiculation of erythrocytes [166]. It is shown that hydrogen peroxide and lipid peroxides of mitochondria represent the main factors of the cell apoptosis, induced by UV-radiation, 2—eoxyglucose, cycloheximide, actinomycin D, and other agents [167].

Damages of ultrastructural organization of membranes promotes occurrence of cells with micronuclei, induce protein, fat and hydropic dystrophy of cells up to their lysis [168, 169]. The study of the origin f radical adducts in lipid extracts from cerebrum, spleen and liver of γ-radiated mice has shown that they occur in reactions, induced by radiation impact of radicals with biological molecules, membrane lipids, in particular [170].

Considering the process of membrane system repair, it should be taken into account that membrane represents heterogeneous, self-renewing structure. This heterogeneity is significant for providing stability of membranes. Moreover, to our point of view, *two different levels of membrane repair* should be considered: the repair of separate molecules in the composition of membrane, which were changed by the radiation impact, and repair of the membrane as the entire structure by means of replacement of damaged components of the membranes by newly synthesized ones. In this case, different repair paths will give obviously different rates. In the process of

damaged protein molecule repair a significant role is ascribed to disulfide reductase and lowmolecular thiols. For lipid peroxides, the repair agents are glutathione peroxidase, more precisely, the family of glutathione peroxidase enzymes, which use PL peroxides as substrates, hydrogen peroxide and other organic peroxides [167, 171, 172], and reduced glutathione, as well as lowmolecular thiols. This stipulates the occurrence of correlation between radioresistance of biological objects with the concentration of nonprotein endogenic sulfhydryl groups (see above) and activity of enzymes utilizing peroxides [173].

An important role in repair of oxidized lipids belongs to phospholipases, specifically to phospholipase A_2. In some investigations it was found that phospholipase used lipid molecules possessing peroxy-groups with higher efficiency than usual PL [74, 174]. Probably, proper orientation of the active site of phospholipase requires polar groups. After "releasing" of damaged fatty acid, acyl transferase, Co-A-ligase, Co-A and fatty acids reduce the phospholipid molecule. Interestingly, phospholipases, acyl transferase and specially mitochondrial phospholipase A_2 are resistant to the action of various damaging agents [174], including radiation [175]: the activity of acyl transferase in liver is not decreased even after lethal radiation of animals [128], and the system of fatty acids in rat liver is relatively resistant even to the impact of γ-radiation in the dose range of 25 – 100 Gy [176].

As the structure and composition of membrane are repaired, its functional activity may also be restored. Actually, at injection of synthetic antioxidant (6-methyl-2-ethyl-3-oxypyridine hydrochloride, 200 mg/kg) four hours after X-ray radiation impact (6.5 Gy) on SHK mice, AOA values of nucleus lipids in liver of test and intact mice 5 and 24 h after the impact were practically equal [177]. Meanwhile, a definite normalization of microviscosity of different components in the membrane and temperatures of structural transitions in the liver nucleus suspension is observed [177].

It is generally recognized that lipids affect activity of membrane-linked enzymes by two main mechanisms: activity of some enzymes depends on the amount of lipids – allosteric effectors, and activity of others depends on the change in viscosity of the lipid component of membranes [38, 70, 74, 75]. Moreover, after the radiation impact, sensitivity of the enzyme to regulatory influences may also be changed, as well as the lipid factor determining activity of this enzyme. Activity of enzymes participating in repair of the membrane structure depends of the composition of lipids. For example, fatty acid synthetase activity increases under the effect of PC and SM [38, 178];

phospholipase A_2 activators are PL peroxide species [174]; acyl transferase is activated by LPC [179]; phosphatide acid (PA) and/or lisoPA are activators of phospholipase D [79, 180]; fatty acids modify activity of all phospholipases [76]. Despite the difference in renewal times of membrane lipids and proteins (varying from 2 – 5 to 120 – 140 h) [181], variation of the membrane protein synthesis rate causes interrelated changes in synthesis rates for both lipids and proteins [182], i.e. the reparative membranogenesis is coordinated for lipids and proteins. As a consequence, changes in the composition and structure of membranes after radiation impact induces activation of enzyme repair and intensification of membrane lipid synthesis and interrelated synthesis of proteins, the more so that RNA-polymerases are also lipid-dependent enzymes [113, 125, 183, 184].

Proton-translocating ATPase, located on the internal membrane of mitochondria, is the key agent for ATP synthesis (hydrolysis) in the cell. The functioning mechanism of this enzymatic complex is rather complex and not completely determined yet [185]. It is shown that in norm allosteric activators of Mg^{2+}–dependent ATPase are CL and phosphatidyl ethanolamine (PE) [186 – 188], and the change of synthetase activity correlates with the changes in internal mitochondrial membrane fluidity [189]. However, it has been found [190] that in early periods after Balb/c strain mouse radiation impacting by minimal lethal dose synthetase activity of the enzyme increased at the background of CL concentration decrease in mitochondria, i.e. impacted by radiation CL became an inhibitor rather than activator of the enzyme. It is also shown [191] that after radiation impacting PE, which is normally activator of glucoso-6-phosphatase, becomes its inhibitor. Meanwhile, it should be noted that the regulatory sensitivity of allosteric enzymes is much higher in the presence of inhibitors rather than activators of these enzymes [192]. As shown by comparison of changes in Mg^{2+}–dependent ATPase activity dynamics in mitochondria of Balb/c strain mouse liver with relative microviscosity of the lipid component in membranes, 6 h after the radiation impact a linear dependence between synthetase activity and fluidity of the lipid component is observed, similar to norm (Figure 7, line 1), whereas hydrolase activity of the enzyme is so higher, the lower fluidity of the lipid component is (Figure 7, line 2). As suggested before, in the norm ATPase complex operation is related to the existence of two population species of the enzyme [193]. Subsequently, a hypothesis has been proposed that synthesis and hydrolysis of ATP by enzyme proceed by different ways (mechanisms) [185]. However, different scale of the lipogenesis index variations in rat liver organelles at acute radiation impact

[147] allows a suggestion that one of the reasons is the change in enzyme sensitivity or the type of dependence of its activity on determining factors under the radiation impact. This also correlates with the popular viewpoint that transformation of membranes under the impact f adaptive radiation dose induces adaptive response from biological objects impacted by the exhibiting dose [66, 194].

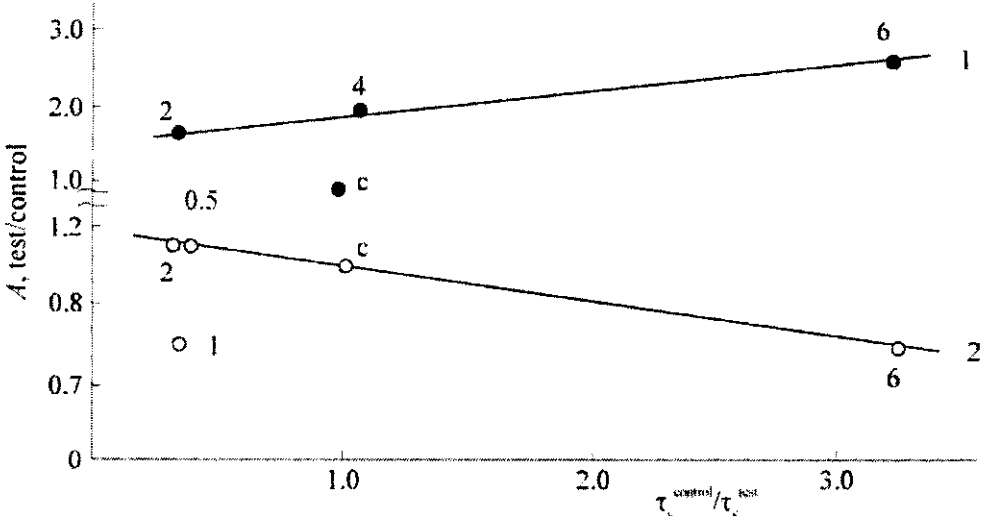

Figure 7. The change of relative synthetase (1) and hydrolase (2) activity of Mg^{2+}-ATPase in mitochondria of Balb/c strain mouse liver, impacted by 6.5 Gy dose of radiation, with respect to fluidity of the lipid component of mitochondrion membrane.
Digits at points show time after impact in hours; C is control

Obviously, there is principally different way of the membrane repair concluding in restoration of the structure and composition of membranes by affecting its functional activity. To all probability, this is the explanation of the mitochondrion structure repair by addition of ADP to mitochondria, extracted from radiated mice [195]. The addition of coenzyme Q_9 in concentration 1 mg/ml suspension of mitochondria, extracted in different periods after X-ray radiation impact (6.5 Gy) from Balb/c strain mice, also normalizes the respiratory control of mitochondria in liver of radiated mice during 5 h after the impact (Table 2). Moreover, addition of coenzyme Q_9 suspension of

mitochondria extracted from intact mouse liver causes no reliable influence on the respiratory control (DC) value.

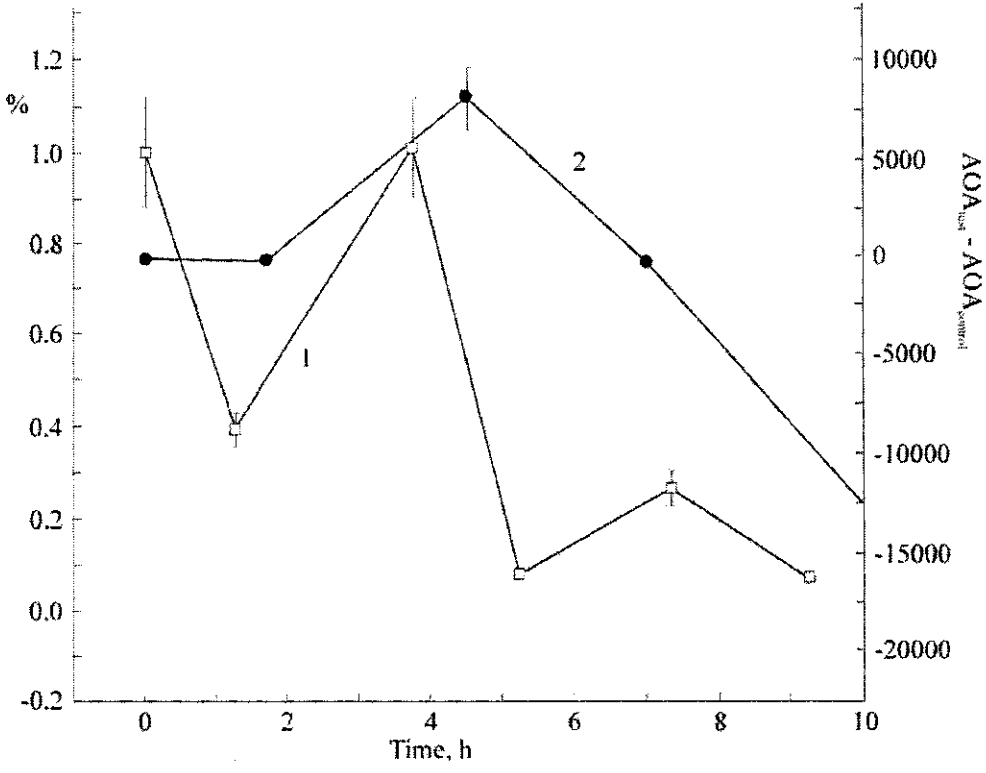

Figure 8. The change in percentage of labeled cells in liver (1) and lipid AOA in liver nuclei (2) of Balb/c strain mice in the early period after X-ray radiation impact in 6.5 Gy dose

It is known that radiation affects the rate of replicative DNA synthesis. The comparison of variation dynamics for AOA of nucleus lipids and percentage of labeled cells in the liver of Balb/c strain mice in the early period after the radiation impact (6.5 Gy; Figure 8) indicates a relation between the lipid AOA and reparative DNA synthesis. On HeLa cells and hepatocytes of regenerating mouse liver, it has been shown that decreasing AOA of lipids is important for the beginning of DNA replication [108, 111]. Obviously, reparative DNA synthesis also requires low level of nucleus lipid AOA, which increase during repair decelerates the process, because during reparative

synthesis DNA is linked to membrane in even greater number of points, than in norm [196, 197]. Therefore, injection of synthetic or natural antioxidants in doses exceeding lipid AOA in the initial hours after acute radiation impact in sublethal and lethal doses induces radiosensibilizing effect in animals [13, 41, 44].

Since the membrane structure in DNA complex with lipids in the presence of Ca^{2+} cations, which are mediators, changes [198, 199], and the membrane conditions effect on completeness of DNA repair [200, 201], fluidity in different zones of nuclei in Balb/c strain mouse liver changes significantly under the radiation impact by 6.5 Gy dose. Moreover, the structural state of membranes is not normalized a day after the impact [13]. In accordance with modern ideas about high importance of SM in genome activity [109, 121], of special attention are almost symbatic changes in lipid AOA (Figure 8) and SM content in nuclei in Balb/c strain mouse liver during initial 8 h after the radiation impact (6.5 Gy) [145]. This fact correlates with the data on nucleus lipid AOA increase during DNA synthesis [108] and SM participation in DNA linking to membrane [121, 122]. Moreover, it is suggested that the sites of DNA association with nuclear membrane are the most radiosensitive components compared with bulky DNA, condensed on nucleosomes [202, 203]. Meanwhile, DNA-linked lipids are hypersensitive sites to ionizing radiation and antitumor drugs [122].

Thus the composition of lipids and their physicochemical properties promote functioning of membranes in norm and cause significant influence on formation of biological consequences of the radiation impact. The lipid AOA in spleen – one of the critical organs at acute radiation impact in the dose range inducing the bone-marrow form of death of animals – is the main factor providing natural resistance of the organism to radiation. Generalized experimental data and analysis of the literature induce the conclusions about the important role of physicochemical POL regulation system in membrane repair after ionizing radiation impact in sublethal and minimal lethal doses and two levels of membrane repair. DNA and membrane damaging and repair may make different contributions into radioresistance of the organism with respect to intensity of the radiation impact. Different sensitivity and separateness of parameter repair in time change the scale and directivity of relations between parameters of POL regulation system, coordinated in norm [39, 40, 126]. The presence of signal ways associated with reactive oxygen species testify about the prospect of studying the mechanism of compound impact o redox processes

as a measure for modification of the radiation-induced genome instability and prevention of cancer transformations [204].

Table 1

Acute X-ray radiation impact (6.5 Gy) on microviscosity of different components of nucleus membranes in liver of SHK mice at 22.5°C

Time after impact	$\tau_c^{test} / \tau_c^{control}$	
	Probe 1	Probe 2
5 h after impact	0.75 – 0.77	1.10 – 1.14
24 h after impact	1.65	2.35

Note: For $\tau_c^{control}$, rotary correlation times of probes 1 and 2 in suspension of liver nuclei from intact mice killed simultaneously with the test mice. The data are averaged from three series of tests.

Table 2

The influence of coenzyme Q_9, added *in vitro* in the dose 1 mg/ml of mitochondrion suspension, extracted from Balb/c strain mouse (males) liver in different periods after X-ray radiation impact (6.5 Gy), on the respiratory control (RC) value

Time after impact	RC value for liver mitochondria of impacted mice, test/control (%)	RC value for liver mitochondria in the presence of coenzyme Q_9, test/control (%)
10 min	87.9	97.3
2 h	81.9	96.05
5 h	87.2	95.7
7 h	91.9	90.0
12 h	86.0	91.0
24 h	70.1	82.3

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MOLECULAR AND CELLULAR MECHANISMS OF THE ORGANISM RESPONSES TO DAMAGES AND THEIR MODIFICATION BY COMPOUNDS

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Annotation

For the entire organism, a sequence of responses of the cellular systems of organs an changes in concentrations of some proteins in blood of mice and dogs to radiation and chemical compound impacts were studied using ESR spectroscopy, radioisotope and enzymatic activity determination methods. The response reactions were estimated by time- and dose-dependent post-radiation changes in the intensity of deoxyribonucleotides, DNA and protein biosyntheses in organs (spleen, liver, bone marrow, thymus), changes in Fe^{3+}–transferrin (Fe^{3+}–TF) and Cu^{2+}–ceruloplasmin (Cu^{2+}–CP) pools, superoxide dismutase activity, and inhibitor activity of α_2-macroglobulin in blood of dogs and mice. The standard nonspecific response of cellular systems to radiation manifests itself as the three-stage process:

1) quick SOS-changes, which include activation of a complex of SOS-repair enzymes for radiation-induced DNA damages, immediate increase of RNA and deoxyribonucleotide synthesis intensity, and Fe^{3+}–transferrin pool increase;
2) the increase of radiation-induced damage of cells in organs, caused by post-radiation inhibition of cellular processes, such as synthesis of deoxyribonucleotides, DNA and proteins and as well due to a significant decrease of Fe^{3+}–transferrin pool in blood;
3) further development of compensatory-reduction reactions, the key event in which is integral increase of intensity in the systems synthesizing deoxyribonucleotides and DNA, and the whole protein-synthesizing

apparatus. As a result, protein homeostasis, first of all, of Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin pools, is restored.

It is found that the iron-transport *protein Fe^{3+}–transferrin* pool controlling the rate-limiting iron-dependent stage in the DNA synthesis, representing synthesis of deoxyribnucleotides, is the highly sensitive blood marker. Its character of development and compensatory-reduction reaction intensity was judged about by time- and dose-dependent changes of this index. Temporal changes in Fe^{3+}–transferrin pool were of the phase type with the highest maxima on the second, sixth and 10 – 17th days for all doses of radiation that indicates nonspecific and universal character of compensatory–reduction reaction development in the organism. Intensity of these reactions depended linearly on the radiation dose up to lethal outcome i.e. deeper damages induced more intensive responses of the organism, which is obvious, because evolutionarily, the cells possess protective mechanisms aimed at recovery of the cellular composition of organs and tissues and normalization of cellular processes. However, a "*hyper-response*" which occurs at lethal radiation doses applied puts the organism at the critical edge, when the state of "*biochemical and physiological stress*" of the organism occurs and the risk of reduction reaction "breakdown" and animal death increases. For dogs with initial blood parameters typical of "*suppressed*" or "*activated*" state, radiation in 0.25 and 0.5 Gy doses induced a sharp increase of response intensity, i.e. a deviation from the linear dose–effect dependence was observed.

It is shown that natural antioxidant α-tocopherol and synthetic antioxidant ionol, applied in *definitely defined doses*, stimulate synthesis of deoxyribonucleotides, DNA and proteins in animal organs. These are key processes in the mechanisms increasing nonspecific resistance of the organism to damaging factors. Resulting activation of protein biosynthesis, Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin pools increase, therefore, inducing an increase of ferroxidase, superoxide dismutase, catalase, peroxidase and amine oxidase activities of blood and intensification of synthesis of iron- and copper-containing proteins. It is found that the stimulating effect of antioxidants on biosyntheses is the dose-dependent event. It should be noted that high doses, one-shot received or accumulated in the organism during many impacts, were insignificant for, but in some cases suppressed DNA and deoxyribonucleotide synthesis.

Biochemical markers of the organism resistance, the authors determined from the experimental data, were used in the health examination of

children – the victims of Chernobyl APP catastrophe. Analyzing the changes in Fe^{3+}– transferrin and Cu^{2+}–ceruloplasmin pools in blood, extracellular DNA content in blood plasma, α_2–macroglobulin and antitrypsin activity of blood plasma, superoxide dismutase and glutathione peroxidase activity of blood, we indicated that the initial two biochemical markers may be used for the control of biochemical adaptation of children organisms to changed environmental conditions and forecasting of distant consequences of low radiation dose effects. In blood of the majority of damaged children Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin pools were higher than in control, which indicates mobilization of protective systems of the organism as a response to radiation. However, such mobilization also has negative consequences: long existence the "*biochemical and physiological stress*" state is dangerous for child's organism, and any additional stresses (continued learning, undernutrition, various diseases, drug effects) may cause an exhaustion and "*breakdown*" of operation of the most important systems in organs and the whole organism.

Keywords: organism, damage and protection, deoxyribonucleotide and DNA synthesis, protein synthesis, children Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin, dynamics and dose dependencies, SOS-response, compensatory–reduction reactions, radioprotectors, antioxidants, protector action mechanisms

Everything beautiful on the
Earth is due to the Sun,
and everything good is due
to the Man.

Michail Prishvin
(the famous Russian writer)

INTRODUCTION

The centuries-old history of the natural science development shows that *there is nothing closer to the man except science about himself, the science about man and his environment*. The understanding of this fact and striving of the scientists to understand the essence of vital activity of the whole living being on the Earth defined the occurrence of new scientific branches studying molecular functioning mechanisms of cells and the whole organism, molecular grounds of disease development and molecular aspects of pharmacology, the mechanisms of interactions between the living being and permanently alternating environment. The necessity of experiencing molecular bases of life and the will to protect this life stipulated deep interpenetration of chemistry, physics, biology and medicine, appearing on integrated scientific directions in the researches and establishment of new branches of science, among which *physicochemical biology* or *biochemical physics* should be mentioned. The establishment and development of this direction in N.N. Semenov Institute of Chemical Physics, Academy of Sciences of the former USSR are associated with the name of N.N. Semenov student and follower, Academician N.M. Emanuel (1915 – 1984). *Chemistry and physics of the living being* became the main scientific directions of investigations, carried out in laboratories of the Section for Dynamics of Chemical and Biological Processes, headed by Academician Emanuel. At present, this direction in investigations is headed by his followers: Academician RAS A.L. Buchachenko and Academician RANS E.B. Burlakova.

In these years N.M. Emanuel held several executive posts in Academy of Sciences of the former USSR and combined the research activity with science organization duties. He was deputy Chairman of the Committee of AS USSR for Biological Tests and Selection of Biologically Active Chemical

Compounds. This fact predetermined the choice of the scientific direction of our, the new colleagues of his, investigations: "Development of pure fundamentals and applied aspects of creation of effective radioprotectors and radiosensibilizers of the new generation". The works carried out by N.M. Emanuel and the scientists from laboratories of his Section included studies of biological activity of many chemical compounds from various classes. These works were finished by synthesis of dibunol and nitrosomethylurea compounds. Dibunol represents typical antioxidant and inhibitor of radical processes; nitrosomethylurea belongs to the group of supermutagens. For these two compounds and subsequently some other antioxidants containing NO-generating groups, we were lucky to determine new aspects of their molecular and cellular protector mechanisms (Avdeeva *et al.*, 1979; Sharf *et al.*, 1981, 1986; Pulatova *et al.*, 1985, 1995; Filatov *et al.*, 1991). For the first time, it has been shown that antioxidants are able to induce deoxyribonucleotide (dNTP), DNA and protein synthesis in cells, whereas NO-generating compounds highly suppress this synthesis. N.M. Emanuel was also interested in investigations related to searching for and synthesis of compounds increasing the organism resistance to extreme factors, including ionizing radiation. In the period of 1970ies the attention paid to radiobiology was sharply risen, which was caused by many objectives reasons (the Report of UN Scientific Committee, 1988). In this direction, it has been found that, for example, an impact of strategic nuclear weapons will cause total γ-radiation of about 30% of urban regions' population, 80% among which might receive sublethal doses. Similar situation may occur at terroristic or accident destruction of nuclear fuel cycle enterprises, which was confirmed by Chernobyl APP accident. People working with radioactive sources also have high risk to be radiated. Therefore, radiobiological problems were tightly related to the question of national security. Emanuel has stated the following scientific directions of the investigations:

1. Creation of new indication methods for radiation damages in broad radiation dose and intensity ranges and determination of molecular and cellular mechanisms of the organism response to acute and prolonged radiation impact;
2. Determination of molecular and cellular mechanisms of radioprotector effect realization by compounds of different chemical structures;
3. Setting the fundamentals and development of applied aspects of the end use radioprotector and radiosensibilizer synthesis.

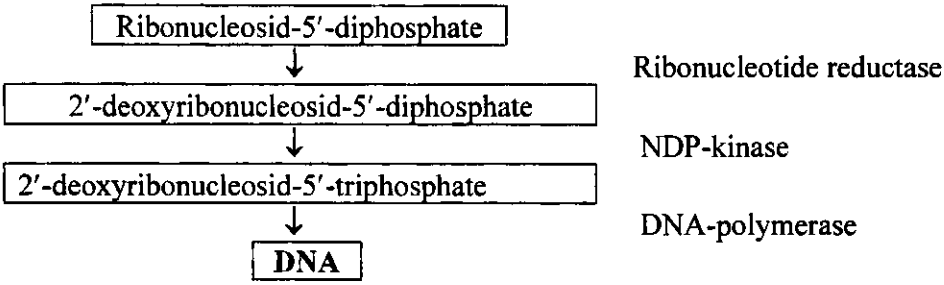
The implemented works resulted in suggestion of the unified selection principles and optimal injection modes for perspective radioprotector compounds of various design, basing on their ability to stimulate dNTP, DNA and protein synthesis in critically radiosensitive hematopoietic organs and liver. These long-term investigations were carried out in cooperation with the State Scientific Centre – Institute of Biophysics, Ministry of Health of the Russian Federation, the 7th Scientific Research Institute of Aviation and Space Medicine, Ministry of Defence of the Russian Federation, the Centre on Chemistry of Medical Compounds All-Russian Research Chemicophysical Institute, and Institute of Chemical Physics, Academy of Sciences of the former USSR. These works resulted in the synthesis of the following compounds: *indraline* – the first-aid radioprotector, highly effective at acute radiation impact in lethal doses, and *indomethaphen* – the compound designed for increasing organism resistance to low dose rate radiation impacts. As is indicated, indraline and indomethaphen increase cellular resistance of organs by the moment of radiation impact via induction of radioprotector-stimulated activation of dNTP synthesis in bone marrow, spleen and thymus. In hematopoietic organs of animals protected by this compound, high balanced pools of four dNTP are preserved during 30 – 36 hours after radiation impact, which is important for urgent repair of radiation-induced DNA damages and prevention of new DNA damages, usually formed in organs of unprotected animals due to dNTP synthesis, suppressed in the early post-radiation period. It is also shown that the second stage of dNTP, DNA and Protein synthesis is activated due to development of compensatory-reduction reactions in cellular systems of organs.

1. DEOXYRIBONUCLEOTIDE AND DNA SYNTHESIS SYSTEM RESPONSE IN THE ORGANISM RESPONSE TO RADIATION IMPACT AND THEIR MODIFICATION BY CHEMICAL COMPOUNDS

1.1. Responses to radiation impact

Modern ideas about protective organism responses to impacts of DNA-damaging factors suggest the presence of any SOS-like response aimed at

urgent elimination of the radiation-induced DNA damage and the following compensatory–reduction response, which should recover cellular structure of organs and blood, and normalize metabolic processes. Due to evolution, cells have protective mechanisms providing for repair of genetic material damages. The SOS-response activates a complex of urgent repair enzymes (Sakai, Okada, 1984; Elledge, Davies, 1987, 1989; Fornace *et al.*, 1989; Pulatova *et al.*, 1995; Pulatova *et al.*, 1999), the key unit in which is induction of ribonucleotide reductase (EC 1.17.4.1, RR). The accuracy of DNA duplication in repair and replication processes depends on the activity of enzymes participating in these processes and, which is most important, on the ratio and concentration of their substrates – the four types of dNTP. The synthesis of 2′-deoxyribonucleosid-5′-diphosphates *de novo* is catalyzed by RR enzyme and represents the rate-limiting stage of DNA synthesis (Scheme 1.



Scheme 1. Metabolic path for ribonucletide inclusion into DNA molecule

At this stage of DNA synthesis, all four types of dNTP are delivered continuously, because both quality and rate of the process depend on their concentration and the ratio of pools, and according to the substrate regulation mechanism, DNA-polymerase activity depends on dNTP pools. The dependence of DNA synthesis rate and DNA-polymerase activity on the total pool and separate pools of each dNTP type is of sigmoidal character. Therefore, even insignificant variations in the substrate concentration or their imbalance cause high suppression of DNA synthesis (Cory, Sato, 1983; Cory Carter, 1986; Pulatova *et al.*, 1986, 1990, 2003a; Pulatova *et al.*, 1989, 1997, 1999). According to results obtained by Sakai and Okada (1984), in the radiated cell culture, 99% of radiation-induced DNA damages are SOS–repaired during initial 70 minutes after the impact. The SOS-response to

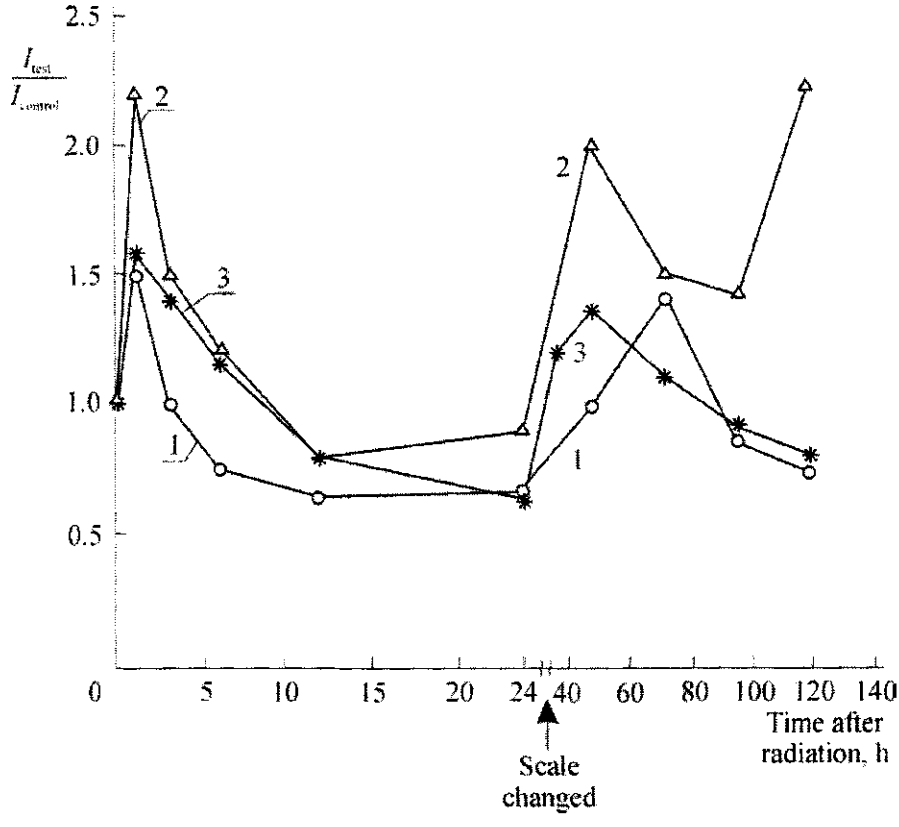


Figure 1. Relative changes ($I_{test}/I_{conctrol}$) of RR-activity in mouse spleen (1) and RNA synthesis rate in mouse spleen (2) and liver (3), irradiated by 6.5 Gy dose. The control group included intact mice. RNA synthesis rate was estimated by the rate of radioactive label inclusion (pulse/min·mg); RR-activity was estimated by ESR method.

impacts induced by various DNA-damaging factors is well studied at the cellular level (Snyder, 1984; McClanahan, McEntee, 1984; Walker, 1985; Elledge, Davies, 1989; Fornace *et al*, 1989; Choy *et al.*, 1989; Hurta, Wright, 1993) and much poorer for the whole organism. Figure 1 shows typical dynamics of ribonucleotide reductase activity (RR-activity) changes in spleen after irradiation of mice by 6.5 Gy dose. In the primary period (0.5 – 1.0 h) after the radiation impact RR-activity in spleen was 1.5-fold increased, and RNA synthesis became 2.2 times more intensive in spleen and 1.6 times –

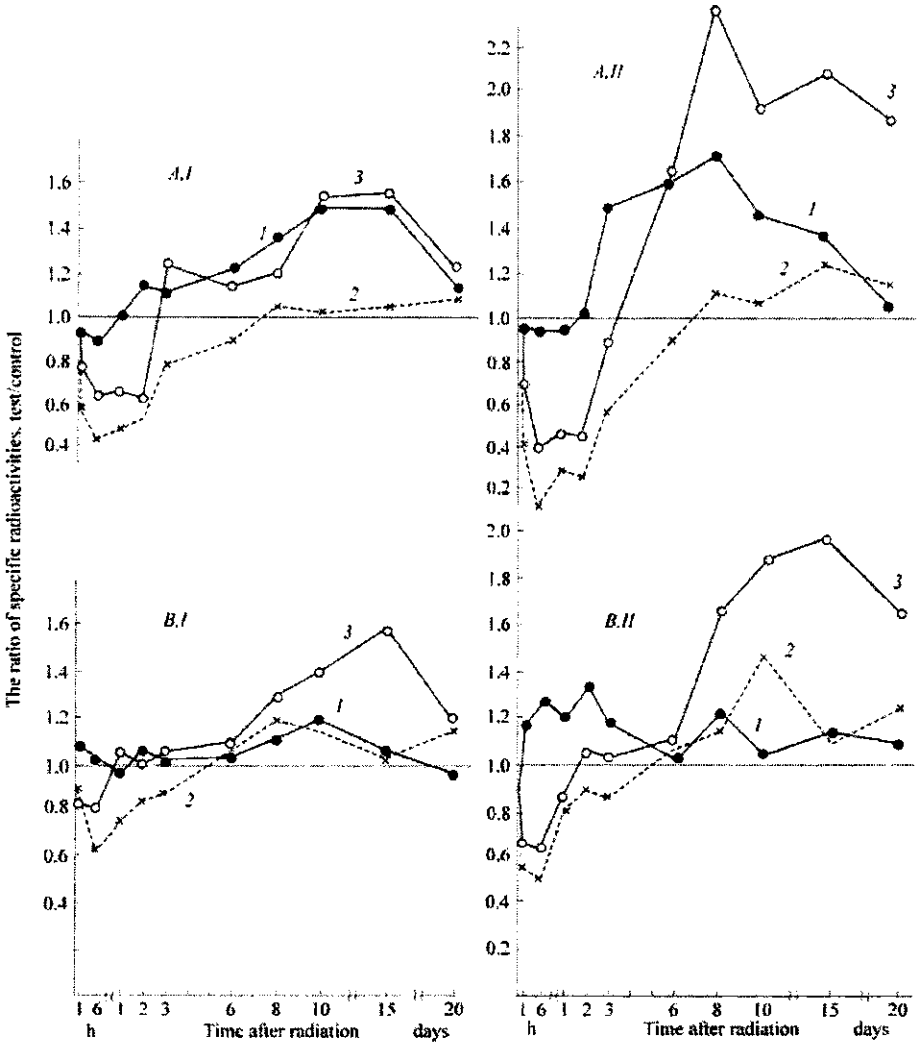


Figure 2. Reactions of protein (I) and DNA (II) biosynthesis systems in mouse spleen (a) and liver (b): 1 – after injection of radioprotector indraline (100 mg/kg); 2 – after irradiation in 6.5 Gy dose; 3 – after irradiation in 6.5 Gy dose at the background of radioprotector indraline (100 mg/kg), injected 15 min prior to the radiation impact.
The rate of radioactive label inclusion ($I_{test}/I_{conctrol}$) was measured (pulse/min·mg). The control group included intact animals. Each experimental point represents the value averaged by 15 mice.

in liver. In the period from 3 to 12 h RR-activity decreased to 60% of the control value, and RNA, DNA and protein synthesis was suppressed (Figure 1; Figure 2, curve 2). On days 2 and 3 of post-radiation periods, dNTP, DNA and RNA synthesis was restored.

Therefore, the response of cellular systems in animal organs can be divided into three stages:

1) the early SOS-activation of RNA and dNTP synthesis in spleen and liver;
2) subsequent stage of RR-activity and RNA, DNA and protein suppression;
3) the stage of reduction of these parameters.

The number of transcription factors in organs during SOS-response involves activation of transcription and accumulation of RR enzyme pool and, as a consequence, the increase of dNTP synthesis. As translation is suppressed by specific inhibitor cycloheximide (CHI), no SOS-activation of RR happened (Pulatova *et al.*, 2003b). The results of experiments with prokaryotes and eukaryotes in the cell culture testify about that occurrence of radiation-induced DNA damages is the signal for RR activation induction (McClanahan, McEntee, 1984; Walker, 1985; Elledge, Davis, 1987, 1989; Fornace *et al.*, 1989; Choy *et al.*, 1989; Mai *et al.*, 1989). The immediate RR activation was observed in hematopoietic organs (bone marrow, spleen, thymus) of animals impacted by radiation or a definite class of chemical substances (Pulatova *et al.*, 1995; Pulatova *et al.*, 1999). The mandatory condition of SOS-activation of dNTP synthesis is the necessity of repairing the increased volume of DNA damages. According to data from Table 1, in response to impacts of DNA-damaging agents a protective effect is observed in splenocytes and tumor cells: dNTP pools are increased (Pulatova *et al.*, 1999) and, consequently, DNA synthesis is intensified (Cory, Sato, 1983).

Table 1

SOS-stimulation of ribonucleotide reductase activity $\left(\frac{I_{test}}{I_{control}}\right)$ in animal tissues after impacts of radioprotector indraline and various DNA-damaging agents

Agents and doses	Test objects	Time after impact, h	RR–activity, $\frac{I_{test}}{I_{control}}$
γ-radiation:			
6.5 Gy	Mouse spleen	1.0	1.60 ± 0.28*
8.0 Gy	5-day newborn rat spleen	1.0	1.55 ± 0.18
Indraline (100 mg/kg)	Mouse spleen	1.0	1.60 ± 0.10
	Bone marrow	0.5	1.15 ± 0.11
Indraline (100 mg/kg) + γ-radiation (6.5 Gy)	Mouse spleen	3.0	1.19 ± 0.09
		1.0	1.95 ± 0.20
Local tumor radiation by 30 Gy dose after NF-100 injection	Spleen of mice with sarcoma S-37	0.5	1.53 ± 0.07
Local radiation of tumor by 30 Gy dose	Spleen of mice with sarcoma S-37	2.0	1.20 ± 0.10
Nitrosomethylurea, 80 mg/kg	Mouse spleen	1.0	1.38 ±0.06
		6.0	1.41 ±0.04
Metronidasole, 1 mg/g	Spleen of mice with sarcoma S-37	0.5	1,20 ±0,10
		1.0	1.42 ± 0.11
Nitrofuran, (NF-100), 0.1 mg/g	Sarcoma S-37	1.0	1.25 ± 0.15
		0.5	1.17 ± 0.05
		1.0	1.30 ± 0.16
	Mouse spleen	0.5	1.15 ± 0.05
	Spleen of mice with sarcoma S-37	1.0	1.27 ± 0.10

Note: * The values of RNA synthesis rate in spleen of irradiated and control mice were measured: $\frac{k_{test}}{k_{control}} = 2.2$.

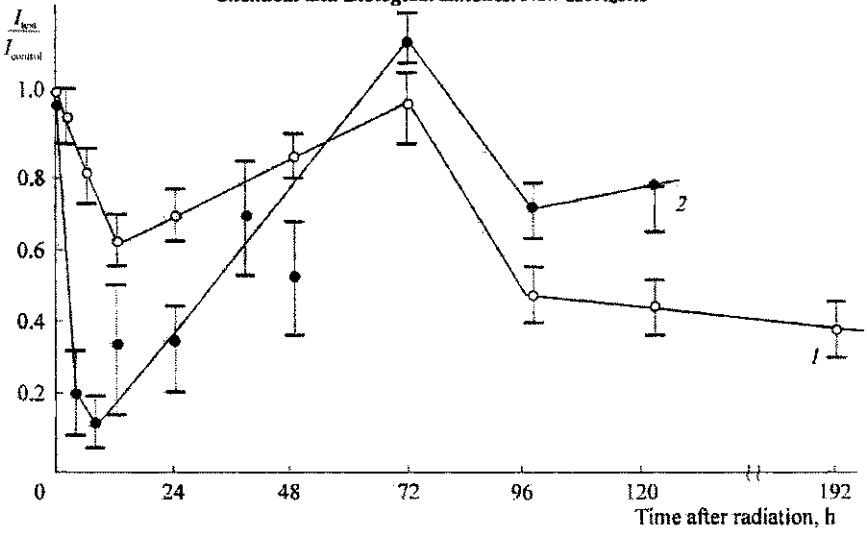


Figure 3. Relative changes ($I_{test}/I_{conctrol}$) of RR-activity (1) and DNA synthesis rate (2) in mouse spleen with time after γ-radiation impact of 6.5 Gy dose.
DNA synthesis rate was determined by the rate of radioactive label inclusion (pulse/min·mg)

Thus, for the entire organism, the fundamental event of SOS-response to DNA-damaging impacts in cells of the vitally important organs was found. It was also shown that SOS-response included the early activation of RNA and dNTP synthesizing systems, which duration is below 1 h after the damaging agent impact on the organism. SOS-activation of dNTP synthesis – the substrates of DNA synthesis enzymes – is the most important stage in the mechanisms providing for cellular stability.

In the period from 3 to 12 h dNTP synthesis in organs of irradiated mice was suppressed by 40% that also induced DNA synthesis rate decrease by 75 – 80% (Figures 1 and 3). As compared with RR-activity decrease, higher suppression of DNA synthesis was observed in all experiments, which is expressed by sigmoidal dependence between these parameters (Chernov *et al.*, 1994, 1996; Pulatova *et al.*, 1989, 1997, 1999; Pulatova *et al.*, 2003a). The suppression of dNTP synthesis reduces DNA synthesis rate and, first of all, affects the quality of DNA damage repair and replication synthesis (Snyder, 1984; *Genetic consequences* , 1985; Elledge, Davies, 1987, 1989; Meuth, 1989; Hirota *et al.*, 1989; Sargent *et al.*, 1989; Wilkinson, McKenna, 1989). This is testified by the results of experiments using specific RR inhibitors indicating the presence of relation between dNTP pool decrease, occurrence of DNA double-strand breaks and cell death (Cory, Sato, 1983; Snyder, 1984; *Genetic consequences* , 1985; Cory, Carter, 1986; Meuth, 1989; Sargent *et al.*, 1989; Hirota *et al.*, 1989; Wilkinson, McKenna, 1989). At the background of low concentrations of four dNTP types and their imbalance, defective repair and replication synthesis due to dNTP inclusion errors and occurrence of gaps responsible for metabolic formation of DNA breaks in the post-irradiation period are implemented. The inhibition of RR-activity in animal organs is stipulated by the radiation-induced suppression of protein synthesis (Figure 2), subunit M2, in particular. Active RR represents a complex of two subunits M1 and M2, the lifetime of which equals 15 and 3 h, respectively. RR activity is regulated by *de novo* synthesis of protein M2. The suppression of protein biosynthesis induces degradation of short-living protein M2 and RR inactivation (Pulatova *et al.*, 2003b). Actually, at CHI impact in the dose able to suppress translation by 95 – 97%, RR activity in spleen of rats was preserved at the control level only within initial 3 hours after injection, and then quickly decreased to 30% of the control value 6 h after (Figure 4). Low RR-activity was preserved up to 12 h due to low concentration of subunit M2,

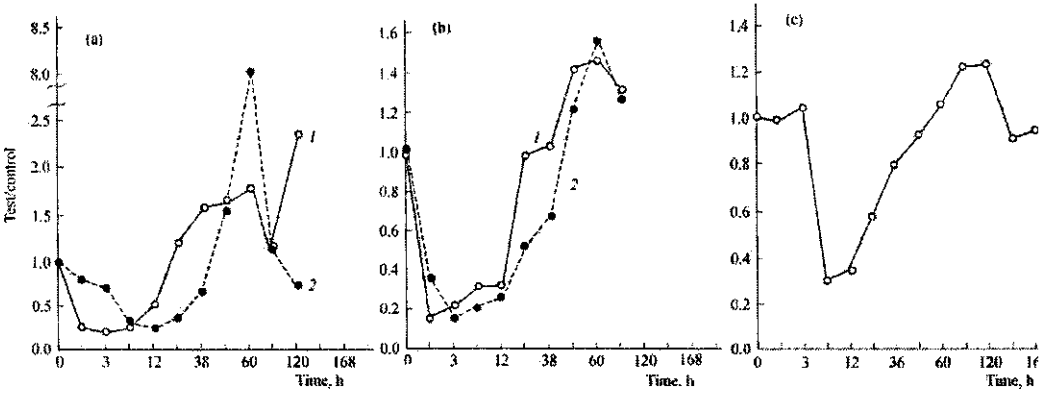


Figure 4. Dynamics of protein (1) and DNA (2) biosynthesis rate in liver (a) and spleen (b) and ribonucleotide red activity in spleen (c) of rats after injection of sublethal CHI dose (3 mg/kg).
The axis of ordinates: (a, b) – specific radioactivity, test/control; (c) – ESR signal intensity from active containing ribonucleotide reductase, test/control
The abscissa axis: time after CHI injection (t, h)

because at that time protein synthesis in spleen and liver was suppressed by 85% (Figure 4). In the same period from 3 to 12 h after translation blockade, DNA synthesis was also suppressed indicating similar dynamics of the current process and RR inactivation. Time dependencies of RR-activity reduction in spleen and protein synthesis in spleen and liver during the second-third days after CHI injection to rats were also similar.

Thus the mechanisms providing for cellular stability include activation of four type dNTP synthesis, required for DNA damage repair during the early post-radiation period and intensification of replication synthesis during regeneration of cells in radiosensitive organs of mammals. Therefore, in the searching for effective radioprotectors of special attention were chemical compounds able to induce RR-activity. Meanwhile, the radioprotector-stipulated increase of the RR-activity must occur during radiation impact and in the first day of the post-radiation period, when radiation induces maximal suppression of dNTP synthesis in organs of unprotected animals.

1.2. The influence of indraline and indomethaphen on deoxyribonucleotide synthesis in spleen and bone marrow of animals

The immediate action radioprotector indraline was injected intraperitoneally in 100 mg/kg dose to $(CBAxC_{57}BL)F_1$ strain mice, and intramuscularly in 15 mg/kg dose to dogs 10 – 15 min prior to the radiation impact. The long-term radioprotector indomethaphen was injected to dogs and mice perorally in 30 mg/kg dose 5 days prior to the radiation impact. These doses and injection rules gave the highest survivability of animals (Il'in *et al.*, 1994; Chernov *et al.*, 1994, 1996; Pulatova *et al.*, 2003a). Indraline demonstrated high protection ability in the survivability test: 85% ($p < 0.001$) of the control level for mice with $LD_{95/30}$ and 93% ($p < 0.001$) for dogs with $LD_{100/45}$. The highest radioprotective efficiency of indomethaphen observed equaled 90% for mice with $LD_{85/30}$ and 80% for dogs with $LD_{80/45}$. Indraline injected to intact mice in the radioprotective dose of 100 mg/kg 1.-fold increased RR-activity of spleen, and 1.2-fold – bone marrow activity in the initial hours after injection. The irradiated animals possessed increased RR-activity of hematopoietic organs, which is important for repair of the radiation-induced DNA damages. Indomethaphen provided for the highest survivability of animals, if the radiation impact was applied on the 5th – 7th day after its injection (Chenov *et l.*, 1996). Also, in this period the highest indomethaphen-

induced RR-activity of spleen and bone marrow was recorded. This index was increased by 2.3 times on the 5th day and by 2.7 times on the 6th day after indomethphen injection. Therefore, despite different injection regimes, both radioprotectors increased RR-activity of tissues of hematopoietic organs by the radiation impact. Also, high level of radioprotector-induced RR-activity of tissues in the organs was observed during initial 3 days of the post-radiation period. For instance, within 6 – 24 h after the radiation impact RR-activity of spleen in mice protected and unprotected with indraline differed by 1.9 times (Figure 5a). In the bone marrow of protected mice, a significant weakening of the inhibiting radiation effect on RR-activity was observed. In the subsequent periods the difference in RR-activity of bone marrow for protected and unprotected mice became higher (Figure 5 b). These data indicate that indraline is a powerful RR-activity inductor in the heterogeneous cellular system of bone marrow. According to this parameter, 6 days after the radiation impact radioprotection effect equaled 1.5 – 1.7. These data coincide with the experimental results, carried out on mice, guinea pigs and dogs (Vasin *et al.*, 1996). The latter have shown that indraline increases radioresistance of the bone marrow and spleen stem cells with FUD approaching the level of 1.5 – 2.0, and decreases the number of chromosomal aberrations in bone marrow cells. As a result of stem cell protection, repopulation in the bone marrow and spleen proceed much earlier. Obviously, the spleen mass in radiation-impacted mice, preliminarily protected by indraline, was restored much faster than in unprotected mice (Pulatova *et al.*, 2003a). For protected mice, RR-activity in the bone marrow was restored much earlier (2 days after the impact) compared with the control (unprotected) group (4 days after). As observed from the dose dependence, the highest RR activation in spleen and bone marrow was detected for the radioprotector dose of 100 mg/kg (Pulatova *et al.*, 2003a). An hour after injection of this dose of indraline to intact mice RR-activity in spleen was 1.6-fold increased, and 3 h after – 1.4-fold increased (Table 2). Also, for the bone marrow, this parameter was also increase, on the 6th day giving 6-fold higher value compared with the control group. These results indicate that during the radiation impact and several hours after it radioprotected animals possessed high level of dNTP synthesis in hematopoietic organs.

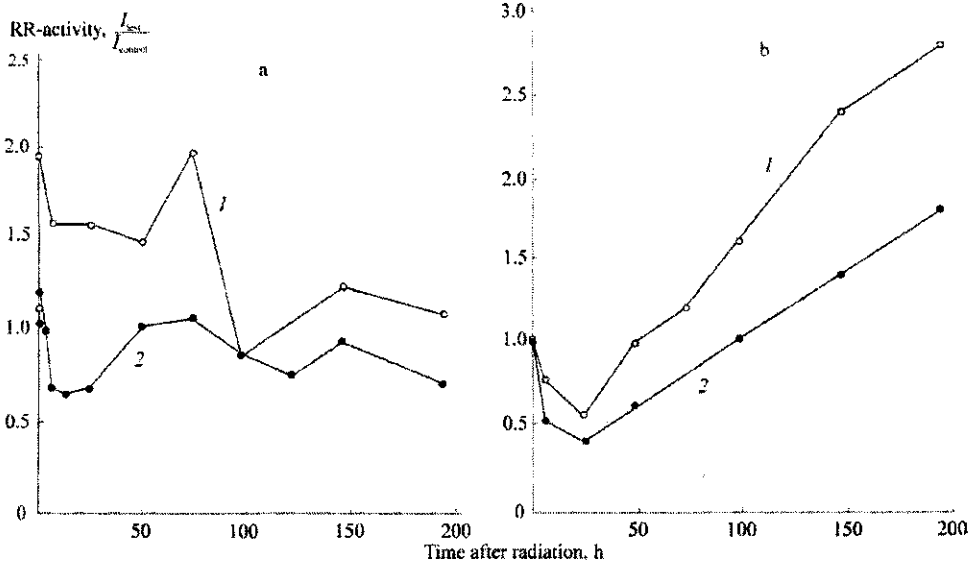


Figure 5. Dynamics of RR-activity change ($I_{test}/I_{control}$) in spleen (a) and bone marrow (b): 1 – after total γ-irradiation of mice by 6.5 Gy dose. Prior to the radiation impact (15 min), these mice were given indraline in radioprotective dose (100 mg/kg); 2 – after total γ-irradiation in 6.5 Gy of mice, unprotected by radioprotectors. The control group was formed by intact mice.

It should be noted that for unprotected animals dNTP synthesis is significantly suppressed in the early post-radiation period (Table 2). In organs of protected animals high balanced pools of four dNTP types provide for qualitative and greater repair of radiation-induced DNA damages, prevent new DNA damages during the post-radiation period, suppress the inhibiting effect of radiation of DNA synthesis, and provide for the earlier removal of radiation block from DNA synthesis. The second peak of radioprotector-stimulated RR-activity increase with simultaneous increase of DNA and protein synthesis promotes earlier and more intensive development of compensatory-reduction reactions in the cellular systems of organs.

Table 2

Relative changes in ribonucleotide reductase activity of spleen and bone marrow in mice for different periods after indraline 100 mg/kg dose injection to animals irradiated by 6.5 Gy dose

(the control group of intact animals physiological substance was given)

Radioprotector and radiation dose	Time after impact, h	RR-activity, test/control	
		Spleen	Bone marrow
Indraline 100 mg/kg	0.5	1.20 ± 0.07	1.15 ± 0.11
	1.0	1.60 ± 0.10	1.17 ± 0.10
	3.0	1.40 ± 0.10	1.19 ± 0.09
	6.0	1.35 ± 0.05	1.54 ± 0.15
	24.0	1.55 ± 0.10	1.81 ± 0.25
	48.0	-	2.50 ± 0.37
	72.0	1.48 ± 0.10	3.00 ± 0.46
	96.0	-	3.69 ± 0.40
	144.0	1.00 ± 0.10	6.30 ± 0.96
	192.0	1.10 ± 0.16	3.80 ± 0.50
Radiation 6.5 Gy	0.0	1.00 ± 0.06	1.00 ± 0.06
	0.5	1.20 ± 0.04	-
	1.0	1.20 ± 0.05	-
	3.0	0.98 ± 0.07	-
	6.0	0.68 ± 0.08	0.52 ± 0.12
	24.0	0.65 ± 0.04	0.40 ± 0.13
	48.0	1.00 ± 0.12	0.60 ± 0.06
	72.0	1.04 ± 0.18	0.80 ± 0.13
	96.0	0.85 ± 0.03	1.00 ± 0.05
	120.0	0.74 ± 0.04	-

	144.0	0.91 ± 0.07	1.40 ± 0.06
	192.0	-	1.80 ± 0.14
Indraline	0.0	1.00 ± 0.06	1.00 ± 0.06
100 mg/kg	0.5	1.20 ± 0.04	-
and	1.0	1.95 ± 0.20	-
radiation	6.0	1.60 ± 0.05	0.75 ± 0.06
6.5 Gy	24.0	1.55 ± 0.10	0.55 ± 0.27
	48.0	1.45 ± 0.10	0.98 ± 0.16
	72.0	1.95 ± 0.05	1.21 ± 0.15
	96.0	-	1.64 ± 0.26
	144.0	1.20 ± 0.10	2.40 ± 0.36
	192.0	-	2.80 ± 0.40
Indomethaphen*	120.0	2.30 ± 0.28	-
30 mg/kg	144.0	2.69 ± 0.30	-

Note: * Indomethaphen induced high RR-activity on the 5th – 6th day after injection to mice. Radiation of indomethaphen-protected animals in this period produced optimal protection by the survivability test.

Therefore, radioprotectors indraline and indomethaphen, effective according to the survivability test, are powerful inductors of dNTP, DNA and protein synthesis, which are the key processes for DNA damage repair and intensified development of reductive reactions of the organism. These are the effects defining high survivability of lethally irradiated animals and represent the most important stages in the protector mechanism of studied radioprotectors.

1.3. The responses of deoxyribonucleotide and DNA biosynthesis system to the influence of substance possessing antioxidant properties

In the researches of compounds increasing resistance of the organism, we became interested in substances with antioxidant properties, possessing a broad spectrum of biological actions (Alessenko *et al.*, 1980; Kosaganova *et al.*, 1984, Sies, Cadenas, 1985; Pulatova *et al.*, 1985, 2004; Slater, Cheeseman, 1988; Burlakova, 1992, 2002; Dyumaev *et al.*, 1995; Kasaikina *et al.*, 1998). Among the basic biological effects of antioxidants, their ability to protect biological membranes against peroxide oxidation of lipids is of the greatest interest. The classical theory of antioxidant action considers commonly known

antioxidants α-tocopherol and ionol (2,6-di-*tert*-butyl-4-methylphenol) as molecules capable of terminating the POL chain via interaction with hydroperoxide radicals of fatty acids. In these reactions, α-tocopherol transfers hydrogen atom to peroxide radical, therefore, converting it to corresponding hydroperoxide and forming chromanoxyl radical, which, in its turn, affected by reducers, may transform to α-tocopherol. It has been shown that, besides antioxidant action, these substances are capable of activating some molecular-cellular processes, among which protein and DNA biosynthesis is of the greatest importance (Alessenko *et al.*, 1980; Kosaganova *et al.*, 1984; Pulatova *et al.*,1985; Kasaikina *et al.*, 1998). As indicated in these works, ionol doses of 30 and 100 mg/kg accelerated proliferative processes in regenerating mouse liver via activating protein and DNA synthesis.

The present work shows results testifying about the ability of natural antioxidant α-tocopherol to increase intensity of dNTP and DNA synthesis in spleen, thymus and bone marrow in mice, and synthetic antioxidant ionol to increase intensity of DNA and protein synthesis in organ tissues in mice. In twin-80, α-tocopherol was single-time injected to (CBA x C_{57}BL)F_1 strain mice intragastrically in the dose of 17 mg/kg (for a mouse weighted 15 g the dose equaled 0.255 mg) or daily during 4 days (total dose equaled 68 mg/kg). Figure 6 shows temporal changes in RR-activity (I) abd DNA synthesis intensity (II) in spleen, thymus, bone marrow and liver, induced by α-tocopherol injection in mice. Six hours after single dose injection RR-activity in the bone marrow increased by 1.5 times, but one day after was found below the control level and remained at this level by the experiment end (Figure 6a, curve 1). However, the following second, third and fourth dose of α-tocopherol had no effect on RR-activity in bone marrow during initial five days after the first dose injection. The value of this parameter increased on the 6th – 8th day after the beginning of the experiment, i.e. two days after antioxidant intake finish. At single-time injection of α-tocopherol, RR-activity was increased in another organ (spleen) on the 2nd – 3rd and the 6th – 8th day (Figure 6, Ib). Apparently, the second peak of RR-activity in organs occurred after excretion of the main part of α-tocopherol from the organism. Within 24 h after one dose intake of antioxidant RR-activity in spleen and thymus was suppressed (Figure 6, Ib and Ic). After 6 h in thymus this decrease reached 50% of the control level, and 20% in spleen. In these periods DNA synthesis was also suppressed in both organs (Figure 6, IIb and IIc). Further on, independently of the regime of α-tocopherol intake, RR-activity in spleen and thymus was significantly

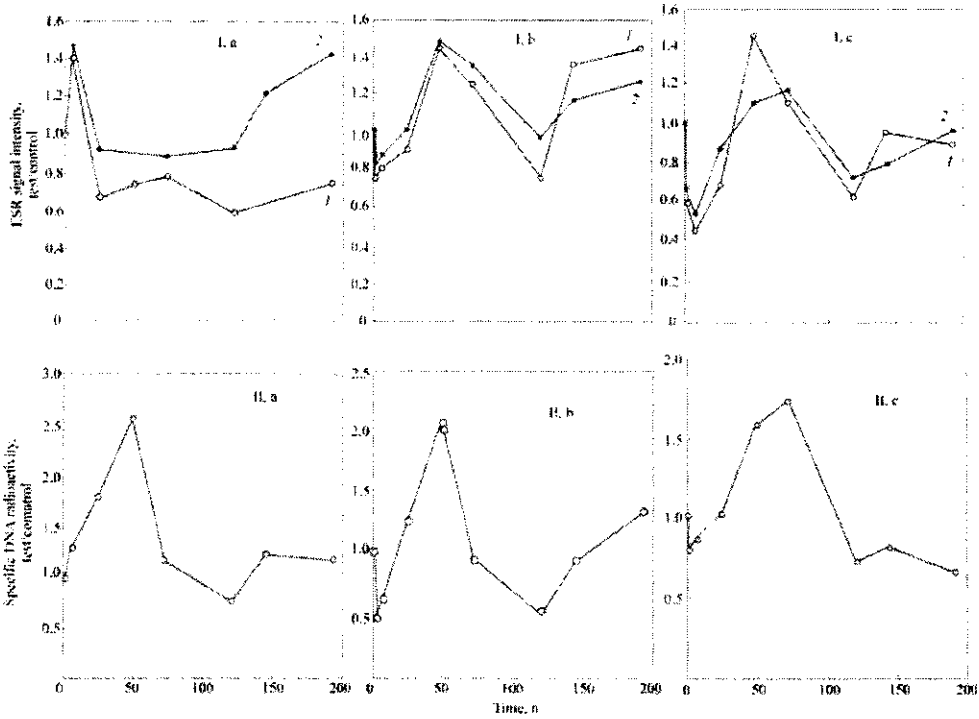


Figure 6. Temporal changes of ribonucleotide reductase activity (I) and DNA synthesis rate (II) in bone marrow (Ia), spleen (Ib and IIb), thymus (Ic and IIc) and liver (IIa) of mice after one-time (1) and four-time (2) intragastric injection of α-tocopherol in twin–80. For one-time injection of antioxidant, the dose equaled 0.25 mg per mouse; at multiple injections, total dose equaled 1.0 mg per mouse. The rate of DNA synthesis was determined from the rate of radioactive label inclusion (pulse/min·mg DNA)·10^{-3}. Each experimental point value is averaged by 15 mice. Maximal deviations from the average value are below 15%.

The axis of ordinates: (I) – the relation between ESR signal intensities of active ribonucleotide reductase in experiment and in control; (II) –the relation between DNA synthesis rates in experiment and in control (intact animals).

The abscissa axis: time after one-time (I, 1) or first dose (I, 2 and II) intake of antioxidant

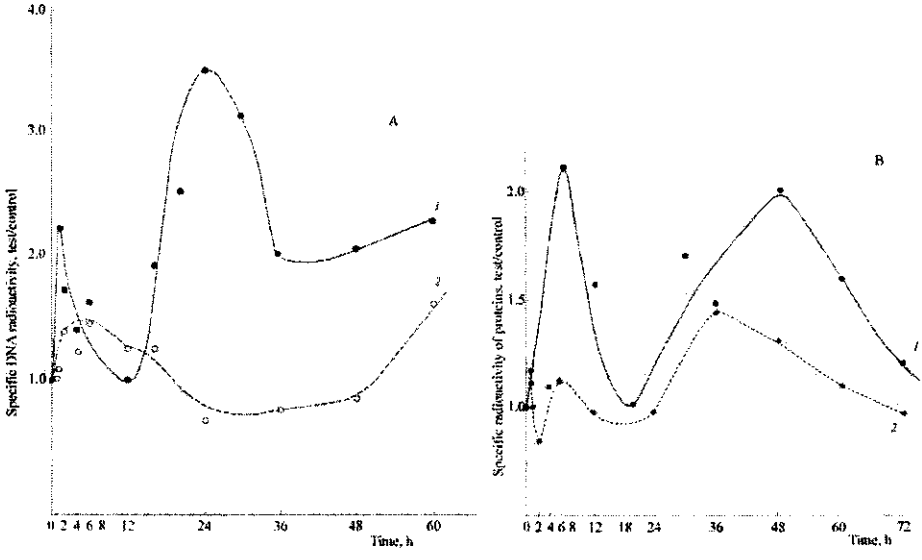


Figure 7. Temporal changes in DNA (a) and protein (b) synthesis rates in mouse liver after one-time intraperitoneal injection of ionol in 60 mg/kg (1) and 200 mg/kg (2) doses. Control animals received olive oil injections. The rates of DNA and protein synthesis were determined from the rate of radioactive label inclusion (pulse/min·mg DNA)·10^{-3} (a) and (pulse/min·mg protein)·10^{-2} (b), respectively. Each experimental point is averaged from data on 10 – 12 mice. The average value differed from the individual value, measured for each mouse, by 15% (a) 20% (b), respectively.
The axis of ordinates: specific radioactivity of DNA (a) and proteins (b), (test/control).
The abscissa axis: time after ionol injection

increased reaching its maxima on the 2nd – 3rd and 6th – 8th days. Since the first day, the intensity of DNA synthesis in all studied organs significantly increased and reached the maxima on the second day (liver and spleen) and on the 2nd – 3rd day (thymus). In spleen, DNA synthesis intensity also increased on the 6th – 8th day, which was related to the increase of dNTP synthesis volume. In thymus on the 6th – 8th day, the level of dNTP synthesis was below the control that stipulated low intensity of DNA synthesis in this period. The increase of dNTP synthesis intensity in bone marrow 6 h after α-tocopherol injection is caused by antioxidant-induced activation of protein synthesis in organs of mice. Actually, injection of the second antioxidant (ionol) to mice induced a sharp increase of protein synthesis intensity in the liver of mice directly in this period (Figure 7).

Thus, α-tocopherol injection to animals increases RR-activity and stimulates DNA synthesis in organs, inducing phase changes of these parameters with the maxima on the 2nd –3rd and 6th – 8th day. Comparative analysis of data on the stimulating effect of antioxidants and radioprotectors on dNTP and DNA synthesis in animal organs allows a conclusion that these are determining processes in the protector mechanism of these substances. Moreover, as follows from these data, a single dose intake of the antioxidant is enough for reaching optimal values of these parameters, typical of every individual, and is determined by the volume of cellular reserves required for purposes of synthesis. Therefore, the following doses of α-tocopherol did not cause additional stimulation, and in some cases even suppressed dNTP and DNA synthesis.

For the second antioxidant (ionol), the stimulating effect on protein and DNA biosynthesis in liver also depended on the dose injected to animals (Pulatova *et al.*, 2004). After injection of 60 mg/kg dose of ionol DNA synthesis was activated in the periods from 1 to 6 h (1.5 – 2.0 times higher than in the control) and from 24 to 36 h (3.5 times higher) (Figure 7a). This parameter was also 2.5 higher in the period from 36 to 72 h (till the end of the experiment). The protein synthesis in liver was 2-fold more intensive than in control both 6 h and 2 days after ionol injection in mice (60 mg/kg). The dose of 200 mg/kg did not induce any significant change in DNA synthesis rate in liver during initial two days. In more distant periods an increasing tendency of this parameters is observed. This dose (200 mg/kg) stimulated protein biosynthesis in liver, but the rate of the process was lower compared with the lower dose (60 mg/kg). These data confirm a conclusion that *the stimulating action of antioxidants on biosynthesis of macromolecules is the dose-dependent*

process, and large doses either cause no effect on DNA synthesis rate, i.e. no stimulation is observed, or even inhibit DNA biosynthesis. Only in distant periods, when the main part of compound was already excreted from the organism, a tendency to increasing DNA synthesis rate was observed. The ionol-dependent activation of protein and DNA synthesis was also observed in liver regenerating after hepatectomy operation at the background of ionol injection in 100 mg/kg dose (Pulatova *et al.*, 2004). DNA synthesis rate reached its maximum in the period equal to the control (36 – 48 h after the operation), but it was 1.5 times higher than in control (without ionol). Also, ionol 2.0 – 2.5-fold increased the protein synthesis rate in the prereplication period (6 – 30 h after operation). In the post-replication period (40 – 50 h after operation) the protein synthesis rate in test and control groups were almost similar, but for the test group maximal rate was observed 6 h later than in control. It can be concluded that ionol stimulates protein and DNA synthesis at early stages of liver regeneration.

Thus, α-tocopherol increases RR-activity and stimulates DNA synthesis, whereas ionol activates protein and DNA synthesis in organs of animals. Possible mechanisms of the stimulating action of antioxidants on biosynthesis processes in the whole organism are discussed (Pulatova *et al.*, 2004). One of the main mechanisms of the stimulating action may be associated with strong induction of xenobiotic detoxication enzymes in response to antioxidant injection. Obviously, dynamics of ionol-induced protein and DNA synthesis in liver – the organ possessing the most powerful detoxication system – reflects the mass increase of protein-synthesizing and energy producing compounds. Ionol belonging to the group of spatially hindered phenols is perceived by the cell as a typical xenobiotic, for which 20 – 30-fold increased content of mRNA biotransformation enzymes is indicated (Saprin, 1991). This is confirmed by our data on the inhibiting action of α-tocopherol on dNTP and DNA biosynthesis in spleen and thymus in the early period of its application (Figure 6). However, the above reasoning cannot explain the observed differences in high and low dose actions of antioxidants. Apparently, detoxication system is unable to consume "high" doses of antioxidants and, therefore, the injected compounds produce toxic effects.

Thus, besides the antioxidant properties, α-tocopherol and ionol are able to increase RR-activity in organs of animals, subsequently increasing dNTP synthesis volume and, therefore, DNA synthesis rate, and then, finally, the resistance of cells in organs. Provision of high dNTP pools by antioxidants

creates conditions for high-quality repair of DNA damages and restoration of the cellular composition in organs in case of their damage.

2. THE INFLUENCE OF TOTAL γ-RADIATION, RADIOPROTECTORS AND ANTIOXIDANTS ON FE^{3+}–TRANSFERRIN AND CU^{2+}–CERULOPLASMIN CONTENT IN BLOOD AND DEPENDENCE OF RIBONUCLEOTIDE REDUCTASE ACTIVITY IN TISSUES OF ANIMAL ORGANS ON THESE PARAMETERS

The synthesis of dNTP in hematopoietic organs – bone marrow, spleen and thymus – is catalyzed by iron-containing ribonucleotide reductase (Fe^{3+}–RR), which is specifically sensitive to oscillations of iron content in the cell. The requirement of iron for actively fusing cells during DNA synthesis is defined by iron demand for subunit M2 enzyme Fe^{3+}–RR. The transfer of Fe^{3+} ions to cells of various organs is performed by iron transporting blood plasma protein Fe^{3+}–transferrin (Fe^{3+}–TF) via Fe^{3+}–TF complex formation with transferrin receptor (*The Biochemistry and Physiology of Iron*, 1982; Crichton, 1990; De Jong *et al.*, 1990); moreover, the receptor blockade suppresses DNA synthesis. Protein Fe^{3+}–TF controls RR-activity of cells, providing for *de novo* synthesis of subunit M2 containing two iron ions (Thelander, Reichard, 1979; Chitambar, Zivkivic, 1987; Chitambar *et al.*, 1988). The second important protein of blood plasma Cu^{2+}–ceruloplasmin (Cu^{2+}–CP), possessing ferroxidase activity and oxidizing Fe^{2+} ions to Fe^{3+}, provides for inclusion of iron ions into apotransferrin (Frieden, 1981). Being copper-transporting proteins, it controls synthesis of copper-containing proteins. Moreover, this protein possesses superoxide dismutase, peroxidase and amino-oxidase activities and represents one of the main blood antioxidants and regulator of the biogenic amine level (Frieden, 1981). These two proteins, most important for the blood plasma, are chosen as the main estimation criteria for organism resistance changes. The experimental results obtained and clinical test data confirmed advisability of their use for estimation of efficiency of applied anti-radiation protection.

2.1. The influence of total γ-radiation on Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin content in blood of animals

The shape of time and dose dependencies of changes in Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin concentration in blood of irradiated dogs may be judged about by the results of one of 7 series of experiments, shown in Figure 8(a and b). Clearly in response to radiation impact unitypical changes in Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin pools were developed. Moreover, these dependencies were of the phase type and independent of the radiation dose. Maximal Fe^{3+}–transferrin pools were registered on the 2nd and the 6th days after radiation impact. Subsequent maxima were less intensive, extended in time, were recorded from 10 to 17, 25 to 27 and 45 to 60 day after radiation impact. Phase changes in Fe^{3+}–transferrin pool, induced by 6.5 Gy dose radiation (Figure 9a), were also observed in blood of mice at the background of injection of radioprotector indomethacin (Figure 9b) or single injection of indomethaphen to intact animals (Figure 9c). Also, the phase dependence was determined for changes in superoxide dismutase activity, anti-proteolytic activity of α_2–macroglobulin and metHb content in blood (Sharygin *et al.*, 2003). These data testify about nonspecificity and universal character of the organism responses to radiation damage or injection of the compound. Temporal dependence of changes in Fe^{3+}–TF pool in blood of radiated animals was divided into three periods, when the following events took place:

1) SOS-increase of Fe^{3+}–TF pool, required for activation of a complex of DNA damage SOS repair enzymes;
2) post-radiation decrease of Fe^{3+}–TF pool, involving inhibition of iron-dependent processes and intensification of the radiation-induced damage;
3) Subsequent phase increases in Fe^{3+}–TF pool, which determine development of compensatory-reduction reactions – protein, dNTP and DNA synthesis, first of all.

During initial 30 min, SOS changes in Fe^{3+}–TF pool in dog blood were individual and depended on the radiation dose: below 1 Gy, this parameter decreased, and at higher doses – increased. The immediate increase of Fe^{3+}–TF pool is considered as a component of the SOS-reaction of the organism, aimed

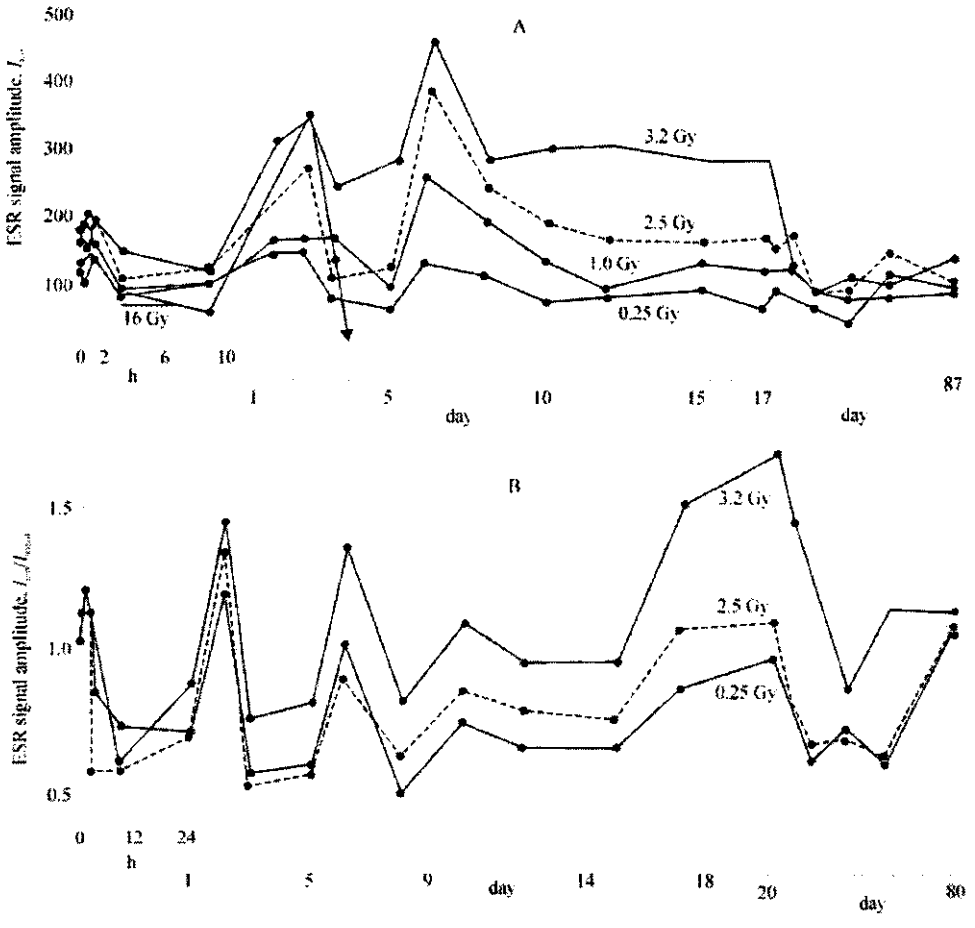


Figure 8. Temporal and dose-dependent changes in Fe^{3+}–transferrin (a) and Cu^{2+}–ceruloplasmin (b) pools in blood of dogs from the initial state (before radiation impact) till death of the animal (16 Gy radiation dose) or by 80 days after radiation impact for survived animals (radiation doses are 3.2, 2.5, 1.0 and 0.25 Gy). Data of one sequence of experiments are shown, each curve related to one dog. The arrow indicates the time of dog's death.

The axis of ordinates: a – ESR signal amplitude (in conditional units), normalized by the standard signal; b – the ratio $I_{test}/I_{initial}$, where $I_{initial}$ is the ESR signal amplitude before radiation impact, and I_{test} id the ESR signal amplitude in the post-radiation period.

The abscissa axis: time after irradiation impact

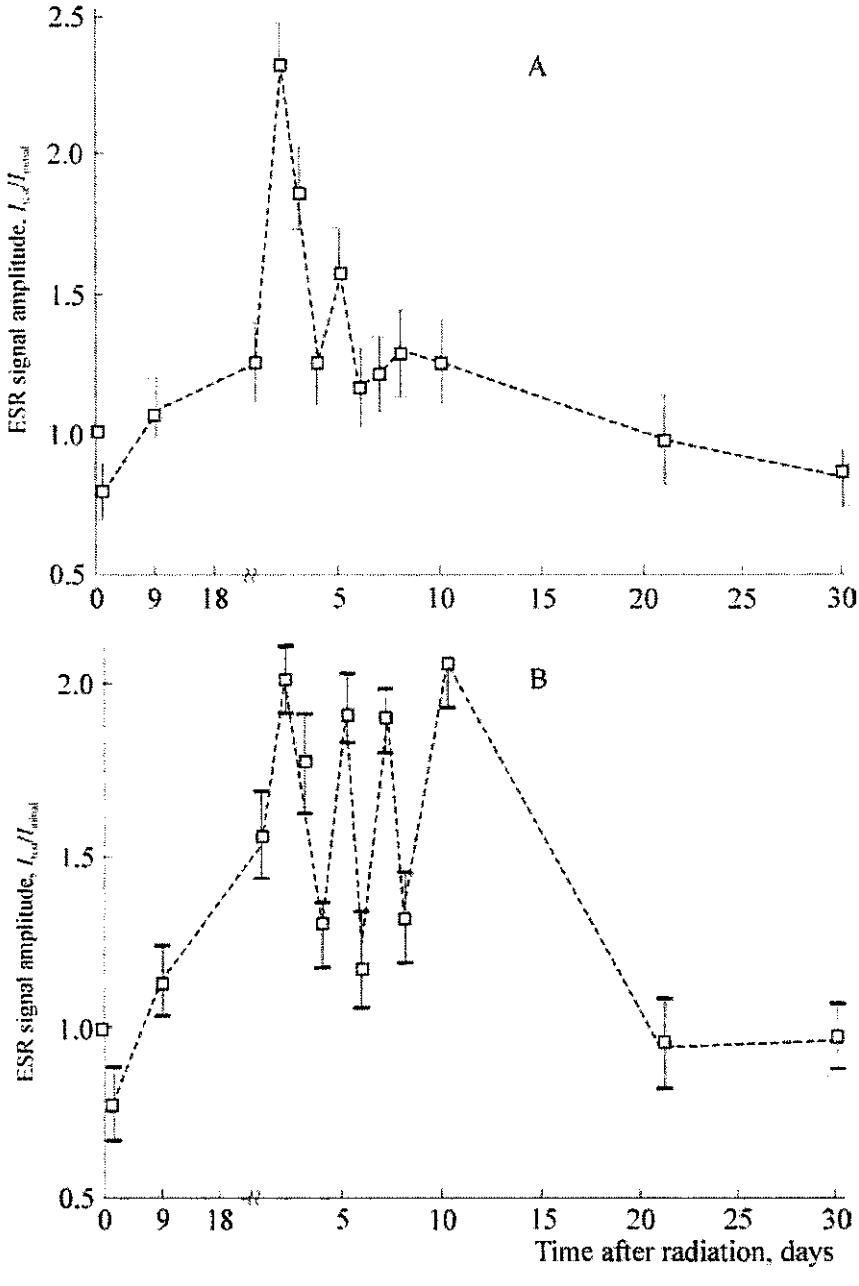

A
B
ESR signal amplitude, I_{rad}/I_{total}
2.5
2.0
1.5
1.0
0.5
0
9
18
5
10
15
20
25
30
Time after radiation, days

Figure 9 (A, B)

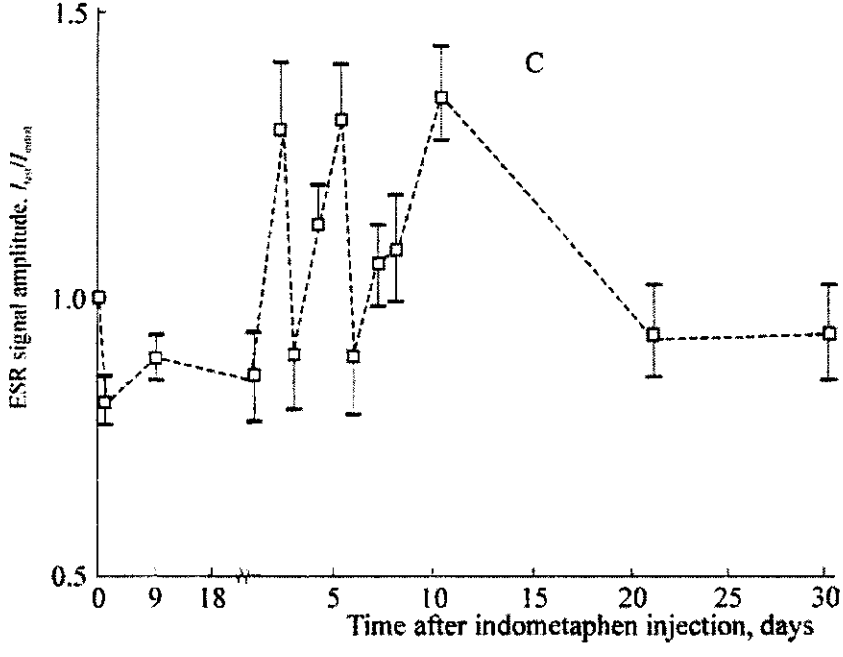


Figure 9. Temporal changes of Fe^{3+}–transferrin pool in blood of mice: (a) after radiation impact by 6.5 Gy dose; (b) after radiation impact by 6.5 Gy dose at the background of injected radioprotector indomethaphen in 30 mg/kg dose; (c) after indomethaphen injection to intact mice in 30 mg/kg dose

The axis of ordinates: ESR signal amplitude for Fe^{3+}–transferrin (the ratio $I_{test}/I_{control}$)

The abscissa axis: time after radiation impact (a and b) or indomethaphen injection (c)

at mobilization of protective functions of the cellular systems as a response to occurrence of DNA damages. For dogs, 1 Gy radiation dose is the threshold for the SOS-response formation. Therefore, the signal for activation of a complex of enzymes for SOS repair is occurrence of a definite volume of DNA damages. The necessity of increased DNA damage repair stipulates SOS increase of ribonucleotide reductase activity of hematopoietic organ tissues – spleen, bone marrow and thymus (Pulatova *et al.*, 1999; Pulatova *et al.*, 2003a). For doses below 1 Gy, Fe^{3+}–TF pool decrease was stipulated by radiogenic stress and occurrence of a significant amount f catechol amines in blood, which reduced iron ions in Fe^{3+}–TF protein leading to the loss of Fe^{2+} ions and formation of apotransferrin and lowmolecular iron complexes, which concentration significantly increased in this period.

$$Fe^{3+}\text{-}TF + e^{-}\ (adrenaline) \rightarrow Fe^{2+} + H^{+} + adrenaline\ radical + apotransferrin$$

The release of iron ions from Fe^{3+}–TF is accompanied by accumulation of adrenaline radicals and overacidation of the environment. Previously, such effect of pH reduction was observed in isolated hepatocytes in rats at Fe^{3+}–TF reduction by means of NADH (deJong *et al.*, 1990; Crichton, 1990). Meanwhile, the loss of Fe^{2+} ions by proteins and NAD^{+} occurrence was registered. The decreased pH values induced changes in physicochemical properties of transferrin receptors (conformation changes, strengthening of bonding to transferrin), as well as H^{+}/e^{-} ratio, Na^{+}/H^{+} exchange transport, and Ca^{2+} mobilization.

The pool of Fe^{3+}–TF in dog's blood in the period from 1 to 9 h after radiation impact is decreased by 20 – 50% with respect to the radiation dose. This causes suppression of iron-dependent dNTP, DNA and iron-containing protein synthesis, which increases the radiation damage. At the same time, peroxide oxidation of lipids is activated and the operation of bioenergetic and detoxicating systems breaks. Low Fe^{3+}–TF pool is preserved by the end of the first day. This may be reasoned by the loss of anions HCO_3^- or CO_3^{2-} from apo-transferrin, required for tight bonding of Fe^{3+} ions (Crichton, 1990; de Jong *et al.*, 1990), degradation of its liposomal enzymes, activated in decreased pH environment in the same periods. Simultaneously, biosynthesis of total

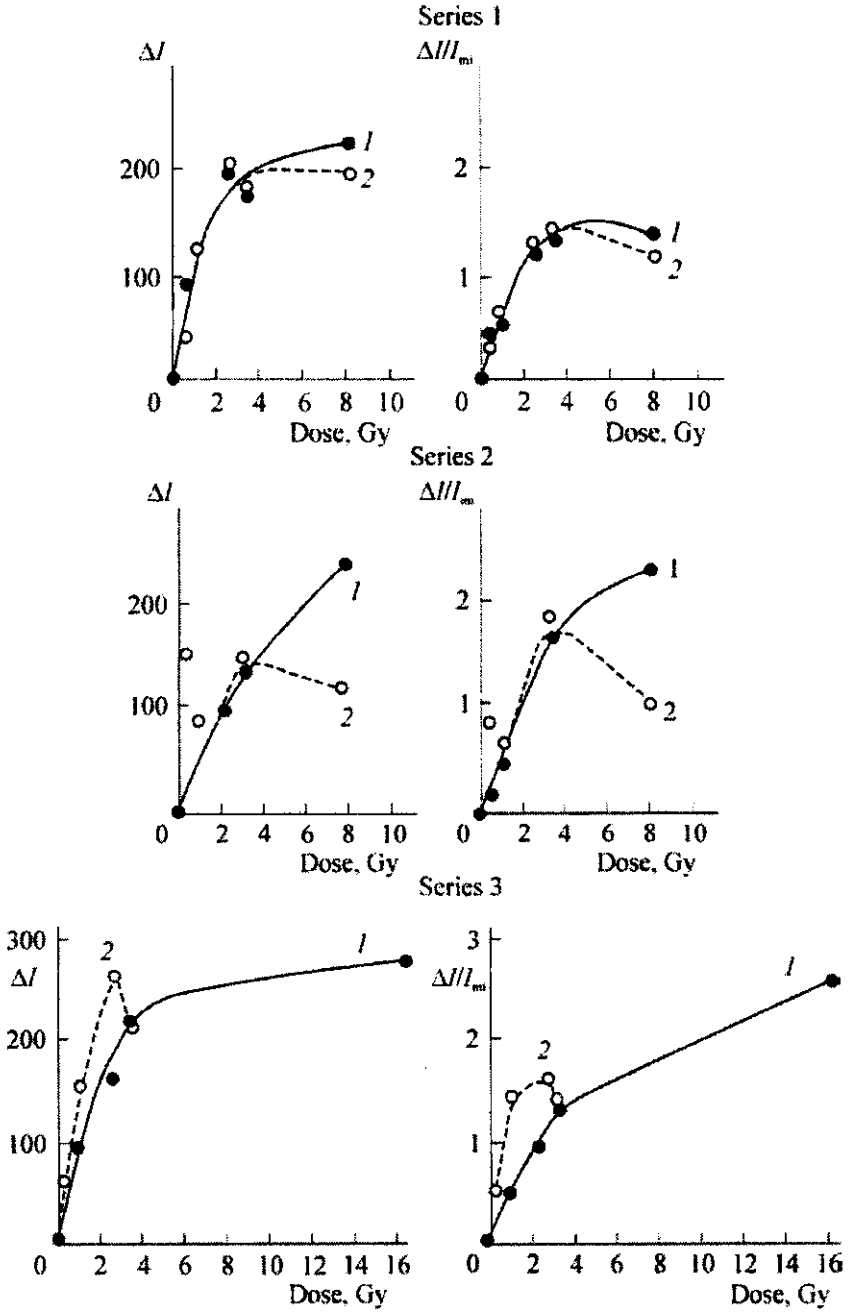

Series 1
ΔI
ΔI/I_m
200
100
2
1
0 2 4 6 8 10
Dose, Gy
Series 2
Series 3
300
3
16
1
2

Figure 10

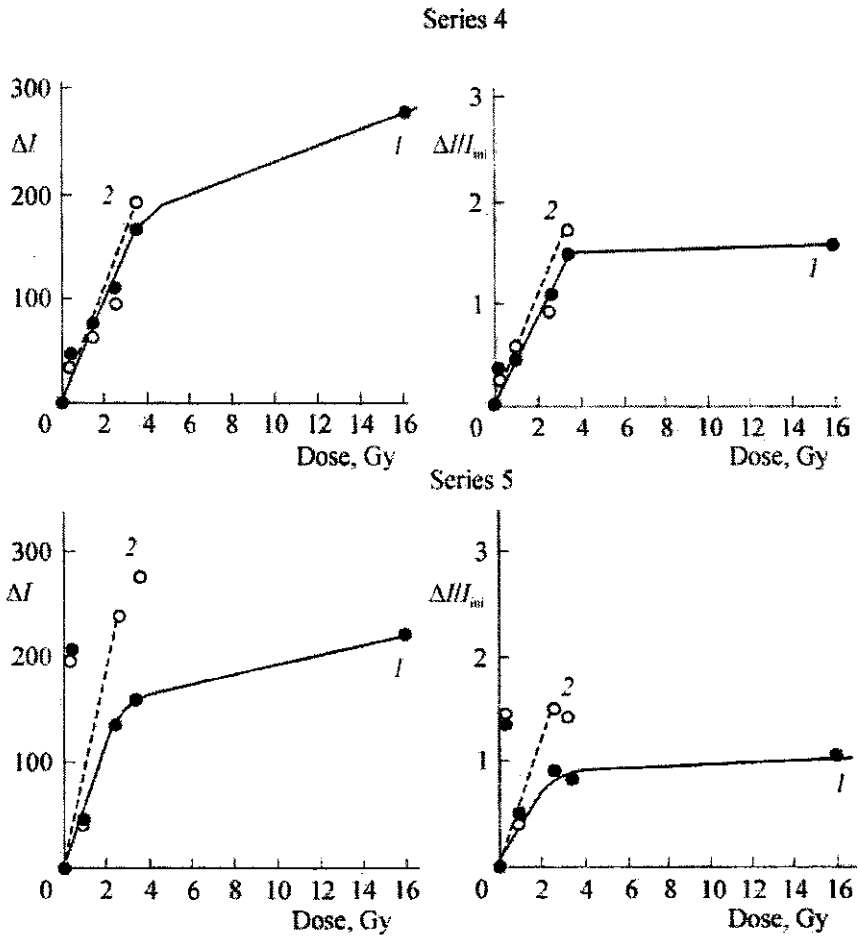


Figure 10. Dose dependencies of Fe^{3+}–transferrin pool increase in blood of irradiated dogs in the period of maximal compensatory-reduction reaction rate: in the second (1) and the sixth (2) day after radiation impact. Data on five test sequences are shown. Every experimental point relates to one dog.

The axis of ordinates: to the left – $\Delta I = I_{max} - I_{min}$, where I_{max} is ESR signal amplitude for Fe^{3+}–transferrin pool in blood of dogs on the second (or sixth) day after radiation impact; I_{min} is ESR signal amplitude on the 9th hour after radiation impact, when it reaches its minimum; to the right – the ratio $\Delta I/I_{initial}$

The abscissa axis: the radiation impact dose, Gy

protein is suppressed by 60% in spleen and by 40% in liver (Figure 2). The translation inhibition and biosynthesis of DNA was preserved during 6 h.

Later (since the first day and further on) changes in Fe^{3+}–TF content are determined by the iron-transport function. This protein controls synthesis of iron-containing proteins, including hemoglobin and ribonucleotide reductase. The latter controls *the rate-limiting iron-dependent stage in DNA synthesis.* The changes in Fe^{3+}–TF and Cu^{2+}–CP pools in blood were used as the markers of compensatory-reduction reaction development and setting time and dose dependencies of these reactions. Unitypical changes in Fe^{3+}–TF and Cu^{2+}–CP pools were developed in irradiated animals in time. They are characterized by the phase dependence and are independent of the radiation dose. The rate of these changes in the period of their maximal effectiveness (on the 2nd and the 6th days) demonstrated linear dependence on the radiation dose: the higher the dose, the greater Fe^{3+}–TF and Cu^{2+}–CP pool increase (Figure 10). The response in dogs to different radiation doses were estimated by Fe^{3+}–TF pool increment on the 2nd (or the 6th) day to its minimal value, which was recorded 9 h after radiation, i.e.

$$\Delta I = I_{max} - I_{min},$$

where I_{min} is Fe^{3+}–TF pool ESR signal amplitude 9 h after the radiation impact; I_{max} is the value of this parameter on the 2nd or the 6th day after radiation impact. The value ΔI showed linear dependence on the radiation dose up to the lethal level. For superlethal doses, no further increase of ΔI was observed, i.e. protective potential of the organism was fully realized. A *hyper-response* occurring in case of lethal doses induces the state of the maximal *biochemical and physiological stress* in the organism, long preservation of which induces exhaustion of bioenergetic, biosynthetic, neurohumoral and immune reserves, subsequently increasing the risk reduction reaction breakdown and causing the animal death. Time and rate of the reduction reaction breakdown depend in the initial protective potential of the organism. The breakdown rate was estimated from Fe^{3+}–TF pool decrease rate (by tangent value). It has been shown that the cross point of the straight line characterizing Fe^{3+}–TF pool decrease and the abscissa axis (time after radiation impact) gives an accurate time of animal death (Figure 8a, the dose is 16 Gy). The key event in development of compensatory-reduction reactions is integral increase of protein-synthesizing cell apparatus rate. This promotes for normalization of protein hemostasis,

reduction of blood and organ cellular composition and their functions (Figure 2).

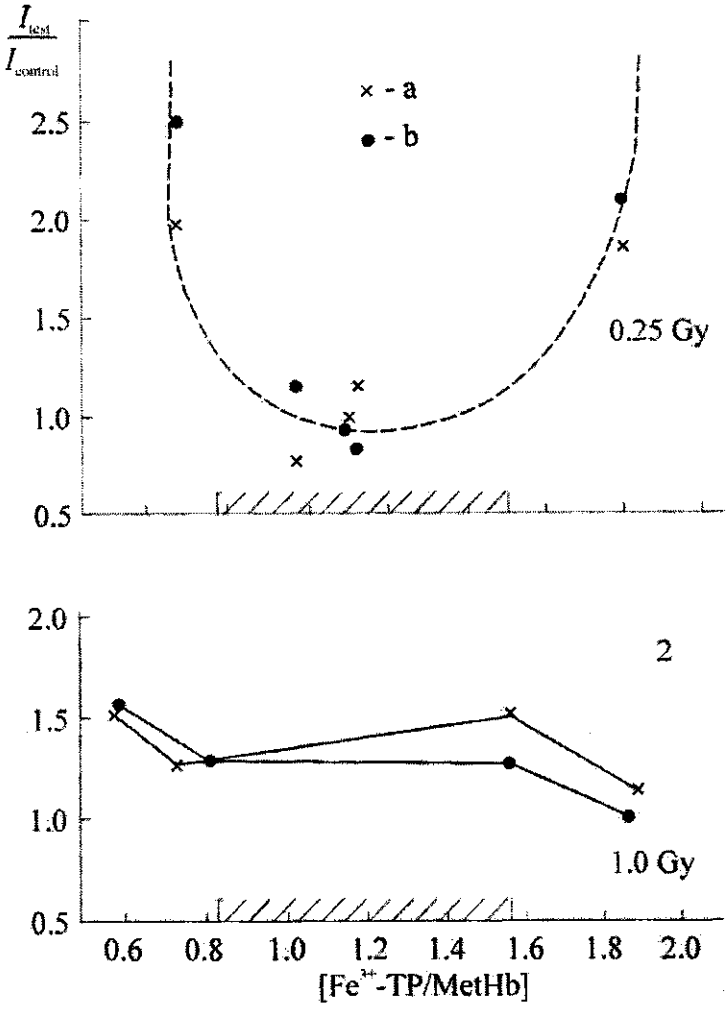


Figure 11

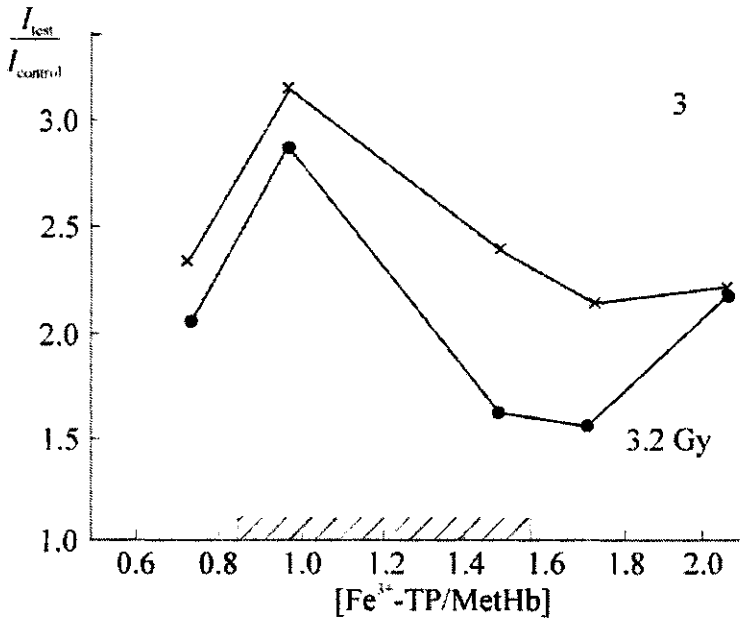


Figure 11. Dependence of response to the radiation impact of 0.25, 1.0 and 3.2 Gy on initial individual blood parameters. The response was estimated by Fe^{3+}–transferrin pool increase in blood on the second (a) and sixth (b) day; the initial state was determined the ratio of Fe^{3+}–transferrin and MetHb pools
The axis of ordinates: ESR signal amplitude for Fe^{3+}–transferrin ($I_{test}/I_{initial}$). Each experimental point relates to one dog.
The abscissa axis: $[Fe^{3+}\text{–transferrin/MetHb}]_{initial}$

In response to nonlethal (0.25 and 0.5 Gy) radiation impacts, compensatory-reduction reactions were less intensive, though increased Fe^{3+}–TF pool in blood of dogs was observed during initial 10 days. Further on Fe^{3+}–TF pool gradually decreased reaching 50% level by the 90th day after radiation impact. This indicates suppression of cellular renewal and hematopoietic tissue restoration (*The Biochemistry and Physiology of Iron*, 1982; de Jong *et al.*, 1990), which is the negative consequence of low-dose radiation impact.

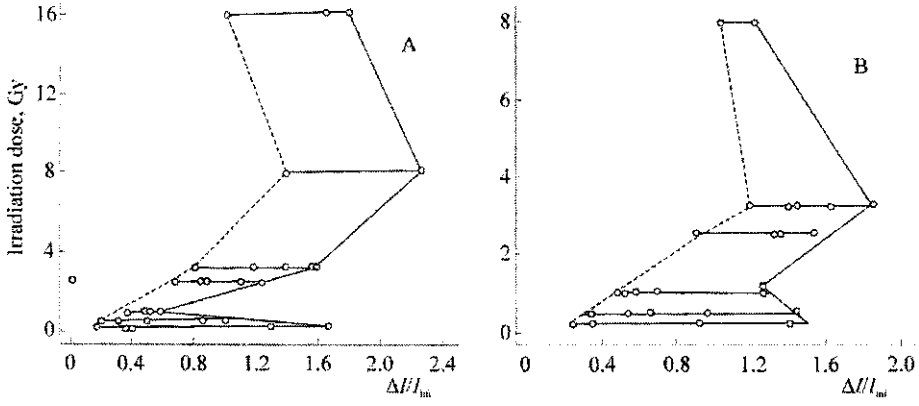


Figure 12. The dose/effect dependence by Fe^{3+}–transferrin pool change index in blood of dogs according to data obtained on the second (a) and sixth (b) days after radiation impact
The axis of ordinates: the radiation impact dose, Gy
The abscissa axis: $I_{test}/I_{initial}$ ratio, where $\Delta I = I_{max} - I_{min}$ represents Fe^{3+}–transferrin pool increment in blood of dogs

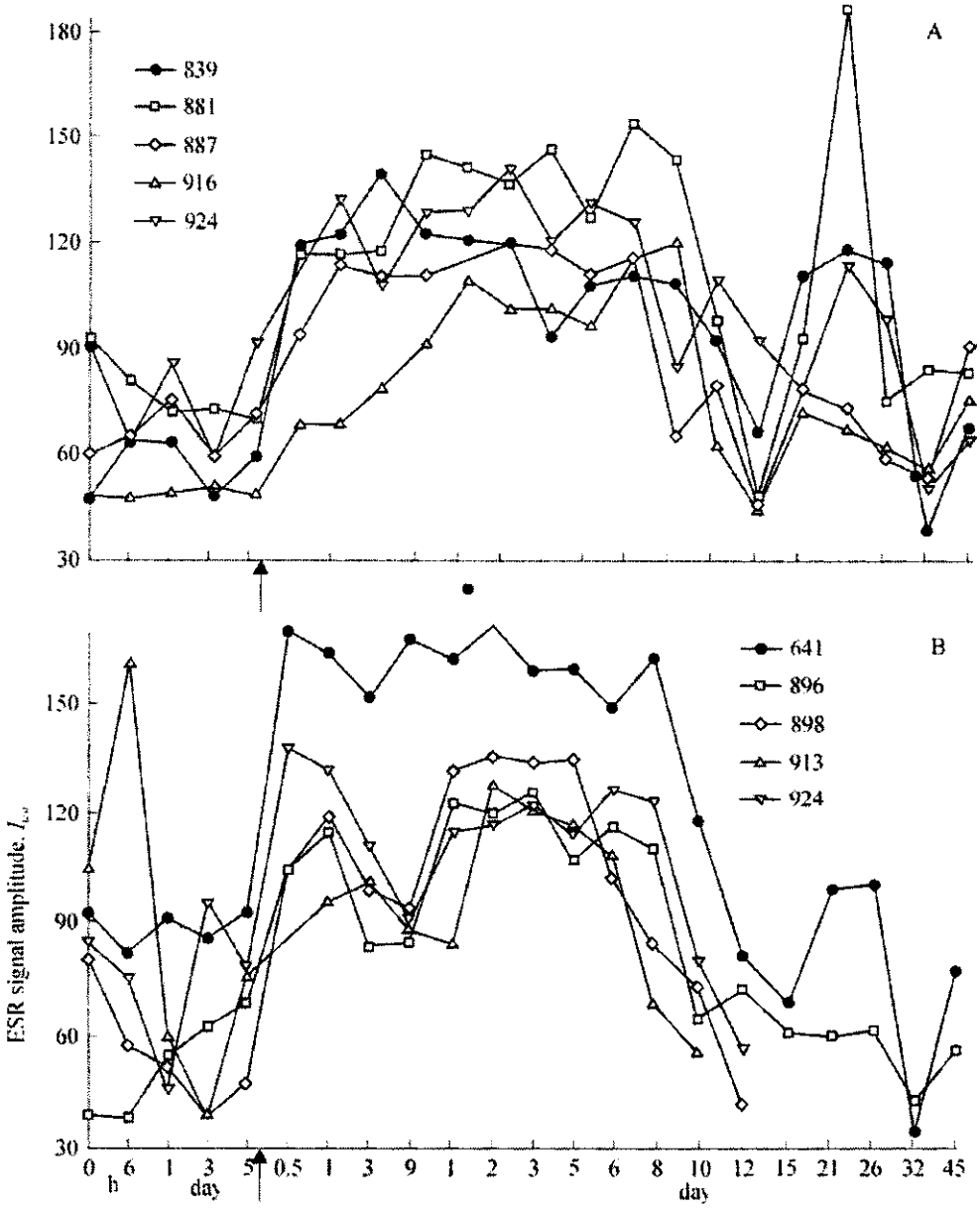
Demonstrations of radiobiological effect at the level of the whole organism significantly depend on the initial individual parameters of systems defining potential of the compensatory-reduction abilities of the organism. A relation between initial reactivity of the organism and its individual radiosensitivity is known. We show that the type of responses in dogs to low-dose radiation impact is defined by individual differences in the initial state of animals (Figures 11 and 12). The initial state was characterized by $(Fe^{3+}–TF/MetHb)_{ini}$ ratio in blood, which takes into account both synthesis and decrease of functional hemoglobin. For healthy dogs, this parameter falls within the range of 0.9 – 1.6. For dogs in the *active* state (for example, in case of drug intake of stress), this parameter was higher, whereas for *suppressed* state – lower. Figure 12 shows dose/organism response dependence, when responses were estimated by Fe^{3+}–TF pool increase in blood of dogs on the 2nd (a) and the 6th (b) days. Minimal and maximal responses showed linear dependence on the radiation dose up to 3.2 Gy, which is lethal dose for dogs. However, application of 0.25 and 0.5 Gy doses indicated deviations of the dose/effect dependence from linearity e.g. cases of sharp Fe^{3+}–TF pool increase. Such unusual responses are typical of dogs, showing *suppressed* or *activated* state before radiation impact. These dogs possessed transformed initial resistance of the organism and response intensity, inadequate to the radiation dose. Such animals are belonged to the group of increased radiation risk.

The initial level of ceruloplasmin in blood is the most important individual characteristic and defines the antioxidant status of blood plasma. However, the pool of this protein should not exceed some border value, because Cu^{2+}–CP also participates in neutralization of catecholamine pool in blood, excessive for the organism. As shown in the experiments, this increase must not exceed 40% of the initial level, otherwise this may lead to a significant decrease of catecholamine pool, which may be lethal for the organism. Each organism has a definite basic quantity of ceruloplasmin, which provides for antioxidant protection of cellular systems and regulation of optimal concentration of active catecholamines. The latter relates to immunomodulating effect of ceruloplasmin. For lethal dose of radiation or higher, Cu^{2+}–CP pool in blood increased by 2 – 2.5 times compared with the initial value in the period prior to the animal death or in the most severe period of the radiation disease, particularly, on 15th – 20th day after impact (Figure 8b). In this period, Cu^{2+}–CP pool increase in blood is related to intensive oxidation of Cu^{1+}–CP and the change in ratio of their pools due to complete

exhaustion of reducer depot and accumulation of products possessing high oxidative activity. In this period, a significant increase of Cu^{2+}–CP pool indicates the exhaustion of reserve hormonal system power in the irradiated organism and suppression of adrenal gland functional activity, and may be one of the reasons of the compensatory-reduction reaction breakdown. In this case, iron ion transfer from Fe^{3+}–TF to cells is hindered, because this process requires the presence of reducers – the hormones. As a result, iron consumption by cell for the purposes of biosynthesis of vitally important iron-containing proteins is disturbed.

2.2. Dynamics and dose dependence of blood parameters in dogs at prolonged radiation impact at 0.96 cGy/min dose rate

After severe radiation pathology, dogs from the test group were characterized as follows. The dogs irradiated by 7.64 Gy dose (LD_{80}) displayed bone marrow syndrome of various severity. For 2Gy dose, clinicohematological changes in animals were less expressed, whereas for 0.5 Gy were practically absent. Hematopoietic system represents an important part of hemostasis and deviations in it are realized at formation of early and distant effects of long-term low-dose radiation impact. At prolonged radiation impact on animals in a broad range of radiation doses, proliferative activity of cells increased in stem hematopoietic precursor pools (Muksinova, Mushkacheva, 1990; Lebedev *et al.*, 1999). Proliferative activity of cells is, in many cases, is defined by dNTP pools, and their synthesis is the rate-limiting iron-dependent process in DNA synthesis. Ribonucleotide reductase activity of tissues is controlled by Fe^{3+}–TF pool in blood and the presence of conditions for iron ion transfer for biosynthesis purposes. Temporal changes in Fe^{3+}–TF pool in dog's blood impacted by 7.64 Gy radiation dose are shown in Figure 13 indicating a significant increase of this parameter immediately after the impact (first time measured 30 min after the impact end). This parameter remained increased during 8 – 10 days. Further on, for all dogs in this experiment an abrupt decrease of Fe^{3+}–TF pool in blood was observed, which is related to development of the acute phase of radiation disease that caused death of experimental dogs No. 898, 913 and 914. One of two survived dogs (No. 614) showed the repeated increase of Fe^{3+}–TF pool in the period since the 21th till 26th day. This is critical period for restoration of hematopoiesis and predetermines survivability of death of the animal.


A
839
881
887
916
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B
641
896
898
913
924
ESR signal amplitude, I_{ES}
180
150
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90
60
30
0 6 1 3 5 0.5 1 3 9 1 2 3 5 6 8 10 12 15 21 26 32 45
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day
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Figure 13

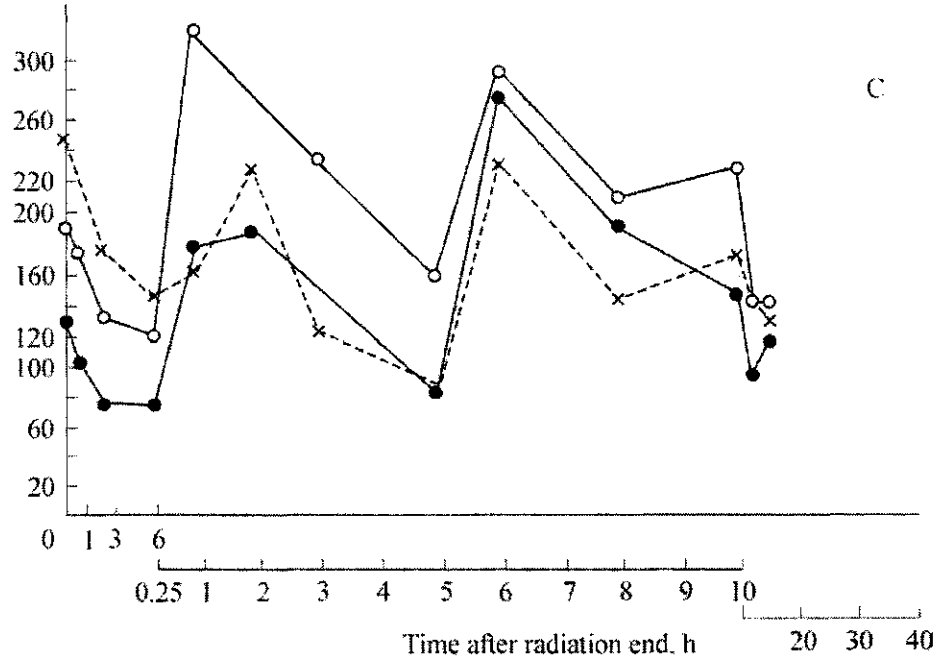

Figure 13. Temporal changes of Fe^{3+}–transferrin pool concentration in blood of indomethaphen-protected (a) and unprotected (b) dogs after total prolonged radiation impact of 7.64 (a and b) and 0.5 Gy (c) at the dose rate of 0.96 cGy/min and the impact duration of 13 h 30 min (a and b) and 50 min (c). Indomethaphen (30 mg/kg – (a)) was injected to dogs 5 days prior to radiation impact
The axis of ordinates: ESR signal amplitude for Fe^{3+}–transferrin in blood. Each curve relates to one dog. Numbers of dogs are indicated.
The abscissa axis: time after radiation impact

After the end of irradiation of dogs by 2 Gy dose, the increased Fe^{3+}–TF pool was also recorded, whereas for 0.5 Gy dose Fe^{3+}–TF pool in blood was decreased by more than 50% of the initial level. Figure 13c shows dynamics of Fe^{3+}–TF pool change in blood of three dogs, irradiated by 0.5 Gy dose. As follows from the data obtained, the lower the dose and, consequently, the radiation time, the clearer the phase character of post-radiation changes of Fe^{3+}–TF pool in blood. For instance, the period of dog irradiation by 7.64 and 2.0 Gy doses, approximately, equaled 13.5 and 3.5 h, respectively. In this

connection, after radiation impact end in these doses, dog's blood samples were tested at different post-irradiation stages. If the end of radiation impact in 2.0 Gy dose coincides with the initiation of inhibition of the most important molecular–cellular processes (the stage of radiation damage increase), at the end of dogs' radiation by 7.64 Gy was the initiation of compensatory-reduction reactions. The phase dependence of post-radiation changes of Fe^{3+}–TF pool in blood was the most clearly observed in dogs, irradiated in 0.5 Gy dose.

Superoxide dismutase activity in blood of dogs is also increased on the 1st – 2nd and 5th – 6th days after radiation impact. Moreover, the second increase was much higher than the first one, which is stipulated by reduction of the protein biosynthesis rate in these periods. Thus, the dog's blood system responds to the radiation impact by increasing antioxidant activity; hence, the highest values of superoxide dismutase activity were registered in periods of the highest Fe^{3+}–TF and Cu^{2+}–CP pools in blood, namely, on 1 – 2 and 5 – 6 post-irradiation days.

2.3. The influence of radioprotectors on dynamics of variations in Fe^{3+}–transferrin concentration in blood of irradiated animals

The above data show that the compensatory-reduction reactions of molecular-cellular systems of organs in animals are nonspecific and universal, demonstrating the phase dependence and being independent of the radiation dose with time. The rates of these reactions in the periods of maximal intensity show linear dependence on the radiation dose up to the lethal level. At lethal doses, molecular-cellular systems of organs and the entire organism sustain the state of *physiological and biochemical stress*, long preservation of which may be the reason for compensatory-reduction reaction breakdown and animal death. Protection of irradiated organism requires prevention of this critical state. We have also found that radioprotectors effective by the survivability test (Chernov *et al.*, 1994, 1996; Il'in *et al.*, 1994; Vasin *et al.*, 1996; Pulatova, Sharygin, 1997; Pulatova *et al.*, 1999; Pulatova *et al.*, 2003) prevent development of response hyperforms in molecular-cellular systems of animal organs, irradiated by lethal radiation doses. Irradiation of dogs, protected by indraline, by doses exceeding 1 Gy has not induced Fe^{3+}–TF pool increase in blood on both 1 – 2 and 5 – 6 days (Figure 14). We suggest that application of radioprotectors provides for preservation of iron ion consumption by cells for

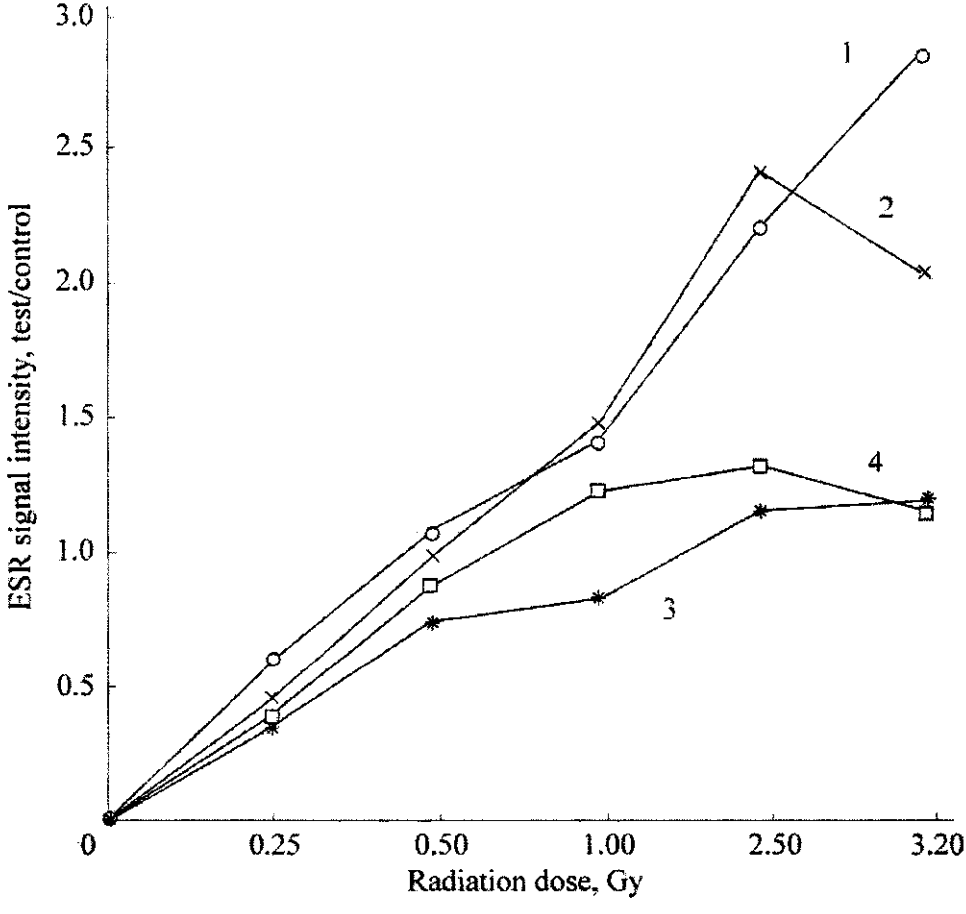

Figure 14. Dose dependencies of Fe^{3+}–transferrin pool increase in blood of dogs in the period of the maximal rate of compensatory-reduction reaction: on the second (1, 3) and the sixth (2, 4) day after radiation impact

1, 2 – impacted unprotected dogs; 3, 4 – impacted dogs, received indraline (15 mg/kg) intramuscularly 15 min prior to the radiation impact. This dose is the optimal radioprotector value

the purpose of iron-containing protein synthesis, firstly, ribonucleotide reductase, hemoglobin, mitochondrion CET and endoplasmic reticulum components. This is confirmed by the data (Pulatova, Sharygin, 1997; Pulatova et al., 1999; Chernov *et al.*, 1996; Pulatova *et al.*, 2003) that indraline and indomethaphen protected animals demonstrate:

1) decreased inhibiting effect of radiation on protein and DNA biosynthesis in the early post-irradiation period;
2) powerful induction of dNTP synthesis in hematopoietic organs for the purpose of DNA damage repair and replication synthesis;
3) development of earlier and intensive proliferative reduction processes compared with the processes in unprotected animals.

Radioprotectors prevent development of hyperforms of molecular-cellular reactions in animal organs (on 1 – 2 and 5 – 6 days of the post-irradiation period) and induce Fe^{3+}–TF pool increase in critical periods of radiation disease development, the main of which if the hematopoiesis period. In the most of experimental dogs, radioprotector indomethaphen induced a noticeable increase of Fe^{3+}–TF pool in blood since 10 to 20 day. Since iron-transporting protein transferrin is the most important participant of hematopoiesis and erythropoiesis, the observed increase of Fe^{3+}–TF pool in blood testifies about activation of hematopoiesis in this period. According to experimental data (Vasin *et al.*, 1996) on anti-radiation effect of indraline on the hematopoietic system of various species of animals, restoration of the cellular composition of blood in indraline-protected dogs, irradiated by 3.84 Gy dose, is initiated on the 15th – 20th day of the post-irradiation period.

2.4. MODIFYING EFFECT OF ANTIOXIDANT α_2–TOCOPHEROL ON FE^{3+}–TF CONTENT IN BLOOD PLASMA OF DOGS

The blood system responses to radiation impact by increasing antioxidant activity of blood and hematopoietic organs, which is the main part of the protective mechanism for repair of damages. Introduction of antioxidants into animals increases their antioxidant status. Antioxidant α_2–tocopherol increases Fe^{3+}–TF and Cu^{2+}–CP pools in dog's blood on the 1st and the 2nd days after peroral intake of 20 mg of the drug (Figure 15). For example, dog

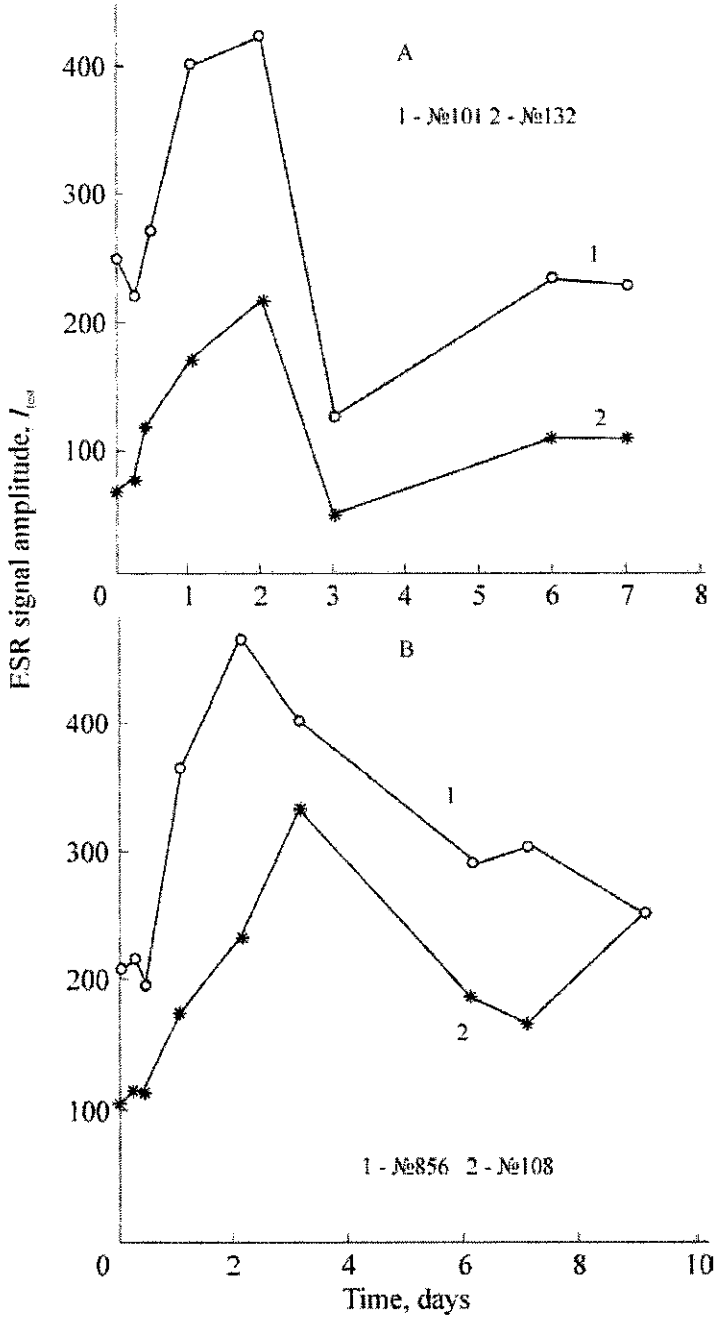
A
1 - №101 2 - №132
1
2
B
1
2
1 - №856 2 - №108
FSR signal amplitude, I_{fcs}
Time, days
0
100
200
300
400
1
2
3
4
5
6
7
8
10

Figure 15

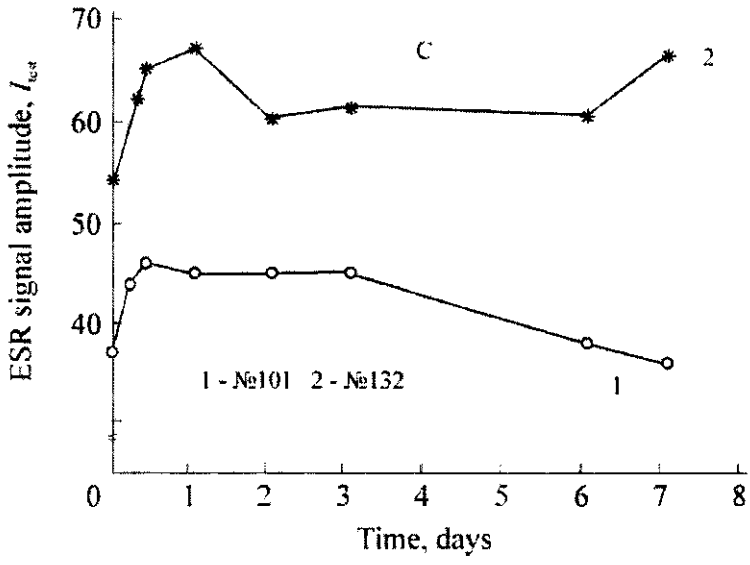

Figure 15. Changes in Fe^{3+}–transferrin (a and b) and Cu^{2+}–ceruloplasmin (c) pools in blood plasma of dogs after peroral intake of 20 mg of α_2-tocopherol (a and c) and for daily intake of 20 mg of antioxidant during 6 days (b). Numbers of dogs tested in the experiment: 101, 132, 108 and 856.
The axis of ordinates: ESR signal amplitude for Fe^{3+}– transferrin pool.
The abscissa axis: time after single intake (a and c) and first dose intake (b) of antioxidant

No. 132 possessed Fe^{3+}–TF pool 2.5 times higher than in control, whereas the rest dogs (No. 101, 108 and 856) had it increased by 1.5 – 1.7 times. Note that the dog No. 132 with the lowest initial Fe^{3+}–TF pool demonstrated the highest increase of it as a response to AO injection. Maximal Fe^{3+}–TF pools in blood of dogs No. 108 and 856, provided with daily 20 mg dose of α_2–tocopherol during 6 days, were observed on the second or third day of the experiment. The reason for Fe^{3+}–TF pool increase was α_2–tocopherol stimulated activation of protein biosynthesis, including apo-transferrin. Antioxidant α_2–tocopherol induces a significant increase of Fe^{3+}–TF pool in blood of animals and, therefore, provides for the synthesis of iron-containing proteins, among which iron-containing ribonucleotide reductase should be mentioned.

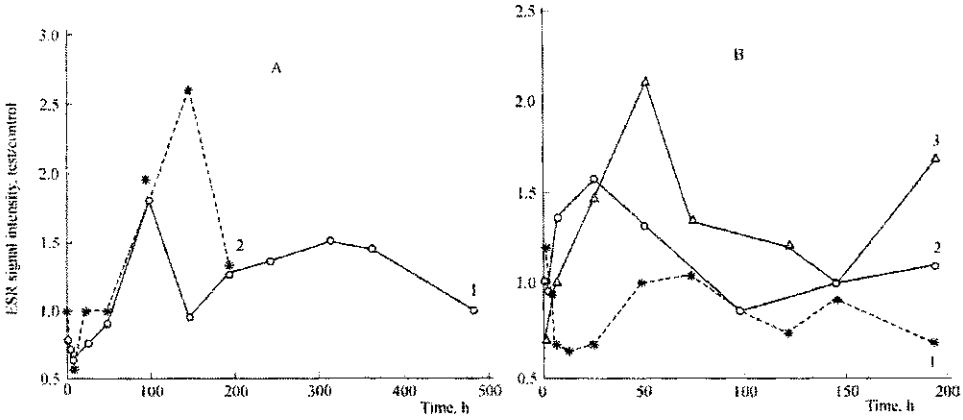

Figure 16. Dynamics of Fe^{3+}–transferrin pool change (a1) in blood and ribonucleotide reductase activity in spleen (a2, b1, b2, b3) in mice.

a – after intragastric injection of indomethaphen (30 mg/kg); b – after total radiation impact in 6.5 Gy dose (1), after radiation impact in 6.5 Gy dose at the background of indraline (100 mg/kg) injected 15 min prior to radiation impact (2), and after single intake of 17 mg/kg dose (0.25 mg per mouse) of α_2-tocopherol (3)

The axis of ordinates: ESR signal intensity ($I_{test}/I_{control}$) for Fe^{3+}–transferrin pool (a1) and active ribonucleotide reductase (a2, b1, b2, b3). The control group was formed by intact animals. Blood and spleen samples were taken from control and test mice simultaneously in all periods of the experiment

The abscissa axis: time after radiation impact (b1 and b2) or after drug intake (a1, a2, and b3)

Actually, RR-activity of tissues of hematopoietic organs increased after injection of radioprotectors indraline and indomethaphen, as well as antioxidant α_2–tocopherol into animals (see Section 1). On the seventh day, the experimental Fe^{3+}–TF pool in blood of dogs reached the initial value. Multiple intake of α_2–tocopherol produced higher Fe^{3+}–TF pool compared with the initial level during the whole observation period. Figure 16 shows that Fe^{3+}–TF pool increase in blood foregoes RR-activity increase in organs. This is the way, how Fe^{3+}–TF pool controls the volume of dNTP and, correspondingly, DNA synthesis. Also, the antioxidant-stimulated increase of Fe^{3+}–TF pool in blood and organs affects the relation of monocytes and macrophages by increasing the immune status of hematopoietic organs (Testa *et al.*, 1987). The antioxidant-induced increase of stability of radiosensitive molecular and cellular processes, including dNTP, DNA and protein synthesis, increases the organism resistance.

The results obtained allows a conclusion that α_2–tocopherol induced the following sequence of reactions in cells of animal organs:

α_2–tocopherol → protein synthesis activation → Fe^{3+}–TF pool increase → RR-activity increase → intracellular dNTP pool increase → DNA synthesis activation

3. MARKERS FOR RADIATION DAMAGE AND DEVELOPMENT OF ADAPTIVE REACTIONS IN CHILDREN SUBJECT TO PROLONGED LOW-DOSE RADIATION IMPACT, INDUCED BY CHERNOVYL ACCIDENT

At present, of special attention are questions of multifactor origin of some distant consequences of low-dose radiation impact on the organism, specifically outlining the oncogenic effect and damaging impact on the hematopoietic system. In this connection, the problem of searching for biochemical markers of radiation-induced metabolic changes, biochemical adaptation, occurrence of lymphoproliferative diseases and tumor processes becomes more and more urgent, because, on the one hand, the number of radiation victims increases and, on the other hand, data on prolonged impact of nonlethal radiation doses, dangerous for the organism, are accumulated. The important aspect of this problem is detection of disturbances in nucleic and

protein exchange. It has been found (Elford, 1972; *Genetic Consequences* , 1985; Gudtsova *et al.*, 1987; Pulatova *et al.*, 1989; Pulatova *et al.*, 1990) that at tumor growth RR-activity increases in tumor cells and spleen of animals, and concentration of polyamines (spemine, spermidine and putrescine diamine) increases in blood plasma. Being the growth factors, polyamines and ribonucleotide reductase are very important for proliferation and cell differentiation. The presence of linear dependence binding RR-activity and malignancy of tumors is shown in the work (Elford, 1972).

Table 3

Mean-group concentrations of Fe^{3+}–transferrin and Cu^{2+}–ceruloplasmin in blood of children of different ages independently of the dwelling zone.

Hereinafter, *n* is the number of children in the group

Age of children, years	Content	
	Fe^{3+}–TF	Cu^{2+}–CP
2 – 7		
Control	46.8 ± 14.8 (*n* = 42)	82.9 ± 14.6 (*n* = 42)
Victims	55.2 ± 15.6 (*n* = 31)	93.3 ± 16.5 (*n* = 31)
8 – 10		
Control	58.0 ± 15.2 (*n* = 34)	86.0 ± 15.0 (*n* = 34)
Victims	63.7 ± 11.1 (*n* = 18)	75.7 ± 13.8 (*n* = 18)
11 – 15		
Control	58.5 ± 14.0 (*n* = 42)	78.0 ± 11.0 (*n* = 42)
Victims	62.0 ± 8.7 (*n* = 19)	58.8 ± 15.7 (*n* = 19)

Table 4

Mean-group concentrations of Fe^{3+}–transferrin in blood of children from dwelling zones with different density of radioactive contmination

Age, years	Control	Zone I	Zone II	Zone III (moved)
2 - 7	46.8 ± 14.8 (*n* = 42)	56.8 ± 8.0 (*n* = 14)	61.0 ± 7.0 (*n* = 32)	72.0 ± 7.0 (*n* = 6)
8 – 10	58.0 ± 15.0 (*n* = 34)	71.0 ± 4.8 (*n* = 3)	69.0 ± 12.0 (*n* = 36)	68.0 ± 11.0 (*n* = 16)
11 - 15	58.0 ± 14.0 (*n* = 42)	62.5 ± 2.5 (*n* = 2)	75.0 ± 14.0 (*n* = 19)	70.0 ± 5.0 (*n* = 13)

Taking into account the dependence of RR-activity in organs on Fe^{3+}–TF pool in peripheral blood, judging by changes of the latter parameter, one may estimate the level of disturbances in cell proliferation and their ability to reproduce. Monitoring of Fe^{3+}–TF pool in blood of children inhabited in Bryansky District (affected by Chernobyl nuclear disaster), in regions with different radioactive contamination, mostly by ^{137}C was also performed: zone I – below 15 Ci/km^2; zone II – 15 ÷ 45 Ci/km^2; zone III – 45 ÷ 75 Ci/km^2 or more. In May 1986, all children from the zone III were evacuated; parents of children born after the accident were also evacuated (the group of two to seven years old). Children with thyroid gland damages, induced by incorporated radio-iodine ^{131}I in determined dose, were united in the separate group. The control group was formed by children, selected to the "*pair–copy*" principle, dwelt in "*pure*" regions of Bryansky District. The mean-group values of Fe^{3+}–TF and Cu^{2+}–CP pools in blood of children of different ages are shown in Tables 3 and 4. As shown by the analysis of Fe^{3+}–TF pool changes in blood of damaged children, this parameter was higher than in children from the control group independently of the dwelling zone. Individual variations of Fe^{3+}–TF pool in blood of damaged children of all ages tended to increase (Figure 17).

The increased Fe^{3+}–TF pool in blood of children provides for protection and reduction of highly radiosensitive hematopoietic and reproductive systems of the child's organism (Chitamber, Zivkovic, 1987; Testa *et al.*, 1989; Crichton, 1990; de Jong *et al.*, 1990) and represents the marker of biochemical adaptation of child organisms to changed environmental conditions. Despite protective function of the adaptive response, it also has *disadvantages*, because the child organism is present in the state of *biochemical and physiological stress*. Long existence in this state due to prolonged radiation impact and additional, even insignificant loads on the organism (stress, undernutrition, diseases, drug intake) may exhaust protective potential and cause breakdown of the most important molecular-cellular and organ systems.

The changes in Cu^{2+}–CP pool in blood is usually considered as nonspecific events, because this protein sharply responses to the damaging impact, inflammatory processes and pathologies. Despite nonspecific character of Cu^{2+}–CP pool variation, it should be noted that such changes affect many cellular and organism processes, because this protein possesses antioxidant, monoaminoxydase and ferroxidase activities (Table 5).

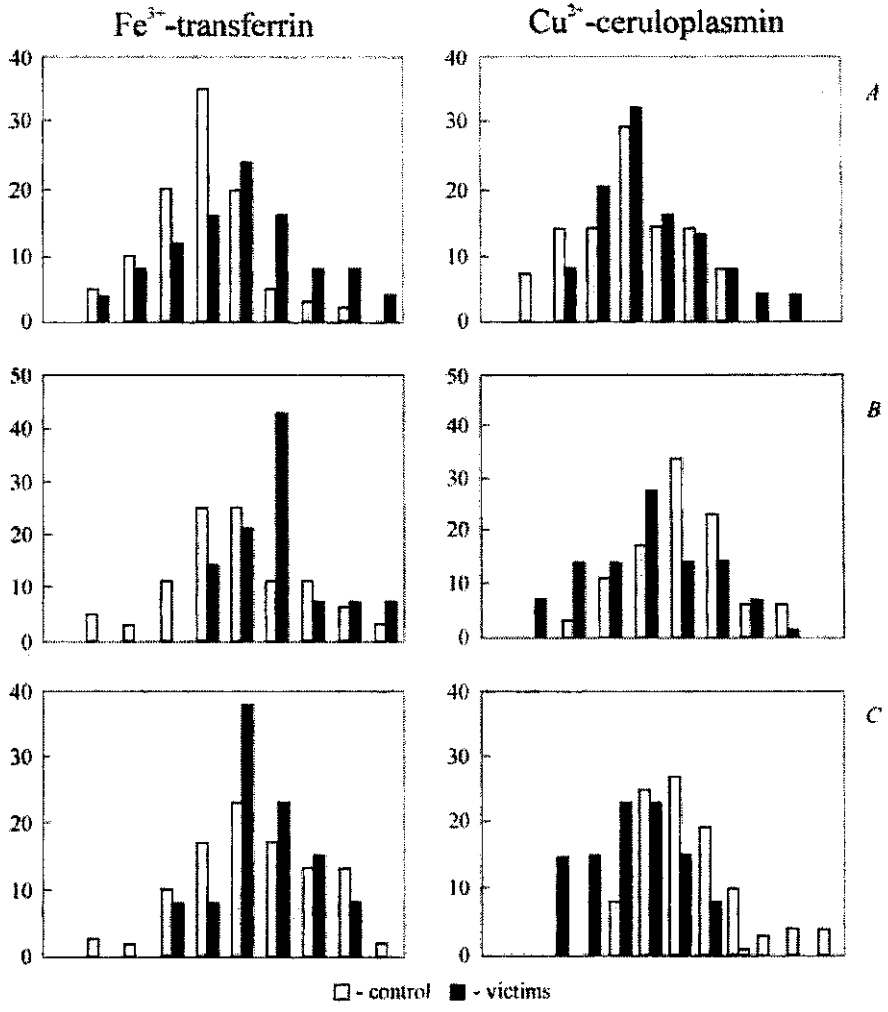

Figure 17. Frequency distribution (in percents of total number of examined children) of Fe^{3+} –transferrin (at the left) and Cu^{2+}–ceruloplasmin (at the right) pools detected in blood of children of various age groups

a – younger group (2 – 7 years old); b – mid-age group (8 – 10 years old); c – senior group (11 – 15 years old). The control group was formed by children from Sizhuch town, Bryansky District. The group of victims was formed from children dwelt in different zones with increased density of radioactive contamination

Table 5

The mean-group Cu^{2+}–CP pool concentration in blood of children dwelt in zones with different density of radioactive contamination

Age, years	Control	Zone I	Zone II	Zone III (moved)
2 – 7	82.9 ± 14.6 (n = 42)	115.0 ± 24.0 (n = 14)	73.0 ± 12.0 (n = 32)	59.0 ± 10.0 (n = 6) born after Chernobyl accident
8 - 10	86.0 ± 15.0 (n = 34)	67.0 ± 4.0 (n = 3)	74.0 ± 12.0 (n = 36)	76.0 ± 12.0 (n = 16) moved
11 - 15	78.0 ± 11.0 (n = 42)	–	77.0 ± 17.0 (n = 19)	75.0 ± 15.0 (n = 13) moved

Growing older, children from the control group displayed some decrease of Cu^{2+}–CP pool in blood, which may somehow be explained by the exceeding of activity of parasympathetic unit above sympathetic one in the vegetative nervous system. For children from the zone I, the same tendency of changes with age was observed, only possessing greater difference between under school and primary school aged children. It should also be taken into account that children older than seven were themselves the objects of the radiation impact, whereas other children were born by women, impacted by radiation, who were pregnant at the moment of Chernobyl accident or after it. For all children from the zone II and the mid-age and elder groups from the zone III, no age dependence of Cu^{2+}–CP pool in blood was observed, and the mean-group values of this parameter (specifically for the younger children from the zone III) were much lower than in the control group. Similar situation was observed for children with thymoid gland damages (with the received dose exceeding 75 rad) (Figure 18). The examination of children for other biochemical parameters of blood (Baleva *et al.*, 1995) also indicates their dependence on the age of children and density of radioactive contamination of the dwelling zone. For example, according to the mean-group and individual values of extracellular DNA concentration in blood plasma, the highest deviations from the norm were observed for the youngest group of children. Low values of this parameter were also observed for many children from the zone II, which is associated with decrease of their immune reactions. Children from the zone III demonstrated a broad distribution in concentration of extracellular DNA in blood plasma, both toward minimum and maximum. High values can be explained by hyperactivation of immune reactions in

response to occurrence of a great number of damaged cells in blood and organs.

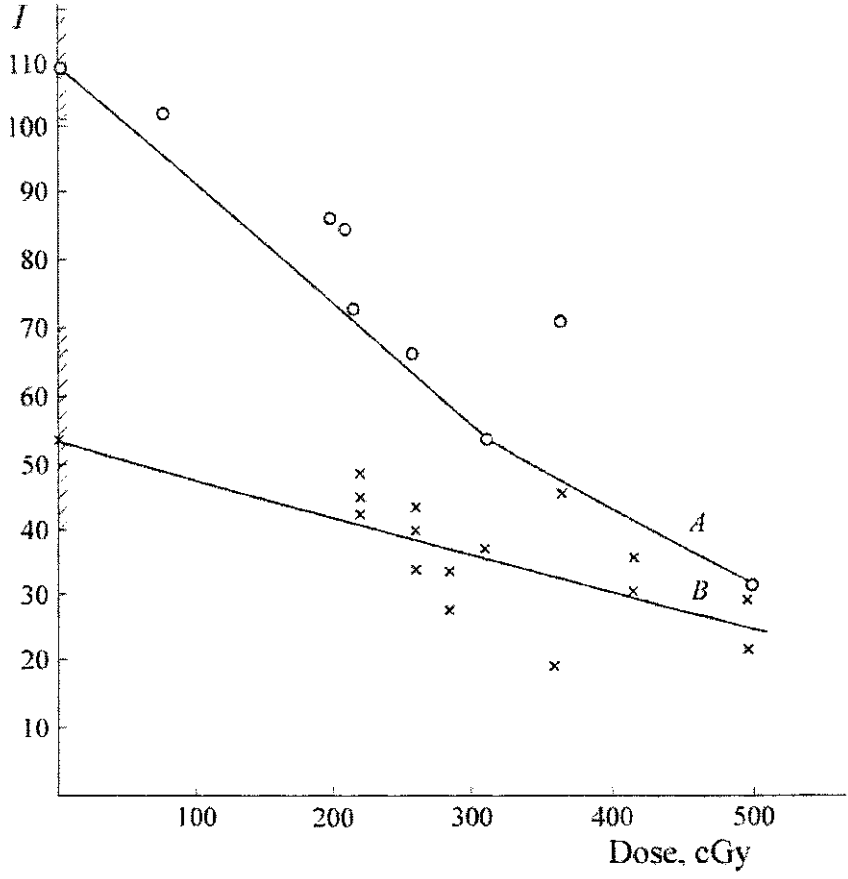

Figure 18. Dose dependence of Fe^{3+}–transferrin pool in blood serum (curve a) and total blood (curve b) in children 7 – 8 years old with radiation damage of thyroid gland due to incorporated radioiodine. The measurements were implemented in 1991

CONCLUSION

Thus, for the entire organism, molecular-cellular response of the blood system and other organs to the radiation or drug impact may be estimated by the change of several metabolic parameters, among which the marker of peripheral blood – Fe^{3+}–TF, highly sensitive and rapidly responding to a damaging impact. This iron-transporting protein is of special importance, because it controls the rate-limiting iron-dependent stage of DNA synthesis – deoxyribonucleotide synthesis. The increase of functional ability of this transport protein and ribonucleotide reductase activity in hematopoietic organs, related to the increase of intensity of the whole protein-synthesizing cell apparatus in response to the damaging effect, is the key event in development of the compensatory-reduction reactions in cellular systems of the organism. These responses are characterized by the phase dependence with maxima at the second, sixth and 10 – 17th days of the experiment. Dynamics of these reactions is independent of the radiation dose that indicates their nonspecific and universal character. Meanwhile, the rate of these reactions shows linear dependence on the radiation dose up to lethal level, if the experiment is carried out on healthy animals, selected by their initial biochemical and radiospecroscopic blood indices. For animals with initial blood parameters typical of *suppressed* and *activated* states, intensity of their response to the radiation impact in relation to the whole organism was inadequate to the dose e.g. deviated from linear dose–effect dependence. It corresponded by value to the response induced by radiation in sublethal or even lethal doses. In this case, selection of a *threshold* permissible dose, which is usually settled according to other parameters of the radiation damage, becomes senseless. This is of special importance for the estimation of distant consequences of low-dose radiation impacts for the population still dwelling on radionuclide-polluted territories. Hence, the detected radioprotector and antioxidant stimulated increase of dNTP synthesis in hematopoietic organs (bone marrow, spleen, and thymus) promotes for an increase of the organism resistance, because high balanced dNTP pools supply the required level of DNA synthesis, provide for the effective repair of DNA damages, prevent formation of new DNA damages, and accelerate development of compensatory-reductions reactions.

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RADIOPROTECTION OF DNA WITH FREE RADICAL REACTION INHIBITORS – A WELFARE OR ? STERIC MODELING

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Annotation

The data of authors' investigations on DNA radiation degradation mechanisms in the presence of free radical reaction inhibitors and possible control of radioprotection processes and formation of 2-deoxyribose residue epimers (potential point mutation sites) are generalized and discussed.

Keywords: Radioprotection and point mutations, free radical reaction inhibitors, computerized modeling

About fifty years ago N.M. Emanuel has put forward a concept that such pathologies radiation disease, cancer and aging are related to intensification of free radical processes in the organism. Therefore, preparations – free radical reaction inhibitors (InH) should be applied as therapeutic means. In the present review, in the framework of this concept (which became common already), the elementary acts of primary stages of DNA radiation degradation in the presence of InH and consequence of its presence in the irradiated system are analyzed.

PHYSICOCHEMICAL STAGES OF DNA RADIATION DAMAGE AND PROTECTION MECHANISMS

Primary physicochemical stages of DNA radiation damage are reduced to degradation of sugar phosphate skeleton and modification of bases [1 – 3]. At the cell radiation, the basic role in these processes is played by water radicals: rate constants of $^{\bullet}OH$ reaction with the bases and 2-deoxyribosyl are

close to diffusion ones, whereas for $^{\bullet}H$ they are by 1 – 2 orders of magnitude lower; e_{hydr} reacts with bases, where in relation to cyclic carbohydrates its reactivity is by several orders of magnitude lower [4]. In reactions of water radicals with nitrous bases, radicals-adducts of bases are formed [2, 5], and their transformations lead to modified bases in the DNA composition. Some of these products represent potential sites of point mutations [6].

At the primary stages of radiation damage of biopolymers (BP) two radioprotection mechanisms are realized in the cell:

1) the mechanism of substance competition, the radioprotectors (RP) for radicals representing products of water radiolysis;
2) inhibition of transformations of BP radicals formed (free-radical reactions, FRR) by radioprotectors (FRR inhibitors – InH, called antioxidants, if radioprotection is implemented in the presence of oxygen, and oxidative processes are suppressed) [7].

In the first case, radioprotection is defined by predominant contribution of water radical reactions with RP; in the second case, the following reaction of radical substitution takes place:

$$R^{\bullet} + InH(RP) = RH + {}^{\bullet}In(RP^{\bullet}), \qquad (1)$$

where $R^{\bullet}$ and $^{\bullet}In$ are DNA and FRR inhibitor radicals [7, 8]. In the general case, DNA radicals (alkyl, alkoxyl, peroxide) can be taken for radicals $R^{\bullet}$ [9].

Realization of the first radioprotection mechanism under the effect of densely ionizing radiation (fractured, alpha-irradiators, protons), when the concentration of water radicals in tracks of ionizing particles is about 1 – 10 M, is completely excluded. In the case of β-, γ- and X-ray irradiation (the average concentration of water radicals in the track is about 10^{-4} M), the first mechanism may also be realized due to endogenic substances in the cell (such as cysteine, cystine, or glutathione), and at application of "*external*" radioprotectors, including synthetic ones, injected to the cell [10 – 12].

In radiobiology and radiation medicine, the forecasting of expected therapeutic effect of RP application as drugs, even a rough estimation of the "*contributions*" of each of these two mechanisms is desirable.

Actually, a question should be resolved, against which particles – oxidation or reduction radicals – DNA should be protected, for example. What is InH able to suppress macroradical transformations, which cause polymer

chain breaks and toxic product formation (aldehydes from carbohydrate fragment, for example [13]) or modified bases and, therefore, potential point mutations? Each of these cases requires the selection of corresponding reagents – acceptors of water radicals, macroradicals, including peroxide ones, if radicals are transformed in microzones of the cell containing O_2 [9, 14 – 16].

It should be outlined that RP application as DNA protective substance implementing the mentioned two mechanisms is far from indicating the necessity of RP accumulation in the cell in unthinkable concentrations (as in a flask, by orders of magnitude exceeding the DNA concentration). The radioprotection effect may also be reached, if RP is present in the place of potential DNA damage beforehand. (As is known, RP application is the most effective in case of its injection into the organism prior to irradiation [10, 17, 18]). Such places in DNA are chemical bonds in 2–deoxyribosyl and bases, attacked by water radicals. A macroradical formed must react with RP, which acts as InH. Such situation may be realized at the application of RP with a set of functional groups, in particular. By some of these groups, amino groups, for example, RP is fixed to the DNA backbone [18], whereas other groups act as radical acceptors.

In accordance with the viewpoint [15, 18], the radioprotection effect is reached not only by interception of water radicals, but to the greater extent, by DNA double strand stabilization due to formation of additional intermolecular bonds between RP and biopolymers. These compounds represent something like "*clamps*" of the polymer structure. The authors believe that degradation of the polymer double spiral is associated with breaks in the sugar phosphate chain induced by transformations of carbohydrate fragment radicals [19]. In carbohydrate macromolecules such process of radical transformation from primary radicals to final products takes from 10^{-9} to 10^{-7} s [20, 21]. We suggest that 2-deoxyribosyl radical transformation also fit the same time range. The characteristic time of radiation damage repair in the cell is from several minutes to several hours [22]. This means that by the beginning of repairing enzymatic system functioning in the cell molecular products of radiolysis have already formed and breaks in DNA strands have occurred. Hence, the role of protectors-clamps is obvious: they must "*preserve*" the polymer structure during the time mentioned.

The substances–cramps are, for example, cystamine, cadaverine and putrescine [18]. It is obvious that these aliphatic diamines can stabilize DNA double strand in a manner similar to DNA interaction with poly-L-lysine [23]. The authors believe that disulfide group of cystamine – the substance-clamp –

might demonstrate the antiradical activity as the competitor of DNA for *e* and as FRR inhibitor (InH) at unpaired electron transfer from the primary macroradical to disulfide bond. Thus, locating on DNA, the RP-clamp cystamine may "*operate*" according to two radioprotection mechanisms.

Generally speaking, preservation of radiation degradable DNA double strand may be stipulated by attachment of RP-clamp molecule to DNA along the sugar phosphate chain within a single strand or transversally, between two strands of the polymer. Discussed in the Section 4 are results of computerized simulation of these two alternatives, shown on the example of diamines and analysis of the condition of antiradical RP activity implementation, the sulfur-containing substance – clamp.

Principally, DNA structure "*clamps*" can be created by chemical and hydrogen bonds between amino, imino-, sulfhydryl, hydroxyl, or oxo-groups of the ligand-protector and corresponding phosphate groups and atoms, bases and 2-deoxyribosyl. Hence, at attractive perspective occurs: prior to irradiation, to locate functional groups – radical acceptors in various places of the DNA backbone by selection of natural and synthetic RP for the purpose of controlling transformations of the polymer radicals.

To forecast consequences of DNA irradiation, it seems significant to estimate the maximal effect of its protection in case of use of one or another radioprotector as FRR inhibitor. The authors believe that for this purpose computerized steric simulation is the most suitable [24].

In contrast with experiments carried out on animal cells, such methodology based on the data about spatial organization of DNA active sites (radicals), protector (hydrogen donor) molecules and the mechanism of the reagent interaction may be useful for estimation of RP efficiency as "*pure*" InH.

The recovery of the initial DNA structure (at atom H transfer from InH to macroradical) is possible only when unpaired electron occurs in 2-deoxyribosyl. If this unpaired electron occurs on nitrous base, the interaction between such macroradical and InH does not repair the initial DNA structure: base derivatives with saturated bonds substitution previously exi8sting double bonds occur in the structure of biopolymer [3, 19].

In this review, we generally discuss the problems of radioprotection on the example of DNA sugar fragment degradation (reduction of the initial polymer structure) and occurrence of mutations (the molecular level) in the presence of FRR inhibitors.

DEGRADATION OF 2-DEOXYRIBOSYL IN DNA AND THE QUESTIONS OF RADIOPROTECTION

Primary damages of DNA sugar fragment induce formation of single-strand (SB) and double-strand (DB) breaks, and alkali-labile sites (ALS), occurrence of free nitrous bases (FNB), and formation of purine 5′,8–cyclic derivatives. DB formation in DNA can be explained by occurrence of two independently formed SB in a definite DNA zone or simultaneous cutting of two polymer strands by ionized particle track [14].

The above-mentioned processes as well as formation of thymine and cytosine dihydroderivatives in DNA irradiated in solutions under conditions, which include reduction of bases by e_{hydr} (for example, solutions are saturated with nitrous oxide [25]), are explained by transformations of five types primary 2-deoxyribosyl radicals and radicals of H atom detachment from C_1 –C_5 atoms [19, 26 – 28].

Formation of SB and FNB in DNA is induced by transformations of radicals by C'_3, C'_5 and C'_4 (in the presence of O_2) [28, 29]; the occurrence of ALS, the sites with 2-deoxyribosyl residues containing aldehyde groups at C_1, C_2 and C_4 atoms, and keto-groups [27, 31] at C'_1, C'_4 and C'_2 (in the presence of O_2) atoms of radicals. The formation of ALS is definitely contributed by apurine and apyrimidine sites (derivatives of bases with saturated bonds after N-glycoside bond hydrolysis in them) [26, 27].

The material balance reduction (with 30% error) by intermediate and final products shows that formation of a single "final" molecular product of sugar chain degradation consumes one primary macroradical [28]. Assuming for 100% "basic" value the total yield of primary radicals from the sugar fragment C'_n (n = 1 – 5) and adjusting efficiency of studied RP (InH) to this value by the access hole method [24], data on the probability of InH interaction with 2-deoxyribosyl radicals (Table 1) were obtained and maximum possible effect of radioprotection in the presence of InH for the transformation of each of 2-deoxyribosyl radicals with recovery of its initial structure.

These data show that the efficiency of radioprotection in transformations of C_2 and C_5 radicals in the presence of shielded phenol is twice higher then for C_3 and C_4 radicals, and is "*zero*" for C_1: the molecule of shielded phenol may not approach C_1 radical with hydroxyl group from the side of C'_3 group of the neighbor nucleotide. In the absence of O_2, the contribution of radioprotection effect by radical C_5 in formation of SB in DNA

equals ~70% [24]. More comprehensive discussion of the radioprotection at SB formation is presented in Section 4.

Table 1

Steric probabilities of 2-deoxyribosyl radical interaction with inhibitors (InH) [24]

Inhibitors	2-deoxyribosyl radical types					Effect*
	C_1	C_2	C_3	C_4	C_5	
Phenol**	0	25.1	9.9	13.1	19.4	R
	10.7	0	12.2	10.7	0	S
MET***	6.6	16.2	11.1	11.4	31.2	R
	5.4	0	10.8	13.3	0	S
L-cysteine	5.1	16.9	12.0	11.3	30.0	R
	3.6	0	10.0	15.5	0	S
Serotonin	7.6	20.0	11.0	10.3	21.2	R
	5.0	0	13.0	13.2	0	S

Notes: * R – protection; S – stereoisomer formation.
** 4-Oxy-3,5-di-*tert*-butyl-α-methylbenzylamine.
*** Mercuptoethanol.

The maximal decrease of ALS in the presence of shielded phenol reaches, for example, ~38% (25.1% for C'_2 plus 13.1% for C'_4, respectively); the formation of stereoisomers of 2-deoxyribose residues by these atoms (refer to Sections 3 and 4) add more 23% (12.2% for C'_3 and 10.7% for C'_4, respectively). Finally, total decrease of the ALS yield related total yield of 2-deoxyribosyl radical yield equals about ~60%.

Basing on the reactivity of nitrous bases and 2-deoxyribose [4, 32], one might suggest that predominant contribution into ALS formation should be made by apurine and apyrimidine sites [32]: potential places of additional SB formation (after alkalization of irradiated suspensions of cells and tissues). Total yield of ALS equals 250 ± 50 bond/(cell·Gr) [33]. At the background of such relatively low value (at high measurement errors) the effect of ALS yield reduction only by transformation of radicals C'_1, C'_2 and C'_4 even by 60% (radioprotection plus yield of stereoisomers) may cause difficulties at determination in the experiment. Nevertheless, maximal contribution of initially damaged 2-deoxyribosyl to the total yield of ALS may be estimated indirectly. For example, according to [27] each act of sugar radical transformations with formation of one ALS type or another is accompanied by

the occurrence of H-adduct base radical, including thymine H-adduct (TH), which is easily detected by the ESR method in cells and tissues irradiated at low temperatures (77 – 190 K) [34, 35]. For example, at spleen irradiation G(TH) = 0.02 radical/ 100 eV. Radical C'_2 transformation is associated with H transfer from C'_1 to C_8 in purines and to C_6 in pyrimidines due to formation of energetically more profitable transition structure of the radical $C'_2{=}C'_1{-}N_1(N_9){-}C_6{=}C_5(C_8{=}N_7)$ [19], in which π- and unpaired electrons are delocalized. According to estimation (with respect to TH registration – the original radical-marker at ALS), the concentration of such process into total G(TH) value is below 1.5% that recalculated per tissue (spleen) gives ~60 bond/(cell·Gr) for thymine and, generally, 120 bond/(cell·Gr) for pyrimidines. It is suggested that at room temperature the transition will not affect the process of H transfer from sugar to the base. As demonstrated by computerized modeling, the distance $H(C'_1)$ C_8 in the transition complex is by 10 – 15% longer for purines rather than corresponding distance $H(C'_1)$ C_6 for pyrimidines. Meanwhile, it may be concluded that purine contribution into H-adduct synthesis is much lower and, generally, sugar radicals contribute 60 – 70% (e.g. ~175 bond/(cell·Gr)) into ALS formation.

As C'_2 interacts with InH (reaction 1), due to sugar radical transformation the ALS yield may maximally be reduced by ~25% reaching ~120 – 130 bond/(cell·Gr) as recalculated per cell. Therefore, the contribution of sugar radicals into total formation of LAS is, at least, comparable with the part of the transformations of bases with unsaturated bonds.

The formation of purine (C'_5–C_8)-cycloderivatives in the DNA composition is induced by radicals C'_5 transformations [19]. Total yield of these products in the cell is below 1% SB. Due to nonrepairness, they are extremely important for radiation mutagenesis [6]. The steric modeling allows for estimation of the maximal efficiency of radioprotection (for example, by shielded phenol) by radical C'_5 (~20%, Table 1). To obtain higher effect, InH with the mask square 5–7 times lower than for the studied phenol is required. The data on C'_5 radioprotection by mercuptoethanol and L-cysteine (the mask area is by 2 – 3 times smaller than for "*phenol*" one) confirm this conclusion: the efficiency reaches 30%.

ABOUT RADIATION MUTAGENESIS (THE MOLECULAR LEVEL)

Stereoisomers of 2-deoxyribose – potential sites of point mutations

As mentioned above, the interaction between InH and alkyl radicals of asymmetrical carbon atoms (or the reduction of a radical by electron or ion of any alternate valence metal) may produce stereoisomers of the initial 2-deoxyribose. This statement is confirmed by the results of experiments of radiolysis carried out on frozen aqueous solutions of carbohydrates – monosaccharides and thymidine nucleoside. As radiation was carried out at T = 195 K (rigid matrix) immovable primary radicals of carbohydrates react with electrons migrating in the matrix [2, 3] and form stereoisomers – the epimers of initial sugars. Irradiation of D–ribose produced epimers by all three asymmetrical carbon atoms:

- D–xylose, D–arabinose, L–lyxose [36];
- epimers by C'_3 and C'_4 atoms of thymine 1–(2–deoxy–β–D– and α–L–treo–pentafuranosyl) for thymidine (thymine 2–deoxy–β–D–furanosyl) [37];
- epimers by C'_1 radical for aqueous solutions of α–deoxynucleotides in the presence of glutathione [38].

For DNA, the rigid matrix for 2–deoxyribose residue radical is the polymer structure itself. As 2–deoxyribosyl epimers occur in DNA at the above-mentioned carbon atoms, this should change orientation of base in nucleotide with respect to rehybridization of orbitals (initial sugar residue : radical : epimer = sp^3 : sp^2 : sp^3). If the repair enzymes have not detected these changes in the structure of DNA molecule nucleotide, modified in this manner, then the consequent acts of DNA replication (a duplex formation) may proceed with the coupling of bases containing stereoisomer of nucleotides with noncanonical (according to Watson–Crick) bases [39 – 43]. The process of base coupling depends only on the fact, if hydrogen bonds are formed between them or not. Thus a conclusion is induced about possible occurrence of potential sites for genetic (point) mutations such as transitions and transversions in DNA by places of 2-deoxyribose residue epimer formation [39]. It should be outlined that these mutations are initiated by primary damage of the sugar fragment rather than nitrous bases of DNA.

Computerized modeling results obtained on location of atoms and rotation angles of N-glycoside bond of the bases in nucleotides containing 2-deoxyribosyl radicals and epimers indicate a significant shift of bases from the initial location up to 46.5 – 49° for C_1 radicals and, minimally, approaching 0° for C_5 radicals. As epimers occur in nucleotide composition, for example, by C'_1 atom, the deviation of bases reaches ~55° from the initial location [28]. It may be suggested that for large shifts of the base at stereoisomers (C'_1 atom) the possibility of hydrogen bond formation between bases is reduced to a minimum and, vice versa, they are formed at small shifts.

According to the estimation, the probability of occurrence of point mutations is only sufficient, when the distance between atoms participating in hydrogen bond formation between bases falls within the range of 2.74 – 3.15 Å [28]. Since atoms C'_2 and C'_5 are not asymmetrical (possessing two C–H bonds each), then occurrence of the mentioned mutations may be expected in the case of stereoisomers by atoms C'_1, C'_3 and C'_4. Therefore, the probability of site formation for potential point mutations must not exceed $2/7^{th}$ part.

Point mutations

Analyzing the data accumulated by 1990ies in the field of radiation genetics, I.B. Mosse noticed different interpretations of point mutations, given by different investigators, and defines it as the "*stable changes at the level f DNA nucleotides*" [44, 45]. Seemingly, such changes in nucleotide are only related to modification or degradation of nitrous bases. According to the authors' point of view, another reason for point mutations is the primary damage of the sugar fragment in DNA.

We cannot yet estimate the contribution of each mechanism of point mutations into total genetic effect. There are just indirect reasons in the literature supporting the existence of "sugar" point mutations (PM).

1. FRR inhibitor effect. In some experiments, carried out on fruit flies and mice, it has been found that their irradiation in the presence of substances acting as radical acceptors in the organisms increases the point mutation yield comparing with irradiation under usual conditions [44, 46 – 48]. If in this case PM were induced by modification of nitrous bases only, then at irradiation in the presence of a substance – RP the yield of point mutations (as well as the yield of base degradation products) would decrease [3, 7].

2. The oxygen effect. As is known, irradiation of DNA solutions in the presence of molecular oxygen induces free-radical formation of thymine and cytosine hydroperoxides in the DNA structure, which then degrade finally producing pyrimidine glycols in the biopolymer [1, 34, 50 – 57, 59 – 61]. The formation of peroxide radicals of pyrimidines in DNA was detected by ESR method directly at low-temperature irradiation of DNA, chromatin DNP, cells and tissues [35, 54 – 57, 60, 61]. Therefore, in the areas where hydroperoxides and glycols were formed, the meaning of DNA codons, to which the initial bases belong, is changed. According to [1], in consequent acts of DNA replication this must initiate point mutations. Pyrimidine glycols are also formed in DNA in the case of hypoxia – due to oxidation of primary radicals of bases, thymine and cytosine OH-adducts [6, 34]. In conditions of organism irradiation in the presence of O_2, as well as in the post-radiation period, the yield of glycols increase and hydroperoxides occur. Therefore, PM yield would increase as compared with irradiation in the absence of O_2. However, no such changes of PM yield were observed in the experiment [62 – 65]. This does not oppose the conclusion about a definite contribution of sugar fragment into PM origination.

3. The dose intensity effect. The dose intensities of 30 and 3000 rad/min produced the same yield of point mutations [65, 66]. Small steps between dose levels do not allow for an unambiguous conclusion about the dose intensity effect on the point mutation frequency. It would be expected that the dose intensity increase suppresses base radical oxidation processes (with glycol formation) and, correspondingly, reduces PM frequency. Hence, the independence of PM yield on the dose intensity (despite the short interval) may be belonged to combined action of two factors: $^{\bullet}OH$ radical reactivity in relation to bases, by an order of magnitude higher compared with 2–deoxyribosyl, and increasing role of base OH-adduct recombination with other radicals. Similar to the previous case, if this reasoning is also true for the broader range of dose intensities, we may prefer PM from the sugar fragment.

Difficulties in PM origin determination from damaged base or sugar are, first of all, explained by the absence of registration techniques for such primary damages (free radicals) in DNA and sequences of elementary acts of their development (stage-by-stage registration of transformations of primary radicals of sugar up to final molecular products in the macromolecule).

ESTIMATION AND FORECASTING OF MUTAGENESIS RADIOPROTECTION EFFECTS AT DNA IRRADIATION IN THE PRESENCE OF FREE-RADICAL REACTION INHIBITORS

Basing on the idea that RP (DNA damage repair by FRR inhibitor) is connected to radical substitution reaction (1) in the sugar fragment and PM occurrence – with substitution of the same radicals with unpaired electron on asymmetrical atoms (C'_1, C'_3, C'_4), we tried to estimate maximum possible values of the target effects in transformations of each radical (radioprotection "R" and synthesis of stereoisomers "S" – PM of the sugar origin, Table 1) at the application of some inhibitors. Obviously, basing on these data a conclusion can be made that possession of a set of InH having different size of the inhibitor functional group mask and its location at the active site (unpaired electron localization places) of the radical, we may control transformations of each of 2-deoxyribosyl radicals and reach the desired effect (RP or PM) in quite broad ranges. For example, as we transit from mercuptoethanol to phenol, the yield of single-strand breaks for radical C_5 changes by 35 times, whereas for radical C_4 (+ O_2) by 18 times (RP effect); the yield of epimers for C_1 is changes by 10 times, for C_3 – by 17 times, and for C_4 – by 27 times (PM formation effect). It should be outlined that the above estimation concerns PM of the sugar origin, and the contribution of PM from initially damaged bases was not taken into account.

Table 2

Distances between oxygen atoms in phosphate and oxo-groups of pyrimidines and carbon atoms of 2-deoxyribosyl in V-DNA, Å

Atom O	C_1	C_2	C_3	C_4	C_5
O_5 phosphate	5.5	4.8	4.2	4.3	3.2
O_3 phosphate	5.1	4.6	3.8	4.8	6.1
O_2 cytidine	2.7	4.0	5.0	4.7	5.5
O_2 thymine	2.6	4.0	4.9	4.6	5.5
O_4 thymine	5.5	6.3	7.6	7.3	7.2

Turning back to the question about the control of RP and free-radical epimer formation processes, let us analyze the relationship of inhibitor functional group possessing antiradical activity and the place of radical potential formation (unpaired electron localization) in 2-deoxyribosyl prior to

polymer irradiation. Mentioned in Section 1 of the current review are sulfur-containing radioprotectors-amines. Basing on purely steric ideas and taking into account RP amino group fixation at DNA prior to irradiation, we have estimated a possibility of participation of functional groups of these molecules, reactive in relation to radicals, in the reaction (1). Two alternatives of amino group fixation ("*anchoring*") at DNA were considered: protonated amino group binding to negatively charged oxygen atom (at DNA dissociation) in phosphate and RP amino group binding to partners by hydrogen bonds (oxo-groups) of nitrous bases. In the first case, ionic chemical bond N–O formation was simulated, and in the second case – hydrogen bond N–H O. Both types of bonds are realized at complex formation between V–DNA and nucleosome – the hystone protein octamer [68, 69]. Table 2 shows distances between oxygen atoms in oxo-groups of pyrimidines (O2 – in cytosine and thymine, and O4 – in thymine) and carbon atoms in 2-deoxyribosyl. With respect to the fact that InH molecules under consideration are attached to DNA by amino groups (hydrogen bonds O H–N), and H (or unpaired electron) transfer from sulfhydryl group to 2-deoxyribosyl radical is only possible at the distance ~1.5 Å, they, principally, at the interaction with all alkyl radicals of 2-deoxyribosyl should produce the RP effect and, moreover, epimers – with radicals C_1, C_3, C_4. Meanwhile, unpaired electron remains in the composition of radical $In^{\bullet}$, the reaction (1). The fortune of $In^{\bullet}$ (cysteamine) will be discussed below.

Data in the Table 2 indicate that InH "*anchored*" by oxygen atom of phosphate might "*serve*" 2-deoxyribosyl radicals (most probably, C_2 and C_5) with sulfhydryl groups (H atom transfer) at the distance of ~1 Å, therefore, preventing SB and ALS occurrence (the RP effect). Thus, for radicals C_1, C_3 and C_4 epimers may also be formed. As amino groups of the same inhibitors are "*anchored*" to oxo-groups (hydrogen bonds) of the bases, the reaction (1) may proceed with all radicals of 2-deoxyribosyl (except for C_3 radical at thymine by O4). Both alternatives under consideration obey conditions of chemical bond formation between alkyl radical and inhibitor's H atom (for above-mentioned inhibitors as well as cysteine and reduced glutathione) in the framework of the ideas of the access hole method [46] (at the reagents with sulfhydryl groups) in the case of approximately equal mask parameters and access hole sizes.

At the estimation of possible interaction between RP molecules with 2-deoxyribosyl radicals, the alternative of unpaired electron transfer from the radical to RP (the radical oxidation reaction) should also be taken into account. For example, such process might proceed at the interaction of alkyl radical

with disulfide bond of the cell endogenic cystine and cystamine, which is RP of the synthetic origin.

As entrapping an electron, disulfide bond in cystine (and cystamine) is broken and then forms sulfhydryl group and a radical with unpaired electron at sulfur atom ("*sulfuric*", thyil radical). This process was detected by ESR method at radiation degradation of cystine (disulfide bonds) saturated protein – wool keratin [70]. In this case, the reactions with disulfide bonds were entered by electrons generated by Co^{60} γ–quanta. Further on [71], only thyil radical may react with double bonds C_5=C_6 in pyrimidines and C_8=N_7 by C_8 in purines with protector–DNA crosslink formation that was already suggested before [15, 16]. As mentioned above, the duration of such process is similar to times of carbohydrate radical transformations. It is found by 7 orders of magnitude lower than the response times of repairing enzymatic systems in the cell on the radiation impact. Finally, the total effect of primary 2-deoxyribosyl radical transformation in the presence of InH proceeding with participation of thyil radical is related to formation of the RP–DNA crosslink.

So far as concerns the second product of disulfide bond break, which is sulfhydryl group anion (or the sulfhydryl group itself of, for example, cysteine after S–S bond break in cystine anchored on DNA), its fortune represents an interaction with any neighboring (closer than ~1 Å) 2-deoxyribosyl radical according to the FRR inhibition mechanism.

Therefore, as DNA is irradiated in the presence of sulfur-containing radioprotectors, thyil radicals may be synthesized according to the following reactions: the interaction between $^{\bullet}OH$ and $H^{\bullet}$ radicals with RP sulfhydryl group and by reaction (1) between RP as FRR inhibitor and DNA radicals (e.g. after H atom transfer from sulfhydryl group to DNA radical), and at the break of disulfide bond formed under the effect of e_{hydr} synthesized for sulfur-containing protectors cystine and cystamine.

The future of thyil radicals depends on the place of amino group fixation at DNA molecule and sulfhydryl group location relative to the polymer double-strand structure. In this connection, with respect to the thyil radical mask parameters, it may react with double bonds in the bases producing thyil radicals-adducts, the reduction of which ($+H^{\bullet}$ or $+ e +H^{+}$) necessarily produces the RP–DNA crosslink. The alternative of thyil radical conversion is recombination with the similar (S–S bond formation) or any other DNA radical (S–C bond). In this case, if O–N chemical bond formation between DNA and RP prior to irradiation (RP amino group anchoring by phosphate oxygen) is taken into account, RP–DNA crosslinks will also be formed.

CONCLUSION

The investigation results on InH application to DNA radioprotection due to the free-radical mechanism induce the following conclusion that the process is carried out by FRR inhibitor molecules, attached to the macromolecule backbone beforehand (prior to irradiation). We have paid general attention to sulfur-containing RP, anchored to DNA by amino groups of the natural origin (cysteine, cystine, glutathione) and synthetic RP (cysteamine, cystamine, chlorophos, etc.).

Basing on the ideas about DNA–hystone proteins bonds in nucleosome, the authors have chosen oxygen atom in phosphate (ionic bond) and oxo-group in bases (hydrogen bond) as locations for attachment to V-DNA. Both these alternatives of RP amino group fixation to V-DNA are confirmed by the experiment: The studies of physicochemical characteristics of DNA interaction with amino groups of sulfur-containing ligands (cysteamine, cystamine, cystaphos, asparagin, β-alanin) have shown [72] that the first case is pH-dependent attachment, and N–O bond may really be formed. In the second case, a hydrogen bond is formed between N atom of the ligand amino group and the base oxo-group. According to other data [69], such place for amino group attachment might be O_2 and O_4 atoms in pyrimidines and O_6 atom in purines. It may also be suggested that sulfhydryl group at O_2 atom in pyrimidines is primarily located in the first hydrate layer of the minor DNA groove, substituting one of the "bridge" water molecules between complementary bases [23].

So far as concerns participation of InH with disulfide bonds (cystine and cystamine, fixed at oxygen atoms of phosphates in different DNA strands of oxo-groups of bases in V–DNA) in radical reactions, according to preliminary estimations, the S–S group of these ligands is located ~6 Å far from carbon atoms of the sugar fragment. In this case, only interaction between S–S bond break products (thyil radical and sulfhydryl group) with double bonds in the bases (DNA–RP crosslinks) and DNA radicals (inhibition of their transformations, RP effect, or epimer synthesis) may be expected in DNA. Further investigations will specify the reaction mechanisms of InH anchored to DNA.

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ACADEMICIAN N.M. EMANUEL AND ESTABLISHING OF THE PRINCIPLES FOR THE CONTROL OF AGING OF THE LIVING ORGANISMS

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"Our writings are imperfect, but
the truth enclosed is great."
L.S. Vygodsky

At present, we can hardly find a man who has not heard about chain reactions, which are specific processes carried out with formation of free radicals – the active intermediates. Academician Nikolai M. Emanuel was one of the scientists who first suggested and then proved experimentally that many biochemical processes proceed with participation of free radicals and studied their role in development of pathological states and the process of natural aging. N.M. Emanuel has ingratiated the worldwide recognition as the pioneer in application of chemical kinetics principles to description of biological investigations and the study of medical problems.

Keywords: Academician Nikolai M. Emanuel, free radicals, retardation of aging, the history of gerontology

N.M. Emanuel developed his scientific creativity in times, when tendencies to unite chemistry and physics, and then biology and medicine became more and more strong. Working in the border fields of science, he has developed his own systematic approach, targeted at the understanding of the object entity. Systematism was dominant in his methodology and the style of thinking; he was in the leading group of scientists who originally and systematically developed the method of control for chemical reactions by effecting on the early stages of initiation [1]. The scientific potential of works of that period is far not exhausted by the problems of chemical kinetics. The fruitful ideas in this field formed the theoretical foundation for a broad cycle of

works on biology in 1960 – 1980ies. Nikolai M. Emanuel has repeatedly outlined that "kinetics as the science about the laws and mechanisms of various process development in the nature has in biology almost unlimited prospects for theoretical investigation and practical applications. Any pathological process in the living organism regularly develops with time; the opposite development and suppression of pathological states under the influence of one or another effective impacts is regular to the same extent".

Consecutive consideration of the main works by Nikolai M. Emanuel induces a conclusion that despite a broad variety of investigation objects and methods, he always preferred searching for the ways of process control, either a chemical reaction or metabolic transformation. In 1982, in the work on "Antioxidants and life prolongation" N.M. Emanuel proved his point of view in the following statements [2].

"Aging of the living organisms represents a complex, multifactor biological process. Biochemical and biophysical processes proceeding in a living organism in norm and pathology are carried out in quite complicated, open, heterophase chemical systems under the effect of multiple chemical and physical factors. From physicochemical viewpoint, solution of the life prolongation question must be reduced to fuller prevention and effective suppression of various unwilling shifts, which occur in the functioning living organism, as well as to prophylaxis and elimination of pathological states. The modern chemical kinetics, which studies the mechanisms and regularities of chemical reaction development with time, to a greater extent, transits from the study of interactions of a small number of simple molecules to investigation of chemical transformations of a great number of substances under the effect of various physical agents. In chemistry, we always have to resolve the problems of controllable behavior of chemical reactions, including deceleration of suppression of unwilling processes. Therefore, the experience gained by studies of chemical kinetics on the chemical reaction control should be used sensibly for the purpose of deceleration and suppression of the processes providing for aging of the living organisms. Interestingly, many unwilling and "harmful" events in chemistry are also called the aging processes. For example, they are processes of the aging of polymers and other materials, various articles from them, food contamination and drug damage, etc. There is a great number elementary processes possessing analogous mechanisms – molecular, ionic, free-radical, which are carried out at aging of the living organisms. In principle, there are similar warrants for optimism in solving the problem of life prolongation (LP), which we have in the field of chemistry, when searching for

effective ways of deceleration and frequently almost complete suppression of unwilling chemical transformations".

N.M. Emanuel headed the investigations, which gave experimental data on free radical participation in the aging process. This allowed for a suggestion that substances which prevent proceeding of the reactions by free-radical mechanism, i.e. various inhibitors and antioxidants, will cause prophylactic or therapeutic effect and increase the organism lifetime.

Aging provides for accumulation of structural and functional disorders at all organizational levels of the living organism. We may indicate at least two principally different sources of "damages and errors" in the cell. The error occurrence frequency at DNA matrix replication, for example, or protein synthesis is defined by imperfection of the method for information transmission from molecule to another and is generally associated with the molecular organization features. Another source of "random" errors is macromolecule free-radical oxidation reactions. Free radicals containing oxygen, O_2^-, $^{\bullet}OH$, or $RO_2^{\bullet}$ are extremely reactive. They are continuously synthesized in the cell, and if there were no inactivation mechanisms, they might induce quick degradation of biological structures. At aging the efficiency of protective systems in the organism decreases, which control the rate of peroxide oxidation of lipids and proteins: concentration of natural antioxidants decreases, the quantity of "inactive" molecules of enzymes destroying peroxides increases, etc. However, diminution of natural antioxidants may be restored by special diets or synthetic substances, which are functionally similar to natural compounds. Primary results on prolongation of animals' life with the help of antioxidants became of special interest for gerontologists, and a possibility was expected to use them for deceleration of human aging. The experience of synthetic antioxidant application shows that some age changes in the cells are reversible or, at least, may be decelerated. Natural and synthetic antioxidants possess a broad variety of biological properties and currently are both widely used for theoretical study of the aging process and applied in medicine.

ANTIOXIDANTS – GEROPROTECTORS

The efficiency of aging deceleration agents, usually applied as food supplements, is experimentally estimated by the number of survived species in

control and test groups of animals. The substances – geroprotectors can increase the average LD value of laboratory mice and rats no more than by 30 – 35% [3, 4, 5].

Table 1 shows some results of experiments carried out in 1970–1980ies, supervised by Academician N.M. Emanuel, in the Institute of Chemical Physics and since 1984 till 2003 by his followers in the Institute of Biochemical Physics, Russian Academy of Sciences.

Table 1

The influence of antioxidants on life duration of laboratory mice [4, 6, 7]

Group, preparation dose	Animal strain, age at the beginning, months	Number of animals	Average LD ± statistical error, months	Difference from control	Maximal LD**
Control	SHK, 2	120	13.0 ± 0.7		25
3–OP, 150	–//–	113	17.3 ± 0.9*	+33.0	28
Control	SHK, 8 – 8.5	156	15.0 ± 0.6		19
3–OP, 200	–//–	156	18.8 ± 0.5*	+25.3	29
Control	C3HA, 2	170	21.9 ± 0.8		32
3–OP, 150	–//–	90	27.0 ± 0.8*	+23.3	41
Control	AKR, 2	50	16.2 ± 0.6		23
3–OP, 150	–//–	50	15.8 ± 1.3	0	24
Control	CBA, 4	50	26.8 ± 0.6		31
Beta-carotene, 0.3	–//–	20	27.9 ± 0.6	+4.0	31
Control	CBA, 4	50	26.8 ± 0.6		31
Coenzyme Q_{10}, 0.4	–//–	20	24.5 ± 1.0	–9.1	31

Notes: Doses of preparations are given in mg per kg of body mass.

* $P < 0.01$; 3–OP: 2-ethyl-6-methyl-3-oxypyridine·HCl.

** Maximal LD is the age at 90% mortality.

Synthetic antioxidants were found perspective geroprotectors, i.e. the substances increasing the life duration of laboratory animals. A low-toxic

water-soluble 2-ethyl-6-methyl-3-oxypyridine chlorohydrate (which is structural analysis of vitamin B_6) was used as a food supplement, added to the diets of SHK laboratory mice since they were 2 months old. The average LD of the animals increased to 17.8 months compared with 13.7 months in the control group. In experiments with 8-month old mice during the following 6 months survivability in test and control groups was reliably identical. Since 15-month age, mortality in test groups significantly decreased, and the maximal life duration reached 28 months compared with 18 months in the control group. Thus the average life duration increased by 23%.

In the Department of Kinetics of Chemical and Biological Processes, N.M. Emanuel headed systematic investigations of synthetic antioxidants of different classes for the purpose of their application as geroprotectors. In these experiments 4 strains of laboratory mice (SHK, C3HA, AKR, and CBA) and several *Drosophila* strains were used, and optimal geroprotector concentrations were determined. A preparation from the class of 3-oxypyridines – emoxypine – was found the most effective and suitable. It served the main tool in the studies of phenomenology of natural aging process deceleration and its FR-mechanisms.

The effect on the aging process depends on many factors: preparation dose, strain and sex of animals, and their ages, as well. To classify the influences which may change the life duration, test results were systematized with respect to changes in the survivability curve shape [8].

A group of animals of the same species may conditionally be presented by two subpopulations of short and long living individuals. Individuals from the first subpopulation die before reaching the age, which characterizes the distribution median. The members of the second subpopulation show longer life duration than the average LD of the whole population. The changes in the survivability curve shape, observed in the experiment, can be presented schematically. In the first case, an impact decelerating the aging process equally increases LD of all members of the population. Therefore, the survivability curve for tested animals is shifted to the right parallel to the curve of the control group. Meanwhile, the average and maximal LD values increase, and mortality rate does not change. In the other case, the presence of several short-living individuals, not responding to the influence, in the group is typical of. Then the initial parts of the survivability curves are identical for test and control groups. The mortality rate in long-living subpopulation decreases with sensitivity to an impact. This causes an increase of the average and maximal LD. In the case, if the aging rate is decreased only for short-living subpopulation, the maximal LD does not increase, but a survivability

"*threshold*" on the test group curve expands, e.g. the age of 10% mortality in test exceeds the corresponding value in the control, but then the test group dies at higher rate than the control group. The results of chemical geroprotector tests clearly illustrate all three types of aging deceleration (Table 1).

The free-radical hypothesis includes a suggestion that aging deceleration with antioxidants is carried out due to the decrease of age molecular damage accumulation rate. It is meant that damages induced by free radicals may occur practically in all cellular structures. The cases for this point of view are considered in detail in many works, and consent with this thesis would require recognition of the antioxidant action versatility. However, such forthright consideration contradicts to the facts. Not all tested antioxidants were found to be geroprotectors, whereas the latter were active in relation to some definite objects only. Antioxidant 3–OP 1.5 – 1.8-fold increased the average LD for mice SHK and C3HA; mice SHK belong to noninbred population with typical high frequency of spontaneous breast cancer. The use of this compound for AKR strain mice damaged with spontaneous leucosis was unsuccessful. Since 18-month age e.g. 16 months after the beginning of the experiment, mice from inbred long-living C3HA strain affected by 3-OP shows a change of mortality frequency distribution shape and decrease of the average mortality rate from 4.7 (in control) to 3.6% per month. The average life duration for these mice is increased by 23.3%, and the maximum LD reaches 41.2 months (Table 1). The particular reasons for unreproducibility of the results are not discussed in gerontological literature, the fact of presence or absence of activity of particular compound is only mentioned. Usually, the methods for the compound dose selection is not justified, at the most, its toxic properties are preliminarily studied. So far as concerns selection of the dose of synthetic antioxidants, the compound interaction with natural antioxidants should be taken into account, because it may either decelerate free-radical oxidation or activate them and induce exhaustion of natural antioxidants in tissues [10].

The influence of food supplements containing beta-carotene and coenzyme Q_{10} on natural and radiation-accelerated (by irradiation of heads with 10 and 12 Gr doses) aging of mice has been studied. In the first case, the use of food supplements (produced by Solgar vitamin Co., Inc., USA) led to unreliably (p = 0.2) small increase of the average LD by 4%; in the second case, a noticeable decrease of LD was observed (9%, p = 0.2) in relation to intact group of animals. The application of natural antioxidants to irradiated mice gave positive results, displayed in full compensation of radiation

shortening of life. It is not an overestimation to expect positive effect of these food supplements on health and LD of people from the so-called "*risk groups*".

Since 1950ies and up to date the statement about the key role of free-radical damages in the aging mechanisms of living organisms is widely discussed in the literature (and has almost become an axiom). Actually, it has been observed in multiple investigations that the efficiency of protective systems decreases with age providing for intensification of free-radical damage of macromolecules. For example, for several strains of laboratory mice a monotonous age decrease of the antioxidant activity of lipids has been shown. For long-living strains it did not exceed 20 – 25% of the initial level and reached 50% for short-living strains [10]. On the other hand, according to R. Cutler [11] the average life duration by species arranges a positive linear dependence on antioxidant concentration in blood plasma. Here it should be noted that the life duration of short- and long-living individuals (of the same species (the cohort members) does not obey this law.

Among substances from other classes of biogenic compounds, geroprotectors are, for example, short peptides [5], hormones [12], etc. Obviously, natural antioxidants are of the greatest importance for normal vital activity, but it is well known for long time that their overdose is dangerous [13].

The coming "epoch of food supplements" requires greater attention to the practice of prophylactic use of antioxidants. In order to approach correct explanation of the results obtained, let us indicate the following known facts. Firstly, the individual variability of vitamins A, E, C, and carotene content in human (and, possibly, animal) blood plasma is very high, up to the 6-fold level, and the age changes are weakly expressed. Are these the reasons for the absence of a correlation between the life duration of an individual and antioxidant concentration in the blood plasma? Secondly, old animals with high carotene concentration in blood showed its deficiency in organs. In this relation, the results by H. Massie [14] are very indicative. They show the reliable independence of the life duration of young mice (+5%) at enrichment of their diets with carotene and 12% decrease of LD for the old animals. Meanwhile, beta-carotene content in blood increased by 60%, but remained unchanged in organs.

The doses of nutrition used in the experiments included antioxidants within the physiological norm, and they can be defined as "*supporting*" concentrations, i.e. additional injections may not induce the case of oxidative stress.

It is known that beta-carotene is not synthesized in human or animal organism, and a complex of carotenoids taken with food possesses much broader biological properties and is not related only to A vitamin activity.

From this point of view, food supplements containing complexes of natural antioxidants, which include not a single compound, but several analogues (for example, not only alpha-tocopherol, but all four natural tocopherols in their original ratio), will be capable of finer control for the efficiency of antiradical protection systems and would possibly have more reasons to be called geroprotectors rather than standard vitamin complexes. To our point of view, the current totality of facts may correctly explain the absence adequate influence from the side of beta-carotene on the life duration of intact mice.

So far as concerns the results of experiments with coenzyme Q_{10}, they were more expected than unexpected. The above note about the advantage of natural complexes is also correct for this case. At least 10 analogues of ubiquinone possessing different side chain length ($N = 0 - 10$) and interacting in the linear and cyclic shapes are known. Contrary to carotene, coenzyme Q_{10} represents endogenic antioxidant and is synthesized in human and animal liver.

The age decrease of energy supply in tissues is accompanied by a noticeable change of mitochondrial DNA. It has been suggested that this phenomenon is just a part of universal, genetically programmable process of aging. In tissues of old people only traces of mtDNA are observed. The loss of mtDNA induce other events unfavorable for the cell: the activity decrease of cytochrome–oxidase C and other components of the respiratory chain and, finally, reduction of bioenergy supply of mitochondria. The model experiments on prevention or deceleration of the mitochondrion aging program by adding coenzyme Q_{10} are unsuccessful still. As shown by G.P. Zhizhina [15], the ability of ubiquinone Q_{10} to inhibit endogenic oxidation of DNA is 2 – 4 times lower than for melatonin and beta-carotene. Though in our experiments the dose of Q_{10} falls within the physiological range, its continuous intake might form conditions for its participation in nonenzymatic reactions with biosubstrates, which products are "*biogenic slugs*" accumulated in the organism and promoting for the aging acceleration [16].

One more very interesting hypothesis reflecting "*positive*" participation of oxygen free radicals in metabolism should be mentioned. It is not so widespread comparing with the hypothesis about free-radical damages, but is already experimentally confirmed and, therefore, is rather prospective.

The question is that "among chemical processes proceeding in the organism, the most suitable sources of energy may be reactions of reaction

oxygen species (ROS)". As two $O_2^{\bar{\bullet}}$ recombine, 1.0 eV is released, whereas at ATP hydrolysis this quantity is below 0.5 eV, i.e. besides ATP, the organism possesses an alternative and much more sufficient source of energy, which redistributing and accumulating in macromolecules may act as the metabolic process initiator. Moreover, it has been found that free radicals containing oxygen represent messengers of the gene activity [17].

So far as concerns the increase of the life duration for animals, irradiated by 10 and 12 Gr doses, which received the same antioxidant supplement with food, this positive result is completely related to the favorable effect on the irradiated mechanism, in which fine regulatory mechanisms were damaged.

THE EFFICIENCY OF GEROPROTECTORS AND VIABILITY OF DROSOPHILA MELANOGASTER POPULATIONS

In the sequence of drosophila generation dynamics of the basic index of viability, which is the average life duration of the population (cohort), is of clear irregular type. To put it differently, the average LD value in populations of parents and their close and far descendants does not remain constant; it may 2-3-fold increase or decrease with irregular oscillations. This conclusion is based on the observations of 128 consecutive generations of Canton-S wild strain, cultivated under standard conditions, accepted by all specialized laboratories all over the world, and cannot be assumed as an artifact. This question was discussed in a broad series of works, carried out since 1989 till 2004 in N.M. Emanuel Institute of Biochemical Physics, Russian Academy of Sciences. Oscillation changes in the viability are highly reliable; complete mathematical description of them and correlation analysis of the relation between parameters of geomagnetic field and LD of drosophila populations are given [18 – 20]. Using strict statistics criteria, the qualitative similarity of aging mechanisms in the sequence of descendants has been confirmed. Great differences between average LD values are caused by different rates of aging of generations having general progenitors and being the members of the same genetic strain. Besides simply heuristic understanding of the novelty and importance of these facts for modeling of oscillation processes proceeding in the living tissue and complex biophysical systems, they are of special importance for experimental aging biology, which together with the medical

practice tends to design means for health protection and life prolongation of people. In this connection, the well-known facts of weak reproducibility of the experiments on LD increase with the help of geroprotectors and their limited 30 – 35% effectiveness obtain different description. All these phenomena may be directly associated with non-random oscillation changes in LD generation by generation. It has been found that extreme average LD values for the control strain define the permissible range of LD values, within which the average LD values for experimental groups after treatment with a geroprotector may occur. All these statements were proved experimentally.

For the geroprotector, spin radical trap 4-hydroxy-tempo (4HT; Sigma, USA) was used. Crystalline powder (0.2 wt.%) was injected to the nutritious medium at the larval stage of drosophila development, and survivability in control and test groups of grown flies was searched for during the following 28 generations. It is indicated that after 4HT influence the average LD value in the experimental groups may both exceed and fall below LD in the control group. The absence of differences was also observed. The results were processed in terms f the oscillation theory, where quantitative differences in the sets were estimated by the Kolmogorov–Smirnov criterion. It is found that LD of the control population, which is nonrandomly changed generation by generation, defines the effect of geroprotector with 76% probability. To put it differently, The ability to increase LD (e.g. decelerate aging) depends on the object viability. For a population with low viability the effectiveness, all other factors being equal, will be higher, and vice versa. The practical contribution indicates that one may hardly expect for the invention of an universal means for life prolongation, and the most preferable way is selection of the most suitable one among their variety (if it is obtained). Popular medical preparations suggested as the drugs for the aging deceleration – melatonin and synthetic peptide Ala-Glu-AspGly, which is the analogue of the active epitalamine essence – are not the exclusion, and their effectiveness obeys the same regularity [21 – 24].

PRIMARY TARGET AND THE AGING STAGES FOR DROSOPHILA MELANOGASTER

The hypothesis about accumulation of damages and errors, including those induced by free radicals, does not oppose the idea about the presence of an internal source for the aging process e.g. programmability. Many authors

believe that the main way for the organism aging is genome degradation. Chromosomal DNA in cells represents the storage of the aging program and, simultaneously, is the main target or the "*initial substrate*" with damaged structure or functional insufficiency of which the aging process at the molecular and cellular levels starts [25 – 27].

The identity of the shapes of survivability curves for people, laboratory mice and rats, and insects indicates similar features of the aging of different populations. Despite great differences in the values of species life duration, we may suggest a unified fundamental mechanism of aging. Nevertheless, it should be noted that since clear physiological signs of aging are observed at the terminal stages of the individual development, then DNA involvement into initial stages of aging should be proved.

It seems desirable to use the features of insect development, because their life consists of strictly separated stages of morphogenesis. For many years, *Drosophila melanogaster* is the main object of investigations for genetics, development biology and gerontology. Nitrosodimethylurea (NDMU) mutagen and geroprotector 3-OP (emoxypin) were used the aging rate modifiers. The doses of these compounds used were nontoxic and caused no effect of the larva development rate. To obtain clearer picture of the aging deceleration, several experiments with varies time of 3-OP compound effect were implemented. In the three alternative experiments with drosophila females the treatment time was gradually decreased by exclusion of the larval stage. Shifting the treatment to later stages of morphogenesis led to a decrease of the life prolongation effect: the average life duration of drosophila females, involved in the experiment at the age of 1 day, increased by 12%. The treatment carried out on 20th and 40th days did not decelerate the aging process. In tests on drosophila males, the treatment time was continuously increased: primarily, it affected only larval stage of morphogenesis and then increased due to initial 10 days of the adult stage and, finally, covered the whole lifetime since ovipositing till death of all imagos. For all three cases, the average life duration increase was similar: 18.0, 22.0 and 20.4%, respectively.

The third age larvae were treated with HDMU (0.001%) during 10 h, which finally led to a significant acceleration of male aging: their average and maximal life duration decreased by 37 and 33%, respectively. The life duration of females under the same conditions has not changed.

At the first glance, the main result seems to be paradoxical: the highest deceleration of aging was obtained by the influence at the larval or young imago stage, when phenotypical signs of aging are absent. The older imagos are, the lower the preparation efficiency is; therefore, the treatment initiated on

the 20th day or older was found abortive. It has been suggested that the tissues of larvae, from which organs of adult flies are formed (nervous tissue, imaginal disks), accumulate damages afterwards inducing the aging. Controlling the rate of damage accumulation with the help of mutagens and geroprotectors, one may affect the rate of adult individual aging. This means that the aging process includes at least two qualitatively different stages: accumulation of damages and their realization. Apparently, the initiation of the realization stage requires not only a definite threshold of accumulated damages, but also time, after which phenotypical signs of aging appear. Since these stages are separate in time, one may suggest macromolecules capable of long-term storage of data on a damaging effect to be the primary target, i.e. for the aging process chromosomal DNA molecules are primary targets. As this threshold is reached, further accumulation of damages has no effect on the aging rate. The information about damage or protection of other macromolecular components of the cell cannot be transferred directly from drosophila larvae to adult species, because all basic macromolecular components of the cell pass through multiple cycles of renovation during metamorphose. More intensive aging of drosophila males in tests with NDMU may be explained by 15% smaller quantity of genetic material in them compared with females [28, 29].

Thus nervous cell DNA of drosophila larvae may be considered as the primary substrate (target) at aging. From the results of some works [26, 27] stages of the aging process can be separated:

1) potential phase, which duration is approximately estimated by the survivability curve threshold length;
2) the realization (terminal) phase e.g. the organism mortality phase.

In our experiments, the larva development was the stage sensitive to the influence.

Similar conclusion can be made from the results of direct observing the secondary DNA structure in mammal cells. At the beginning of the terminal phase, post-mitotic cerebrum cells of mice, neurons, obtain and accumulate defects of secondary DNA structure, recognized by nuclease S_1, generally localized in AT-saturated zones. It is known that AT-saturated areas of DNA are located in many functionally valuable areas of eukaryote genome. Therefore, occurrence of unrepairable defects in them may negatively affect the function of genetic systems of cells. Apparently, the increase of the number of S_1-sensitive areas in genome with age is not a stochastic but genetically controlled process [30, 31]. The transition from potential to terminal phase of

aging is very quick due to realization of accumulated changes, which quantity exceeds the critical level. A mathematical model of such process was also designed. All the above-mentioned coincides with the idea that mortality of individuals and extinction of the whole species of animals during evolution may be based on the same fundamental mechanism acting at a different rate in somatic and genital cells, which provide for material succession of generations [24].

By the ideas discussed, this sequence of works is similar to the early works by N.M. Emanuel, devoted to the control of the chain chemical reaction rate via the influence on the early initiation stages [1].

CRITICAL PERIOD IN THE LATE ONTOGENESIS. LIFE DURATION PREDICTOR

Despite the outstanding progress in recent decades, biology of aging has not designed a method for quantitative estimation of the individual aging rate, which creation might help in predicting life duration of a species. On the other hand, there are no great obstacles for empirical solution of the task. However, the aim of the work must be brought into coincidence to technical methods applied.

The quantitative result of gerontological experiments is often reduced to recording of the "age differences" or "age changes", and though in the literature are frequently equalized, each has its own meaning. The cross-section methods of the studies, the main ones giving empirical facts, allow for determination of the average age differences between groups of animals or people. Analysis of the average values may not give the rate of the individual aging rate and indicate individual features of this process. Only longitudinal observation of the same test animals allows tracking the real age changes, the totality of which gives the adequate dynamic picture of aging.

The present work shows data on the individual age changes in body mass, rectal temperature, functional motor asymmetry index (behavior), life duration and constant potential of cerebral hemispheres in 37 CBA mice-females during the period of March 1993 – October 1995. Despite the fact that biochemical indices are frequently ascribed the meaning of "aging markers", we sensibly excluded them from the consideration. There are two reasons for that:

1) the procedures applied must be strictly noninvasive, i.e. cause no harm to animals;
2) the coefficients of biochemical parameters participating adaptation may vary in a broad range of 20 – 60%, whereas for morphological tests the measurement error does not exceed 4 – 6%.

The functional motor asymmetry index was estimated from the number of spontaneous turns to the right among 10 attempts in a T-shaped labyrinth.

According to modern ideas, the constant potential value, measured in the left and the right cerebral hemispheres, is the integral biophysical characteristic reflecting the unity of exchange processes in the cortex. The value of this parameter is formed by membrane potentials of neurons and glyal cells. The greater the difference in potentials of cerebral hemispheres, the clearer expression of functional and biochemical cerebral asymmetry [32].

One of the tasks concluded in observing the age dynamics of mouse behavior in T-shaped labyrinth and searching for a regular relation between the difference in cerebral hemisphere potentials and the functional motor asymmetry.

There is no doubt that large cerebral hemispheres in mice have lateral specialization, which is the most important principle of the brain functioning in vertebrates. Early, this fact was argued due to the absence of accurate data confirming the presence of the functional motor asymmetry in the majority of animal species. Ontogenetic dynamics of this phenomenon is shortly studied yet.

In recent fifty years, the increased attention of scientists to the brain aging allowed for determination of many morphological and biochemical differences between neurons of young and old animals. With the few exceptions, the authors of such works applied the group comparison and invasive (harmful) experimental techniques, which principally may not provide for a description of aging as the process proceeding with time. To avoid such methodological mistake, we have selected two parameters, which can b changed during the whole life of animals without any harm for their health:

1) constant potentials of left and right cerebral hemispheres potentials (U);
2) the index of functional motor asymmetry, which characterizes behavior of mice in the T-shaped labyrinth (P).

This allowed us to avoid the group comparison and gives an opportunity of kinetic (longitudinal) study.

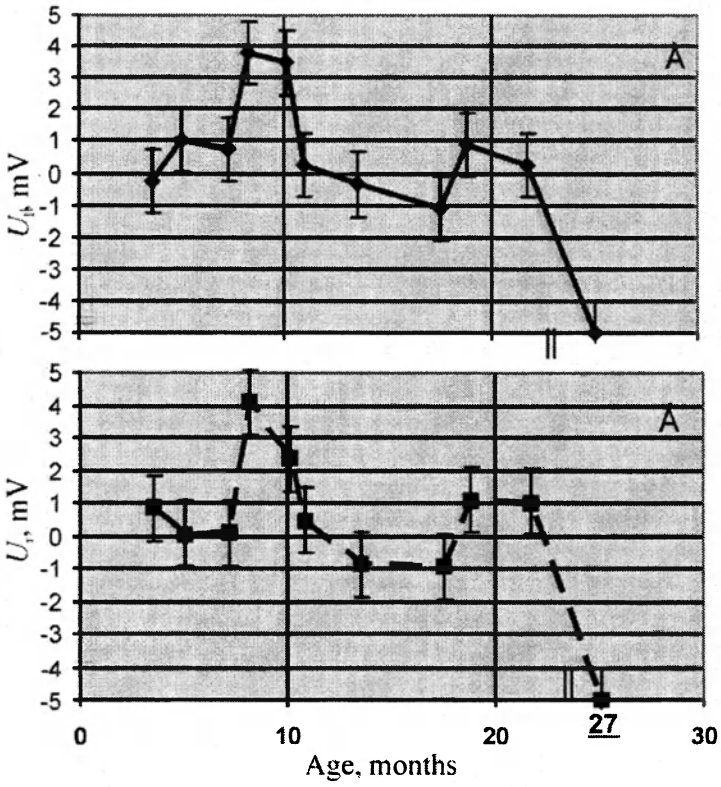

Figure 1. The age dependence of the average constant potential in the left (a) and the right (B) cerebral hemispheres in mice.
The average error id shown on curves

Figure 1 shows the age dynamics of the average U value (mV) in the left (A) and the right (B) cerebral hemispheres of mice. Two specific features of this process should be mentioned:

1) clearly expressed nonlinearity;
2) symbate changes in both hemispheres.

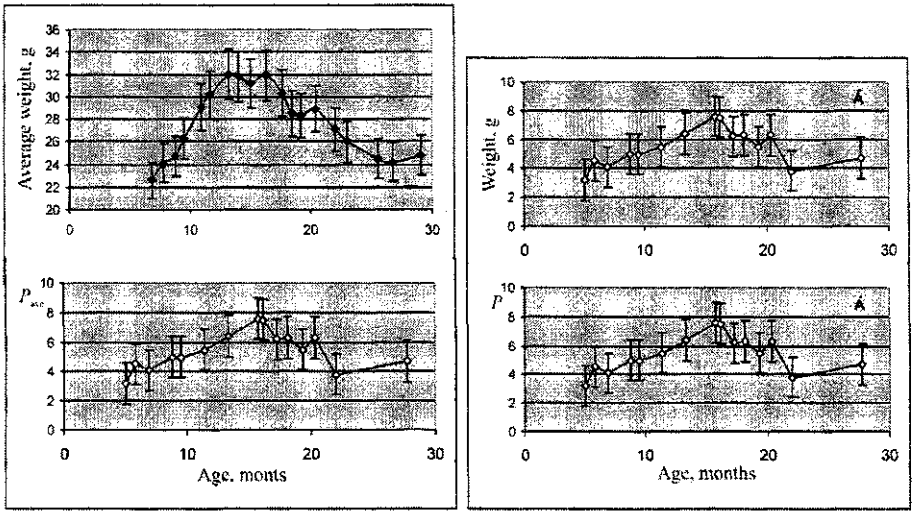

Figure 2. Age dynamics of the average (a and b) and individual (c and d) body masses and functional motor asymmetry index (B) in mice

A narrow area of maximal U values falls on the age of 8 – 10 months, followed by a long period (from 10 to 18 months) of potential decrease from +4 to –1 mV; the senile age since 18 to 22 months shows a small uplift followed by an abrupt decrease of the potential down to –30 mV in the extreme old age.

Figure 2 shows the age changes in the body mass and behavior of mice, which also very complicated. Dynamics of the average and individual values are also shown. Here, the main general features can also be separated:

1) nonlinearity;
2) the area of maximal body masses and P values is broad (since 10 till 18 months) and coincides with the period of decreasing values of constant potentials of cerebral hemispheres.

Lateralization of behavior (P equals 8 – 10) is clearly expressed only in middle age, in younger and older ages individuals–ambidexters dominate.

The relation between motor asymmetry index and inter-hemisphere difference of potentials was studied in mice younger and older 14 months. For both age groups, linear dependencies between these parameters were determined with correlation indices equal –0.63 and +0.88, respectively. Figure 3 shows correlation indices for different age groups. For the age of 3 – 7 months the index is negative (–0.42); then it gradually increases, approaching zero at 10 – 18 months and in the age of 21 – 22 months reaches the value of +0.53. The basic feature of this dependence is the sign change in linear regression. This transition happens not as a sudden jump, but gradually, due to which in the middle age correlation index value approach zero. This is the period corresponding to the zone of the highly expressed lateralization. Apparently, the change in the regression sign testifies about qualitative functional changes in CNS tissues happening prior to occurrence of the phenotypical signs of aging. Also, the change of the linear regression sign indicates the change of hemisphere dominance.

The above results show that the motor control in mice is not strictly related to activity of one of the hemispheres. Moreover, dominance by this sign is relative and does not exclude inversion. The age dynamics of behavior reflects relations between hemispheres, changing in ontogenesis. If one of hemispheres is damaged, by radiation, for example, to compensate the loss the load on the other is increased. It has been observed that in this case the dominant (left) hemisphere is more sensitive [33].

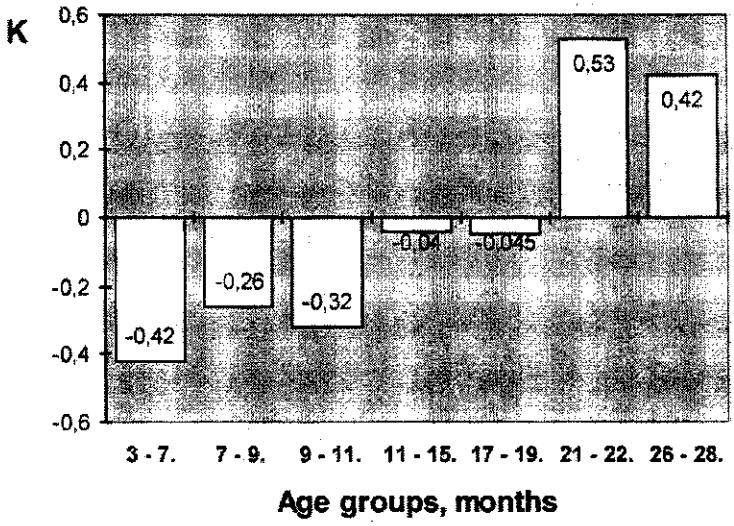

Figure 3. The correlation index (*K*) for the difference in potentials between cerebral hemispheres ad *B* (behavior) for different age groups

The qualitative character of cerebral changes with the age indicate that aging is not cause only by gradual accumulation of damages. The aging is more similar to a phase transition happening in the middle age, when external signs of it are not observed yet. As a consequence, to understand the aging mechanisms, scientists must be interested in this age period.

The age picture of changes in physiological parameters, shown in Figures 1 – 3, clearly indicate nonlinearity of dependencies. Of special attention is not the presence of extremes, but their chronological coincidence, which indicates the presence of peak changes in the age period between 10 and 18 months. Presence of a minimum or a maximum on the curve of age changes in average values may indicate either real nonlinearity of the process or the presence of two subpopulations or more in the test group, which are aged at different rates. In the latter case, even if some individuals show linear function of the aging dynamics, the averaged (reduced) curve may have a peak, the reason for occurrence of which may be determined in longitudinal investigations only.

We have obtained data indicating that besides linear age changes, used for calculation of biological age, there are nonlinear age changes e.g., generally, the aging process is irregular [34, 35].

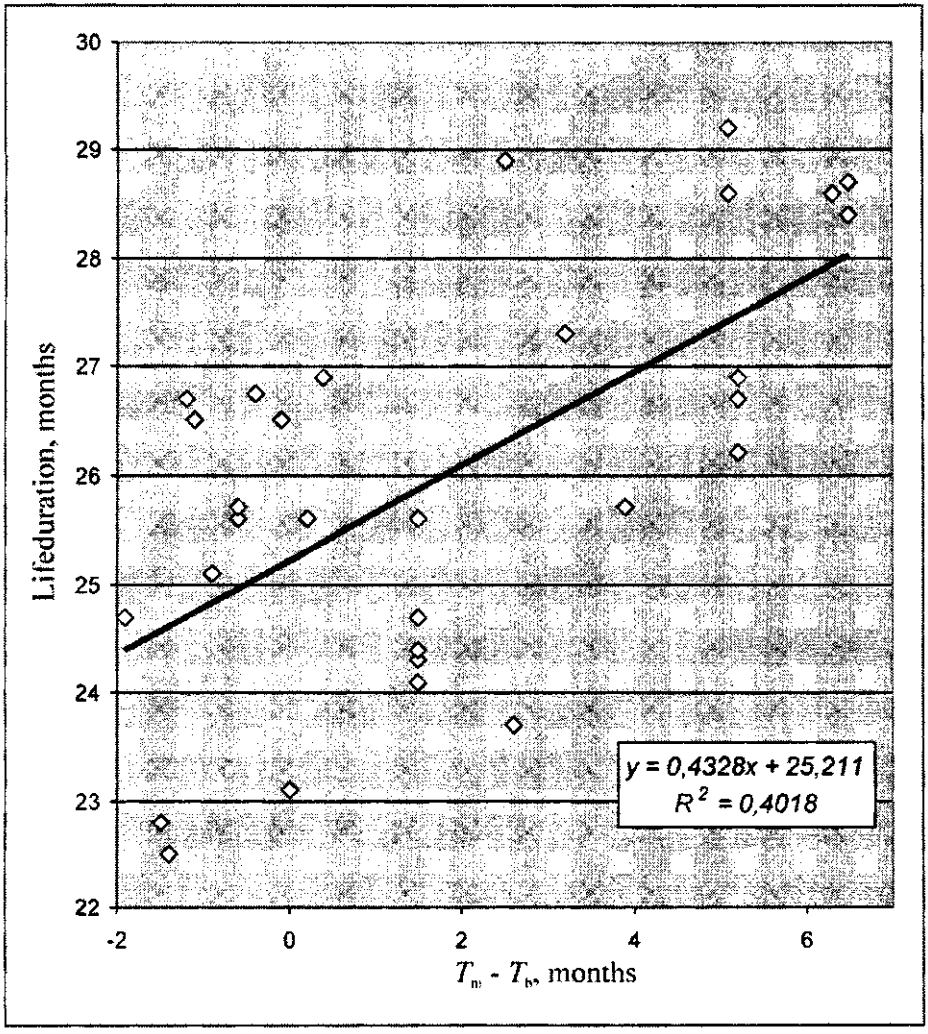

Figure 4. The relation between individual life duration of mice possessing the difference (T_m – T_b), where T_m is the age, at which maximal body mass is obtained; T_b is the age of the maximal lateralization of behavior. The plot shows the curve of linear regression and approximation line

The fact that extreme values observed are related to the age period between 10 and 18 months allows this phenomenon to be considered as non-

random, but as a critical period, which takes sufficient time in late ontogenesis in mice.

In further analysis we have used the following values: age at which body mass reaches its maximum (T_m), and the highest lateralization of behavior (T_b). The correlation analysis as detected a correlation between these values ($r = 0.6$; $p < 0.001$). To our point of view, this correlation may reflect the casual relations, but indicate that both values may be affected by other general factors.

The difference ($T_m - T_b$) shows a linear dependence with the life duration ($r = 0.62$; $p < 0.001$), e.g. this value has a prognostic meaning as a "life duration predictor" (Figure 4). The empirical dependence obtained allows for estimation of the expected change in the life duration after any influence (geroprotectors, radiation of any kind, low-calorie diets, etc.) directly during the experiment.

"Experiment-setting scientist knows well "how", but does not know "why"" – this famous phrase is strictly proved in this case. Meanwhile, primarily we had no task to obtain a good result basing on any theoretical idea (which are multiple) about the mechanisms of aging. The advantage of this approach is in its freedom from mandatory linearity of correlation between age changes and calendar life duration, which is the basis of all method for biological age calculation. This requirement seems to be something like an extrapolation of logical thinking rather than reflection of the reality, which, as follows from the above-said, does not allow simplified ideas about dynamics of the living organism aging.

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WHEN CREATING AN EMBRYO, CELLS ARE SYNTHESIZING TRANSITORY "PERICHROMOSOMAL" DNA-CONTAINING ORGANELLES NECESSARY FOR INTERPRETING POSITIONAL INFORMATION

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Summary

The mechanism allowing cells to translate the DNA linear information into three-dimensional systems of the developing organism is considered. The process is carried out with the help of postulated organelles – printomeres.

Keywords: printomeres, interpretation of positional information in morphogenesis

INTRODUCTION

Morphogenesis is a process of creating the organism on the basis of migrations of embryo's parts, their chemical and mechanical interactions, induction of numerous differentiations and growth. Despite the current progress both in deciphering genes participating in the individual development [1] and in studying the genes involved in segmentation and other morphogenetic events, so far entirely unsolved is the main question – how linearly encoded in the DNA information is recoded into three-dimensional tissue structures.

This communication deals with the role in morphogenesis of postulated transitory nuclear organelles – printomeres. Beside printomeres, in the organism there also exist cognate structures – chronomeres unified by the common for printomeres and chronomeres generic term – redumeres. DNA-containing organelles – redumeres are called so, because, as a result of DNA end underreplication, redumeres become shorter, like telomeres, and lose their genes. Shortage of products encoded by the lost redumere genes causes

replicative senescence. Detailed description of the processes related to the gene loss (phenomenon of "genoptosis") from redumeres is beyond the scope of the present communication; redumere shortening as a primary cause of aging was earlier discussed [2 – 4], see also at the websites:

http://www.chronos.msu.ru/Public/olovnikov_redusomnaya.html;
http://www.medline.ru/thorough/oglav/tom95alx.shtml

Printomere, as any other redumere, is removed outside the bounds of the chromosome (however not extrachromosomal, but so to say "perichromosomal"), a copy of a segment of chromosomal DNA. It has a physical lateral contact with chromosome. Chromosomal original of each redumere, that is a protoredumere, is transcriptionally active only at the moment of creating a redumere, then, during the whole life of the cell clone, protoredumere remains in norm silent. Redumere is created via reverse transcription of RNA transcribed from protoredumere. The set of genes in various redumeres is different, some of genes repeating even within the same redumere. There are different redumeres at different chromosomes of the same cell. Redumeres masked by proteins may be called redusomes. As stated above, redumeres of two types (chronomeres and printomeres) are represented in the organism. Chronomeres control the organism developing and aging in time (temporal events). Printomeres are used to control morphogenesis (spatial events). Each redumere contains relatively short genes coding for small nuclear RNAs participating in the cell regulation. On the whole, redumeres are at the top of the regulatory pyramid of the eukaryotic genome. They are involved into modulation of the activity of structural chromosomal genes and, in particular, into regulation of chromatin configuration.

Each redumere has its own *ori* for replication and a promoter for transcription, this promoter being common for all the genes transcribed in the redumere (this is one of the distinctions of redumere genes from ordinary chromosome ones). Terminal sites of a redumere, playing a buffer role, are designated as acromeres (in order not to be confused with the chromosome ends – telomeres). Acromeres carry a defensive function like telomeres, but acromere sizes are far smaller than those of telomeres. Redumeres have no their own centromere; that is why, the destiny of these postulated perichromosomal organelles totally depends in mitoses on the destiny of chromosomes themselves, serving them as a particular carrier. Redusome "travels" in mitoses on chromosome body like a suck-fish travels on a shark,

though a brainless redusome, as a matter of fact, is devoid of "the liberty of will" of the fish. The transcripts produced on redumeres, to put it differently – on a redusome DNA, belong to small nuclear RNAs (snRNAs). At present, an important regulatory role of multiple and various snRNAs participating in RNA interference, in regulation of DNA methylation and some other important events is known to be getting clearer. Also, in the bulk of snRNAs is a fraction of transcripts coded by printomeres, namely printomere RNAs are assumed to work at the top of the regulatory pyramid of the eukaryotic genome.

PRINTOMERES AS A BASIS OF REGULATORY MORPHOGENESIS AND CELLULAR MEMORY OF THE ACHIEVED STATE OF DIFFERENTIATION

Printomeres, i.e. one of the two variants of redumeres (chronomeres and printomeres), are functioning in dividing cells and are of special importance in embryo's organogenesis helping to convert linearly encoded in the genome information into three-dimensional structures of a developing organism (printomere mechanism of interpreting positional information). Positional information is the information obtained by the cells in some still experimentally non-determined way about their position with regard to the whole (e.g. regarding a source supplying naïve cells with the inducer of differentiation) [5 – 7]. The participation of printomeres in interpretation of positional information is carried out owing to unpacking the strictly determined set of chromosomal segments (protoprintomeres); this happens under a direct or indirect influence of a morphogenetic inducer. Its concentration at the opposite poles of the morphogenetic field of the cells which have to make a decision is "opposed", namely a concentration is high near the source of inducer and is low at the opposite end of the morphogenetic field. This cellular field itself is usually not very big, about a hundred practically identical cells competent to perceive the inducer. As assumed here, owing to this, the cells are able to make a decision according to the binary principle realizing a choice from two alternative possibilities. The essence of the exercised by the cell binary choice is as follows. The cell can unpack one or two alternative protoprintomeres. Immediate unpacking of both protoprintomeres occurs only at the inducer source. On the contrary, unpacking only one of the two protoprintomeres occurs at a low concentration of the inducer near a cell.

Binary decision-making can be exercised by a cell having at least two "competent" protoprintomeres specific for just a given differentiation. The same is true for the cell determination which is nothing but a latent, not proving itself at once, cell differentiation. Each unpacked protoprintomere, remaining a segment of the chromosomal DNA, serves as a template used in the course of creating proper printomeres. The cells having opposite positions at the axis of the morphogenetic gradient will acquire the opposite states of differentiation, out of the two possible ones. How exactly is the choice of the adequate protoprintomeres exercised in this process? Let us designate the two considered protoprintomeres as resistant (R) and non-resistant, namely sensitive (S). The essence is unchanged, whether these R or S are at the same or at the different chromosomes of a cell. They are compacted with "seals" that could be an object of "unsealing" and activation initiated by inducer of differentiation. Let us assume that in cells surrounded with a high or a low concentration of morphogen (inducer of differentiation), there created an increased or, respectively, decreased level of a factor that activates protoprintomeres.

The role of such factor could be performed, e.g. by NO-radical. Participation of nitric oxide in morphogenesis and differentiations, from formation of fruit fly to the bone marrow operation, is widely described [8 – 10]. Why NO and some other factors that are freely spread across the tissue are important in interpreting by cells their positional information in morphogenesis? This question still remains elusive. In case of absence of other possibilities, cells could "seal" protoprintomeres, for example, with participation of lipoproteins and unpack them using destruction of such seals with the help of free radicals. In the absence of other variants, this could be used by cells for nonspecific unpacking of chromatin domains. Though cells, apparently, have more elegant pathways. One of them, for example, is based on activation of guanylate cyclase by nitric oxide. It is well known that, as a receptor of nitric oxide, guanylate cyclase acts as follows. When NO comes into contact with iron of heme, the atom of iron creates access of GTP as a substrate for catalytic site of the enzyme. Hence, under the influence of NO, the enzyme converts from inactive to active state, implementing the function of NO perception as a signal molecule. Thus, arrival of NO to the cell can result in synthesis of cGMP by guanylate cyclase, and this secondary messenger launches a cascade of events resulting in decompaction of certain segments of heterochromatin. It can be mentioned that the influence of cGMP on morphogenesis and differentiation of cells in rodents which occurs with

participation of cGMP-dependent protein kinase type II [11, 12]. One can assume that the above mentioned protoprintomeres of the R and S types should become unpacked and activated, for creation of printomeres, only under opposite levels of cGMP-dependent modification of proteins bound to them in heterochromatin. The fulfillment of this requirement is necessary for printomere-related interpretation by cells of their positional information in the morphogenetic field, when primordium of the organ is being created.

The finely tuned reading by cells of morphogen gradient concentration levels can be performed, probably, if all inducers of differentiation have the following three properties. They, by themselves or through some messengers generated under their control, should be able to:

1) easily propagate across the tissue, including membrane barriers;
2) penetrate into a nucleus;
3) decompact heterochromatin-containing protoprintomeres.

As is known, differentiation of some cell types may be induced by various combinations of cGMP, cAMP, retinoic acid (RA), NO, etc. Some inducers indeed are able to freely migrate between cells. This is true, for example, for RA, NO, prostaglandins and some others inducers. Some protein inducers, probably, exert their activity also only through interaction with such freely diffusable compounds.

As to NO, it acts, for instance, as a mediator of the effects of estrogen in bone, it is involved in mediation of the effects of mechanical loading on the skeleton where it acts along with prostaglandins, to promote bone formation [13]. Though nitric oxide was first identified as a unique diffusible molecular messenger in animals, it plays an important role in diverse physiological processes in plants [14 – 16]. In particular, in plants NO acts as developmental regulator, activates various defense genes; in plants NO can be produced by nitrate reductase [17].

Another widespread inducer of differentiations, namely retinoic acid, or RA, plays an important role of an organizer of segmentation, besides RA regulates the expression of many proteins. The RA supply is performed via stage of diffusion across the tissue [18, 19]. RA is integrated in both anteroposterior and dorsoventral patterning processes [20]. It was shown that the pattern of RA metabolic enzymes controls the actions of RA; and developmental abnormalities result when this pattern breaks down [21]. The localization of enzymes, which metabolize retinoic acid, helps to control the

local action of this acid, and one can assume that a pattern of those enzymes is, among other factors, also under control of printomere-dependent reading by cell of their positional information. At the same time, retinoids are able to attenuate the effect of another inducer; namely, nitric oxide production was decreased in the presence of RA. That is achieved by declining the inducible NO-syntase [22]. Interestingly, another differentiation agent, 1,25-dihydroxyvitamin D3, may also modulate endogenous nitric oxide production [23]. Interaction and competition of protein FGF and RA pathways, which are mutually inhibitory, provide a global mechanism that controls differentiation during vertebrate body axis extension [24]. The existence of sequentially deploying programs of retinoic acid synthesis in the migratory cell groups of the developing organism [25] is a probable consequence of the printomere-cell-migration cycle functioning considered below in the present communication.

Prostaglandins, another class of inducers, are potent lipid hormones that activate multiple signaling pathways resulting in regulation of some differentiations, including cells of bone and bone marrow [26 – 28]. Lipophilic prostaglandins are also freely diffused in tissues through the membrane barriers [29].

The immense variety of differentiation inducers in morphogenesis is not required at all. One can suppose that the relatively small number of inducers is enough, if there is the sufficient set of protoprintomeres, whose auxiliary proteins are responsible for perception of inducible signals. The task of perception at the protoprintomere level can be solved, if the choice (during unpacking of protoprintomeres according to the binary principle, using R and S types of the above mentioned protoprintomeres) is carried out in such a way that proper pairs of protoprintomeres R and S do perceive the signal to unpack only in the strictly defined zones of the inducer concentration gradient. That is, each certain pair of protoprintomeres R and S is able to interpret positional information for the cell only within the limits of its zone of the inducer gradient concentrations. Such requirement can be accomplished, if outside the specified zone, i.e. under an abnormally high level of inducer, the perception of this morphogen is interlocked. Blocking is possible, for instance, when an excess of ligand prevents formation of dimers of receptor proteins in cell, which were necessary for activation of protoprintomeres.

The strictly defined interval of the cell residence in their zone of morphogenetic field is as important as a local concentration of inducer, because an abnormal increase of time of cells' exposure can promote accumulation in the cell of factors acting on protoprintomeres.

The occurrence of morphogenetic field of the distinct sets of printomeres, as important regulators of nuclear genome, in similar cells is capable of eliciting both intracellular changes, as well as changes in sets of the cell surface adhesion molecules. These molecules, influencing on intercellular aggregation, participate then in creation of multicellular novel structures in the course of organogeneses.

Returning to the role of NO, one can take into account that nitric oxide is able to utilize several independent signaling pathways when inducing gene expression [30]. Hence, it is possible to assume that different types of protoprintomeres could be activated under the influence of non-identical NO-dependent auxiliary factors.

For the purpose of morphogenesis, it is of great importance that NO-radical, for example, is relatively stable (its life time is several seconds) and is able to penetrate through the cell membranes. That is why just such molecules as nitric oxide could spread freely along the morphogenetic field. It is important to stress that by the time, when the cell acquires the state of competence, the corresponding segment of heterochromatin has changed its configuration getting ready for a possible act of unpacking of the proper domain.

A molecule of NO is known to exert an anti-proliferating action. This property is very important, since displacements of cells in morphogenetic field prior to the printomere creation and before recognition by cells of their positions, within concentration gradient of inducer, would make impossible interpretation by cells of their positional information.

Near a source of morphogen, where its concentration is especially high, the cell will unpack both protoprintomeres, namely R and S. So, in such cells both R and S printomeres can be created. However, at the far, opposite pole of the same morphogenetic zone, the cells will be able to unpack only one protoprintomere S, since it is sensitive even to low concentration of unpacking factor. Such kind of binary choice is made by different protoprintomeres of the same cell. Finally, the cells, both near the source of morphogenetic gradient and at the opposite end of the morphogenetic field, primarily consisting of identical cells, will acquire quite different cytophenotypes. Owing to this, one multicellular structure will be created near a source of a morphogenetic inducer, while at the opposite pole another structure will be formed. The considered printomeric mechanism (or otherwise redumeric one) of regulating organogeneses and creating various cytodifferentiations should take place in any morphogenetic fields, be realized under the influence of various inducers,

as well as at different orientations of the axes of inducers' concentration gradients.

Morphogenetic movements are typical of embryogenesis. They are migrations of individual cells, as well as shifts of groups of cells relative to one another. They are usually accompanied by appearance of local mechanical tension or relaxations in tissues [31 – 35]. One can assume that most, if not any, significant morphogenetic (i.e. shaping) movements in embryogenesis are every time the consequence of just acquired novel specificity of the corresponding cells, which is controlled by their new set of nascent printomeres. Creation of the next set of printomeres in nuclei, acquisition by cells of the corresponding novel printomere-dependent specificity and the following morphogenetic movement (that is a mechanical event) constitute in the aggregate the universal "mechano-printomere cycle" of morphogenesis. Reiteration with renewal (it occurs due to the appearance of new sets of printomeres or new combinations of former variants of printomeres) represents the basis of any morphogenesis, enabling the developing organism to generate more and more complicated shapes. These mechano-printomere cycles are crucial for translating the linearly encoded genome information to the 3D-shape of multicellular structures of organs and the whole organism.

The considered mechanism is based, as a matter of fact, on the idea that cells, when forming a multicellular organism, create a particular "situational genetic program" of development for themselves. It arises on the basis of an ordinary chromosomal genome. However, it is represented by small "perichromosomal" linear copies of segments of nuclear genome, which are different in the cells of distinct differentiations. Owing to printomeres, development of most eukaryotes gets a huge flexibility in that respect that the process of forming three-dimensional multicellular structures can be realized situationally rather than through a rigid program when the destiny of each cell is predetermined. This means that a non-differentiated cellular group can create for itself a certain set of instructions, in the form of DNA fragments, depending on where these cells (i.e., a part of the whole developing organism) took up their position.

One more great advantage of using printomeres is that they facilitate to resolve an otherwise hardly solved problem of "regulation of regulators" and the coupled problem of the cell memory, i.e. the memory about the achieved state of the cell specialization maintaining afterward in mitoses. The essence of the problem of regulation of regulators is as follows: in order to produce any regulatory RNA or protein, it is necessary to have an earlier regulatory factor

controlling them, whereas such factor should have even earlier predecessor, and so on. Allocation of the copies of chromosomal segments that is the formation of printomeres (or redumeres in general) outside the chromosomal DNA does solve the considered problem at once. Printomeres become, so to say, top regulators of other regulators of a given differentiation. However, these top "managers", being able to replicate, inasmuch as they are molecules of DNA, don't need to keep any earlier regulators which would control them.

Printomere is, as a matter of fact, an instruction necessary for realizing one or several small steps in individual development. The same genetic texts are surely to be present in the chromosomal DNA, however, there these instructions, according to the hypothesis, keep silence, being recorded in those segments of chromosomes, which spend nearly the whole ontogeny in a tightly compacted and transcriptionally inactive state. They are transcribing only at the moment of the very onset of differentiation, in order to their transcript should then be used by a reverse transcriptase for synthesis of DNA copy, which afterwards lands at the surface of an already silenced chromosomal original, or chromosomal "nest" [3]. This copy is just a redumere (otherwise, printomere or chronomere, dependently on its function in a cell). A set of printomeres in a differentiated cell can be viewed as a "situational genetic program", because the particular set of these DNA fragments originates in a competent cell *de novo* according to the position which it takes in a developing organism that is as a specific situation requires. Printomeres, so to say, "adapt" such cell to corresponding environment, endowing it with the proper printomere armament, which is suitable to specific situation, giving possibility to arrange proper syntheses. That is why the set of corresponding redumeres is a peculiar "situational genetic program" of a differentiated cell. This does not at all mean that the cells can easily change their obtained differentiation; the cases of *trans*-differentiations in norm are known, but they are rather scanty. Appearance of a particular set of redumeres in the cell more frequently narrows the range of its future possible specializations.

In the course of cell proliferation bearing redumeres in the nuclei, there occurs, due to end underreplication of these linear DNA molecules, a consecutive loss of redumere genes that causes a gradual decline in the cell activity resulted in a cellular senescence. The loss of genes from shortening redumeres, including printomeres and chronomeres, can be designated as the genoptosis. The term "genoptosis" is composed from words *gene* and *ptosis*, i.e. fall or abscission (by analogy with the names known in the literature – apoptosis and phenoptosis). Thus, genoptosis is the consecutive and inevitable

loss of genes from the ends of "perichromosomal" DNA linear molecules. Thus, this loss is determined by the redumere shortening, whereas the shortening itself may be caused by several mechanisms (DNA end underreplication, DNA end underrepair synthesis, and the so-called "scrupting"); these mechanisms are considered in detail in several publications [2 – 4].

Redumeres are important both for highly differentiated cells and stem cells. As is known, there are various tissue- and organ-specific stem cells [36 – 38]. These regional stem cells are responsible for replacement of differentiated cells within continuously regenerating tissues, such as blood, skin, intestine, etc. For example, hematopoietic stem cells can differentiate into different blood lineages, but normally they produce only cells of the blood system, rather than tissues of liver, heart or brain. Correspondingly, muscle-derived stem cells have been proven capable of regenerating just dystrophic muscle [39]. Taking apart any artificial interventions, the organ- and tissue-specific stem cells give rise exactly to those tissues, organs and systems, in which they normally reside [36, 37, 40]. This means that stem cells already have certain specialization. Though regional stem cells are non-differentiated cells in comparison with their progenitors, such as spleen lymphocytes or hypothalamus' neurons, they nevertheless should differ by their properties from their remote ancestors, e.g. from the cells of a blastocyst. This distinction of regional stem cells from the progenitors and ancestors should be persistently kept in the course of numerous mitoses by a corresponding mechanism of cell memory. The role of such mechanism is probably also played by redumeres, though by their specificity (i.e. by sets of redumere genes) the redumeres of stem cells must differ from other cells. An important distinctive trait of stem cells may be the role of telomerase necessary for maintaining the length of acromeres (buffer sites at the ends of redumeres). This is necessary to prevent redumeres from losing their genes, namely to prevent genoptosis during long existence of stem cells. Telomerase, extending telomeres and acromeres of redumeres, resists to the shortening of redumeres. Though divisions of stem sells are relatively rare, the ends of redumeres during replication are vulnerable, especially under stress conditions. If a stem cell generates its progenitors under the stress condition then the protection of redumere's termini (acromeres) by telomerase is of special importance.

"REDUMEROGENETICS" OR "REDUGENETICS" AND EPIGENETICS ARE THE TWO COMPLEMENTARY REALMS OF REGULATING CHROMOSOMAL GENES

When summing up the role of printomeres (and broader, the role of redumeres) as representatives of the above-mentioned "situational genetic program" in the individual development, the following should be accentuated. An irreversible change in the range of cellular alternatives of any cell clone into which it can normally be transformed during differentiation, usually lead to a successive narrowing of the spectrum of possible specializations. This corresponds to the following succession:

totipotency → pluripontency → oligopotency ..→ terminal differentiation.

As is suggested, the most changes in this sequence are maintained by redumeres, which provide interpretation of positional information, cell memory keeping in many mitoses and solution of the problem of regulation of regulators. According to a current understanding, differential gene activity resulting in phenotypic diversity of cell types, is a consequence of work of the epigenetic mechanisms such as DNA methylation, binding regulatory factors to definite genes in order to alter their activity and, besides, some covalent modifications of chromatin proteins, e.g., their methylation, acetylation, etc. [41 – 45].

Not denying the confirmed role of epigenetic factors, in the context of the present work, along with epigenetics, it is reasonable to state the existence"redumerogenetics", or "redugenetics", for short. This statement is based on the following. Heritable changes within a cell clone, which are not based on changes of nucleotide sequences in cells (a classic example of them is selective methylation of cytosines in DNA) are usually referred as a realm of epigenetics. Under the term "redumerogenetics" one can realize the processes associated with the appearance of redumeric DNA and the sequential loss of genes from redumeres that lead to numerous biological changes in cells and organisms. Redumeric DNA is a perichromosomal, rather than a chromosomal one. To the realm of the redumerogenetics and redumere biology as a whole, one can attribute the control of morphogenesis, as well as regulation of cell determination and differentiation, aging and, probably, some events in cancer cells when the latter are controlled by alterations in sets of redumeres. Thus,

redugenetics does not reduce to epigenetics, since it considers the events caused just by changes in sequences of DNA molecules. Moreover, some epigenetic changes, including those occurring in the course of aging (e.g. an experimentally observed decline of the chromosomal DNA methylation with aging of the individual) can themselves turn out to be caused by the alteration in redumeres. These alterations are the loss of genes from redumeres, i.e. the redumere "genoptosis", as well as even the fall out of the whole redumeres from their chromosomal nests. Thereby, redumeres themselves can initiate and modulate the epigenetic states of eukaryotic genome.

CONCLUSION

Thus, the proposed solution may answer the most ancient mystery of embryology – how the linearly recorded information is being translated in the course of individual development to 3D–shapes. Accepting the printomere mechanism working in morphogenesis, one can suppose that the changes in the course of evolution in the properties of protoprintomere segments of a genome (i.e., changes in structure and in number of protoprintomeres' genes) should be one of the most significant events in creating morphological diversity of eukaryotic organisms. Many processes are well known in the individual development; nevertheless, the basic direction of morphogenesis is origination of printomeres and migration of cells delivering them to a rendezvous with the inducer in order to perform generation of next printomeres and novel differentiations. Anatomy and physiology of a developing organism originate owing to the successive mechano-printomere cycles. Printomeres and cytomechanics are those two wings on which the bird of morphogenesis shoots upward creating a new life. Printomeres are necessary for a morphogenesis, but being simultaneously redumeres and being shortened in mitoses, they carry in themselves the primary cause of aging of dividing cells.

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MITOCHONDRIA: OXIDATIVE STRESS, MITOCHONDRIAL DNA MUTATION, PATHOLOGIES, AGING AND APOPTOSIS

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The intensive studies of free radical (FR) role in various branches of biology and medicine, initiated over 40 years ago in foreground works by Academician N.M. Emanuel and hid school, discovered physicochemical origin of FR interactions with the living system components at all organizational levels and formed the basis for searching for effective ways of counteracting such phenomena as aging, stress state, radiation and degenerative diseases, and many other pathologies, to which FR often make a decisive contribution.

In recent 15 – 20 years, an immense number of works [1] was devoted to the investigations of the oxidative stress and its effect on different organizational levels of the entire organism. However, now we would like to focus attention on the foreground of the events defining development of the oxidative stress in cell, i.e. on cellular organelles, where main reactions of energy metabolism proceed, which are ***mitochondria***.

1. OXIDATIVE STRESS

Traditionally, mitochondria have been considered as "*electric power plants*" of the cell, which produce adenosine triphosphate (ATP). For more than five decades many investigations carried out in organelles were focused on explanation of molecular events related to ATP synthesis via oxidative phosphorylation and biogenesis of mechanisms of this complicated process.

In time, when the fundamental discoveries were made and the studies of these outstanding multiphase energy production processes proceeded, recently, many biologists and biomedics have understood that mitochondria are also invoked in other processes, also extremely important for the life of higher

organisms. Step by step it have become clear that, admiring as it may seem, the events as ***oxidative stress, cell death, aging and mitochondrial diseases*** are associated, partly, at least, with oxidative phosphorylation, structural wholeness of mitochondria and informational identity of their genetic apparatus – mitochondrial DNA (mtDNA). The understanding of this interrelation awoke interest of many scientists to investigations of molecular and biochemical mechanisms of the mentioned pathologies and to finding the ways of their treatment.

Being the main intracellular source of energy delivered in the form of ATP, mitochondria absorb over 90% of total oxygen, consumed by cells of higher animals and a man. Under normal physiological conditions about 1 – 5% of oxygen consumed by mitochondria is transformed to superoxide anion radical ($O_2^{\bullet}$-), which is quite inert itself, but represents a precursor of harmful (or potentially harmful) formations, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). All these compounds are usually denoted as reactive oxygen species (ROS) or ***active oxygen species (ROS)*** [2 – 4]. Mitochondrial chain of electron transport (MCET) is an intensive AOF source. The higher MCET functioning level is, i.e. ATP production, the higher ROS generation level is.

The possibility of oxidative stress development depends on the production level of each ROS, the rate of their relative neutralization and efficiency of casualty action on repairing cell systems. The imbalance of these factors may induce a serious dysfunction of mitochondria. Each individual ROS has its own production and detoxication mechanism and self reaction profile with biological targets that defines specificity of the pathological effect, which depend on shape of invoked ROS.

Under normal physiological conditions, these hazardous secondary products of respiration are usually opposed by protective systems of mitochondria, including a system of antioxidant enzymes: manganese superoxide dismutase (MnSOD), catalase and glutathione peroxidase (GPX), and a complex of endogenic lowmolecular antioxidants. However, both under normal physiological conditions and specially under functional overloads, aging of cells and tissues, and some pathological states, a significant part of ROS can "*obviate*" antioxidation systems and induce oxidative damages of macromolecules (polysaccharides, lipids, proteins, nucleic acids) and their supermolecular complexes of various complexity (RNP, DNP, LONP, LNP, LVP, cellular and subcellular membranes, cellular "*skeleton*" components,

etc.), i.e. induce the oxidative stress state [5 – 11]. As a consequence of these and other damages, bioenergy functions of mitochondria decrease with age and become deficient in patients with mitochondrial diseases, and in critical situations may be invoked in programmable cellular death – ***apoptosis***. All these phenomena are the integral result of both ROS production increase in the chain of electron transport (CET) and insufficient ATP production in mitochondria [4, 11, 12].

1.1. Mitochondrial respiratory chain

Mitochondrial respiratory chain consists of four enzymatic complexes: NADH–coenzyme (CoQ) – reductase (complex I), succinate – CoQ – reductase (complex II), $CoQH_2$ – cytochrome c – reductase (complex III), and cytochrome c – oxidase (complex IV). NADH or $FADH_2$ which are continuously produced by various *dehydrogenases* at biological oxidation of monosaccharides, amino and fatty acids are the sources of electrons for complex I or II of the respiratory chain. Coenzyme Q shuttles electrons between these two complexes and complex III. Finally, molecular oxygen receives electrons from complex IV. Mitochondria produce ATP due to proton gradient, generated by respiration, and its use for ADP phosphorylation by FoF ATPase (complex V, Figure 1).

The respiratory chain transmits electrons from reduced substrates to oxygen and uses the difference in redox potential of transmitters for "*pumping*" protons through the internal membrane forming the gradient of proton electrochemical potential. This gradient is used for starting up ATP synthesis with the help of ATP-synthase in the complex V (Figure 1). This gradient of electrochemical proton potential is summed up from membrane potential and pH gradient. Several defects in complexes I, III or IV (similar to complex V) occur as a resull of the oxidative stress which induces mutations in mtDNA genes. In turn, the latter encode polypetides functioning in in the composition of these complexes (Figures 1 and 3). These defects disturb respiration, reduce the gradient of mitochondrial proton potential and prevent ATP synthesis in mitochondria even when ATP-synthase itself is not invoked [13]. On the contrary, mutations in a single ATP-synthase gene will not affect the ability of mitochondrial respiratory chain to create electrochemical proton potential gradient, but will prevent ATP synthesis (Figure 1).

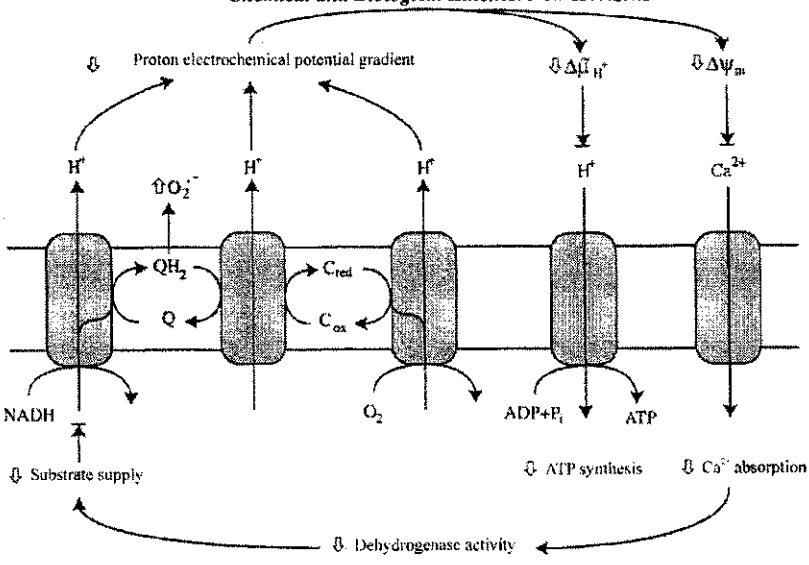
⇩ Proton electrochemical potential gradient
⇩Δμ̃ H+
⇩Δψm
H+
⇧O2·-
H+
H+
H+
Ca2+
QH2
Q
Cred
Cox
NADH
O2
ADP+Pi
ATP
⇩ Substrate supply
⇩ ATP synthesis
⇩ Ca2+ absorption
⇩ Dehydrogenase activity

Figure 1

Complex I (NADH:ubiquinone oxidoreductase)	Complex III (Ubiquinone:cytochrome C oxidoreductase)	Complex IV Cytochrome C Oxidase	Complex V (F_0F_1 ATP synthetase)	Ca^{2+}-transporter
The quantity of mtDNA-encoded subunits				
7	1	3	2	-

Figure 1. The influence of mtDNA mutations on oxidative phosphorylation and calcium absorption. $O_2^{\bullet}$- is superoxide; Q is ubiquinone; QH_2 is ubiquinol; C_{OX} is oxidized ferricytochrome c; C_{RED} is reduced ferricytochrome c: $\Delta\mu_{H^+}$ is the motive force of the proton; $\Delta\psi_m$ is the mitochondrial membrane potential; P_i is the phosphate (adapted from [13])

1.2. Complex I as the source of superoxide radicals.

There are two main areas of respiratory chain, where ROS generation is observed. One area represents complex I [14 – 16] and another is complex III [17]. Under normal physiological conditions, one mitochondrion of rat liver can produce about $3 \cdot 10^7$ superoxide anions per day [6].

According to modern ideas, the greatest contribution into superoxide production is made by complex I which represents the first local zone of oxidative phosphorylation (Figure 1). Complicated, perfect enzymatic complex consists of 43 subunits for mammals, 7 of which are encoded in mtDNA, several prosthetic groups, including FMN and, at least, 7 iron–sulfur clusters, and several coenzyme Q molecules bound to proteins. Each of Fe–S clusters is a single-electron donor transmitting electron to oxygen or one of three zones containing three different bound types of coenzyme Q. since 7 of 13 structural genes in mtDNA encode polypeptides for the complex I, it with high probability may be subject to functional changes [19]. Apparently, this is the place of electron leakage and formation of superoxide anion radicals. Obviously, CET appears slightly "*unsealed*" in this point (Figure 2 [18, 19]).

Recently, a group of Italian authors [19] have suggested an interesting model of particular CET zone localization in the complex I, where electrons leak to the environment. According to these results, the use of a combination of specific inhibitors affecting three different areas of the complex I linking quinones [A, B, C (Figure 2)] intensified superoxide generation indicating that the oxygen recovery zone is located above the areas of the complex linking quinones (Figure 2). Most likely, FeS – cluster – N2 is the donor transmitting electrons to the first molecule of bound ubiquinone [20, 21]. This site (N2), apparently, is also the donor transmitting electrons directly to oxygen or via single-electron recovery of several exogenic quinones [DB, Q–1, Q–2 (Figure 2)]. In line with this hypothesis, the studies of depleted CoQ and recovered mitochondria show that endogenic CoQ is not required for superoxide generation. Therefore, exogenic quinones were preferably recovered in ubiquinone pool, but low though significant part of quinone molecules may directly react at the place of endogenic quinones bound to N2 (Figure 2 [19]).

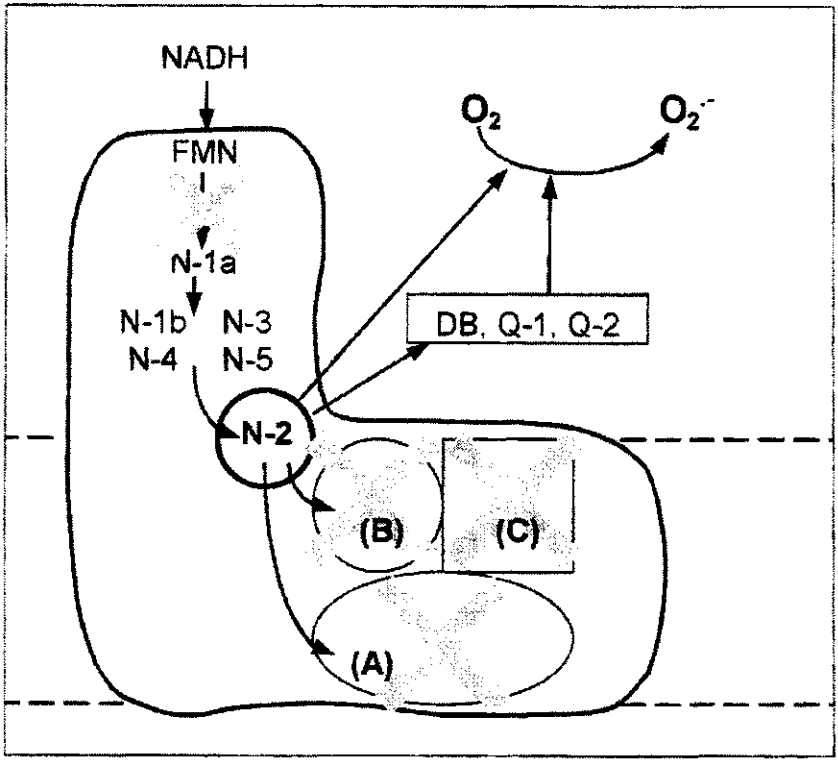

Figure 2. The model of electron transport and an area of the complex I synthesizing superoxide. The scheme uses the Degli Esposti model [43] and depicts FeS-cluster N2 as the source of electrons for bound ubiquinone (Site B) and ubiquinone molecule delivered from the pool (Site A). These two "semi-reduced" ubiquinones (ubisemiquinones) dismutate so that the Site B contains oxidized ubiquinone, and reduced ubisemiquinone (ubiquinol) shifts towards the Site C, from which it is extracted to the pool. The effect of various inhibitors (marked by × in the scheme) and acceptors (FMN, N-1a, N-1b, N-3, N-4, N5) are conjugated with FeS-cluster of N2 as a single electron source for oxygen or exogenic quinones (DB – decylubiquinone, Q–1, Q–2, etc.) which, in turn, implement single-electron reduction of oxygen

Structural changes in the complex I may increase ROS production. This fact was determined by some defects in the complex I, which were induced by nuclear DNA mutations [22, 23], but it is reasonable to suppose that mitochondrial DNA defects affecting complex I, for example, three mutations which induce *Leber hereditary optical neuropathy* (LHON [24]), are even stronger accompanied by increased ROS production.

Complex I is to the highest extent subject to the age changes that leads to a strict limitation of electron transport rate and corresponding increased ROS generation, which are considered as pathogenetic factors of many diseases, aging and apoptosis [19, 25]. As mentioned above, this is because ROS are able to damage vitally important cell structures, especially membranes, where they induce POL processes and messanger macromolecules – proteins, RNA and DNA.

Affected by SOD, radical ($O_2^{\bullet}$) dismutates to hydrogen peroxide (H_2O_2), which then in the presence of bivalent iron (Fenton's reaction) transforms to highly chemically active hydroxyl radical ($^{\bullet}OH$).

In addition to these toxic CET products stipulating the oxidative stress, in the internal mitochondrial membrane enzyme *monoaminoxidase*, being the important source of H_2O_2, catalyzes oxidative deamination of biogenic amines that promotes an increase of stable ROS concentration within the mitochondrion and cytosol matrix [26]. Moreover, ROS reacts with nitrogen oxide inside mitochondria thus increasing concentration of *peroxynitrate* (ONOO– [27]) which has the damaging action.

Step by step we obtain the general picture, according to which subcellular signaling system associated with ROS and AON (active nitrogen species) is important for cellular physiology and pathology. It is obvious that changes in NO or $O_2^{\bullet}$- flow velocity may modify this signaling system, in some cases, creating conditions for development of various pathologies [177].

Concluding this section, we should outline that ROS production essentially the function of oxygen consumption. As a consequence, increased metabolic activity of mitochondria provides for a potential possibility of oxidative stress development in the cell as its antioxidant protection is weakened. Moreover, ROS production is more intensive in many pathological states related to damages of the respiratory chain. Since *mitochondrial DNA*, without which normal oxidative phosphorylation is impossible, is localized directly at the respiratory chain permanently generating ROS, it is more subject to oxidative damages rather than nuclear DNA. The well-studied various mutations of mtDNA relate to aging and progression of both the so-called

mitochondrial diseases and such known diseases as diabetes, cancer, cardiac arrest and many other, as well as to programmable cellular death – *the apoptosis*.

2. MITOCHONDRIAL DNA MUTATIONS

One of the most important targets of oxidative damages in the cells is DNA. First of all, this is because DNA damages are able to accumulate, because other damaged targets such as proteins and membranes can be eliminated during cellular *turnover*. Though DNA may sustain many types of damages, it is suggested that oxidative damages among them are of the prime importance. According to calculations, a cell sustains 10,000 oxidative impacts per day [28]. Oxidative damages of cellular DNA are of special importance. They are related to:

1. Damages of nuclear DNA and its repair mediated by *poly(ADP)polymerase–1* activation [29, 30];
2. Damages of telomeric DNA and their contribution into telomer-controllable cell aging and pathologies [31];
3. Damages and accumulation of mutations in mitochondrial DNA significantly exceeding the level in both nuclear and telomeric DNA [32].

2.1. Genetic map, informational capacity and structural features of mtDNA.

The oxidative phosphorylation system contains over 80 polypeptides, among which only 13 are encoded in mtDNA. All other proteins, permanently resent in the mitochondrion, represent nuclear gene products, which are translated in cytoplasm and then transported into mitochondria [33]. In the synthesis and assembly of enzymes for mtDNA replication, transcription [34], translation and repair [35] mitochondrion depends on nuclear genes. The synthesis of proteins and enzymes participating in heme synthesis, substrate oxidation in tricarboxylic acid cycle (Krebbs cycle), fatty acid degradation by β-oxidation, partly in the urea cycle, and apoptosis control is controlled by nuclear DNA genes, though they are localized and operate in mitochondria [32].

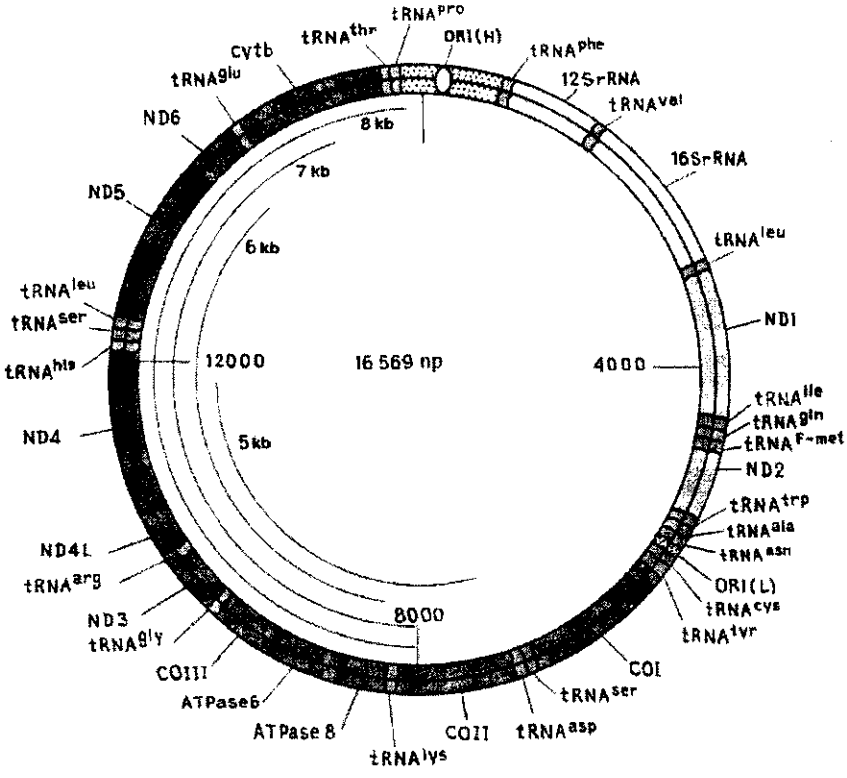

Figure 3. Human mitochondrial DNA. Genes encoding proteins are highlighted by dark-gray. The encoded three large subunits of cytochrome c – oxidase (COI, COII, COIII), cytochrome b (cyt *b*), subunits 6 and 8 of ATPase complex and 7 subunits of NADH-dehydrogenase complex (ND). Genes encoding various rRNA are highlighted by light-gray, and tRNA genes – by narrow light-gray bands.

The areas of replication initiation (*origins*) [ORI (H) and ORI (L)] are highlighted by points (adapted from [212]).Large-scale deletions of 5 – 8 KB peculiar to subjects with mitochondrial diseases and old invalids are most often localized in the approximate zone from 8000 to 16000 nucleotide pairs. Some deletions are schematically depicted by arcs inside the circular mtDNA

The nucleotide sequence of human mtDNA was published in 1981 [36]. It represents a two-strand, circular molecule with 16,569 pairs of nitrous bases (np). In contrast with nuclear DNA, mtDNA has no introns (Figure 3). Shift loop (D-loop) is the unique noncoding zone (1.1 KB, approximately) in human mtDNA, which contains and initiating area (On) for mtDNA replication and transcription promoters for both heavy (H) and light (L) mtDNA strands. As transcription of both mtDNA strands is implemented, polycystron transcripts are synthesized, from which 13 mRNA, 22 tRNA, 2 rRNA – 16S and 13S, respectively [50], are released. In H-strand 2 rRNA, 14 tRNA and 12 polypeptides are encoded, whereas in L-strand 8 tRNA and 1 polypeptide are encoded (Figure 3). Together with polypeptides encoded in nuclear DNA, 13 polypeptides encoded in mtDNA form respiratory enzymes of complexes I, III, IV and V in mitochondria. Only complex II is fully encoded in nuclear DNA. In the gaps of the sequence encoding polypeptides 22 tRNA genes and 2 rRNA genes are present, which products are sufficient for synthesis of proteins in mitochondria (Figure 3). All genes encoded in mtDNA are important for mitochondrial respiration and oxidative phosphorylation, and in future any mutation of mtDNA which disturbs expression of these genes may cause insufficiency of the energy metabolism.

2.2. mtDNA susceptibility to damages and mutations.

Mutations in mitochondrial genome are of special attention, because they occur at a rate 16 times higher than in nuclear DNA in liver in rats (1 damage per 8,000 ps versus 1 per 130,000 ps [6]). ROS plays the prime role in mtDNA damage. Among nucleotide oxidation products the greatest quantity of ***8-hydroxy-20-deoxyguanosine*** (8-OH–dG) was obtained, which presence in DNA is usually used as the indicator of DNA oxidative damages [6]. This extraordinary "*ability*" of mtDNA to mutagenesis was associated with its several specific features. In contrast with nuclear DNA, mtDNA is a "*naked*" compact molecule having no protective histones. It is replicated quickly without correcting mistakes and effective DNA repair systems. It is known that mtDNA is attached to internal mitochondrial membrane, in which respiratory chain permanently produces a significant quantity of ROS [10, 37]. Moreover, it is shown that some zones of mtDNA (for example, D-loop – the control zone of replication initiation) are specifically sensitive to ROS oxidative attack and are affined to increased mutagenesis level [38 – 40]. Many of somatic mtDNA

mutations in human malignant tumors are localized in the D-loop and, especially, along C-tract, called D310. The zone D310 displays variability of polymorphous length in different individuals. It was described as a "***hot spot***" for somatic mtDNA mutations in many types of cancer [174]. For example, multiple mutations in the control zone of mtDNA were observed in human prostate tumor that allowed for a suggestion about existence of unique mitochondrial hyper-mutagenesis, probably mediated by the oxidative stress [175].

These data on high level of mutagenesis in the control replication zone of mtDNA are explained well by the effect of extremely high radiation vulnerability of mtDNA biosynthesis, primarily discovered in 1960 s by A.P. Galkin and I.N. Todorov, compared with nuclear DNA biosynthesis in liver at sublethal dose γ-radiation of rats [41] which, in fact, equals the damaging ROS effect (Table 1).

Table 1

Biosynthesis of nuclear and mitochondrial DNA in liver of irradiated rats [41]

(specific DNA radioactivity in control (nonradiated) animals is assumed equal 100%)

Time after irradiation, h	Nuclei	Mitochondria
	Pulse / min / mg DNA	
Unirradiated	100	100
12	21	4.6
24	40	7.7

It is also shown that for oxidative modification and mutations of mtDNA hot spots are localized in or nearby unusual structures, including twisted, anti-twisted and non-B-structure of human mtDNA [39, 40]. These characteristics make mtDNA vulnerable to effects of ROS and free radicals, continuously generated by electron leakage from the respiratory chain.

Moreover, several mutations and oxidative damages of mtDNA happen more frequently and are accumulated in the upper layers of skin subject to solar irradiation [42, 43], in lungs affected by cigarette smoke [44, 45], and in mouth tissues of individuals having tobacco chewing habit [46]. These observations indicate high sensitivity of mtDNA to oxidative damages and mutations, induced by both endogenic and exogenic oxidative factors. These defects

provide for continuously increasing changes in the structure and functions of mtDNA which promote disease progression and aging [47 – 49].

2.3. Multicopy origin and intracellular distribution of mtDNA.

Basing on the great amount of mtDNA molecules which is estimated as hundreds or thousands per cell, essentially, mitochondrial genetics is the intracellular population genetics related to selection and segregation phenomena. Moreover, intracellular distribution of mtDNA molecules plays an important role in mutation display. Owing to dynamic compartmentalization of mtDNA molecules inside the mitochondrion, which stipulates the ability of these molecules and/or their products to interact with one another, there is an possibility of functional differences between mitochondria localized in different areas of the cell [51]. This is impressively illustrated by functional differences between synapse and neuron cellular body mitochondria [52]. Local differences in the intracellular medium and external stimuli may be defined by *genetic and functional thresholds of organelle sensitivity* with respect to specificity of subcellular compartment – the current residence of mitochondrion [51].

2.4. Intercellular mosaic structure.

Another important factor to be discussed in the analysis of the role of mtDNA mutation in aging, diseases and apoptosis is interstitial distribution of mutations. Usually, mtDNA mutations and mitochondrial dysfunction are detected by:

1) biochemical methods of pieces of tissue or the whole cellular population, extracted from an animal;
2) histochemical or immunohistochemical methods on tissue slices using hybridization *in situ*, laser microdissection, mtDNA analysis from a single cell or muscle fiber via PCR (polymerase chain reaction) mediated sequencing, or electrophoresis in gel.

Obviously, the general analytical approach may determine only average values of parameter under study, whereas the second approach allows for identification of intercellular and in some cases even intracellular mosaic

structure. Recently, the latter methods helped in determining frequent intracellular mosaic distribution of defects in activity of respiratory enzymes [53 – 55] and mtDNA mutations in aged and ill individuals [55 – 58].

Intercellular mosaic structure of myDNA mutations allows for estimation of the effect of current mutation on intercellular cooperation with respect to the functional state of the tissue.

2.5. Maternal inheritance.

Mitochondria and human mtDNA are maternally inherited [59, 60]. In mature oocyte of mammals the number of mtDNA copies increases to 100,000, approximately, whereas spermatozoons contain about 100 copies only [61]. Nevertheless, at fertilization some paternal mitochondria may occur in the ovum [62, 63]. However, even this small part of paternal mtDNA in fertilized oocyte quickly degrades and is eliminated after fertilization and at the early stage of embryogenesis [64]. Therefore, a majority (but not overwhelming) of mtDNA mutations is maternally inherited, but in case of paternal mitochondrial diseases genetic mtDNA defect cannot be inherited by the following generation [65]. Almost all pathogenic point mutations of mtDNA are maternally inherited. However, according to unknown reasons, large-scale deletions, insertions and tandem duplications of mtDNA which are mostly sporadic are not even maternally inherited [66, 67].

2.6. Heteroplasia, graduation, and threshold effect.

A typical human cell contains hundreds of mitochondria and thousand copies of mtDNA [68]. Theoretically, all individual mtDNA molecules are identical. This state is called ***homoplasmia***. However, two or more different mtDNA genotypes (about 10 per mitochondrion) may coexist within the same mitochondrion, cell, organ, or individual. This phenomenon is called ***heteroplasmia***. Almost all pathogenic point mutations of mtDNA caused by Leber hereditary optical neuropathy (LHON) are of homoplasmia type, the majority of pathogenic mutations of human mtDNA are of heteroplasmia type [69]. The heteroplasmia degree of mtDNA pathogenic mutations may vary among mitochondria, cells and tissues of the individual (the mosaic structure). It has been shown that in damaged tissues of patients with mitochondrial diseases the level of mtDNA with pathogenic mutation is usually above 70%

[49, 70]. However, there were several cases, when peripheral tissues contained more mtDNA mutations than target tissues [49].

As a result of mitosis, various parts of mutant mtDNA may be randomly transmitted to daughter cells. Random graduation of mtDNA molecules during mitosis and development may cause both high level of mutant mtDNA in one part of cells and low in the other part [71, 72]. Unequal distribution of mitochondrial genomes during cytokenesis may induce differences in mitochondrial genotypes of daughter cells and random genetic drift [72]. Moreover, heteroplasmia degree in cells of the same tissue may vary during the life of individual [10]. Often in patients an increase of mutation load induces progress of mitochondrial diseases. Damaged mitochondrion having high concentration of mutant DNA may proliferate by an unknown mechanism and, therefore, increase the contribution of mutant mtDNA in postmitotic tissue cells. Vice versa, concentration of mutant mtDNA in quickly dividing cells is relatively lower [66].

Usually, large-scale deletions, insertions and tandem duplications of mtDNA are not detected in blood cells, and mtDNA level with point mutations in these cells is lower than in muscles of patients with mitochondrial diseases [10, 49]. As a consequence, the absence of mtDNA mutations in blood samples may not be the basis for excluding mitochondrial disease [73]. Vice versa, high level of mtDNA mutations is usually detected in postmitotic tissues, such as cardiac and skeletal muscles and skin of patients. Large-scale deletions or point mutations of mtDNA are detected in muscle biopsies in, approximately, 70% of patients with mitochondrial diseases [49, 73].

It is clearly observed that mitochondrial functional defects are insignificant until mutant mtDNA contributions reaches high level, determined on the basis of the "***threshold effect***" concept. In skeletal muscle the level of **A3243G** mutation is strictly associated with strokes, epilepsy and dementia in patients with MELAS syndrome [49, 74, 75]. Similarly, the level of **A8344G** mutation correlated with cerebellar ataxia level and *myoclonia* in patients with MERRF syndrome [76]. Molecular genetic analysis of mtDNA mutations in muscle biopsies usually allows for more precise diagnostics of mitochondrial disease [49]. However, the relations between mtDNA genotype and clinical phenotype are more complex for the majority of pathogenic mutations [49, 70, 77].

It should be noted that significant differences in heteroplasmia degree is usually observed in generic carriers of pathogenic mtDNA mutations. A woman of this genus with heteroplasmic mtDNA mutations transmits a variable amount of mutant mtDNA to each of her children [49, 75]. This may

lead to various, in a rather broad range, clinical phenotypes in the following generations: from asymptomatic individuals to highly damaged descendants with progressive fatal neuromuscular disease [75].

The threshold effect of mtDNA mutations are shown up by mitochondrial disorders [75, 78]. However, the influence of mutant mtDNA molecule concentration on biochemical defects in heteroplasmic populations unnecessarily follows from linear correlation [79]. As soon as mtDNA mutations reach the critical level phenotypes at cellular and tissue levels are quickly changed from normal to anomalous state [75]. It has been found that at large-scale deletions the threshold for biochemical defect expression equals 65%, approximately [41, 80], and for point tRNA mutations in mtDNA – up to 95% [77, 81]. Moreover, organs with high energy demand – skeletal and cardiac muscles and central nervous system – are more frequently subject to mitochondrial diseases [49, 70]. Therefore, the threshold effect depends on both mtDNA mutation type and energy demand of individual cells and tissues [49, 73].

Thus, variability of heteroplasmic mtDNA mutations in various tissues of affected individuals together with tissue-specific differences in the threshold effectof mtDNA mutations and energy demands of various tissues may produce highly variable clinical phenotypes, observed in patients with mithochondrial diseases [49, 70, 78].

3. CLASSIFICATION OF mtDNA MUTATIONS RELATED TO MITOCHONDRIAL DISEASES[1]

Since mitochondrial diseases represent a hereditarily diversified group associated with the disturbance of oxidative phosphorylation [67, 69, 82], patients can display a broad variety of clinical signs in different combinations [49, 83, 84]. These diseases may occur in any tissue of a body, but most

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[1] Universally adopted abbreviations of mitochondrial diseases[99]:
MELAS – mitochondrial encephalomyopathy, lacticemia and cases similar to stroke; MERRF – myoclonic epilepsy with red coarse fibrils; CPEO – chronic progressive external ophthalmoplegia; LHON – Leber's hereditary muscular optical neuropathy; NARP – neurasthenia, ataxia and pigmented retinitis; KSS – Kearns-Sayre syndrome; NIDDM – insulin-independent diabetes mellitus.

frequently in brain and skeletal muscles, obviously, because of their much higher metabolism compared with other tissues.

Mitochondrial respiratory system disorders often induce *mitochondrial myopathy*, which may be the primary clinical manifestation or associated symptom of a mithochondrial disease [62, 67, 82]. Moreover, damages of oxidative phosphorylation in patients are frequently associated with general neurological disorders, including *eye muscle paralysis, pigmented retinitis, deafness, ataxia, strokes, dementia and peripheral neuropathy* [70, 83, 85 – 92]. Moreover, patients having no neurological symptoms may display *cardiomyopathy, diabetes or endocrine dysfunction* [49, 75, 93 – 98].

Table 2

Point mutations in mtDNA genes of programming proteins related to mitochondrial diseases [32, 99]

Local mutation of mtDNA gene		Amino acid variation	Disease
ND1	T3308C	Met-Thr	MELAS
	G3316A	Ala-Thr	NIDDM; LHON; CPEO
	T3394C	Tyr-His	LHON
	T3394C	Tyr-His	NIDDM
	G3460A	Ala-Thr	LHON
	G3496T	Ala-Ser	LHON
	C3497T	Ala-Val	LHON
	G3635A	Ser-Asn	LHON
	A4136G	Tyr-Cys	LHON
	T4160C	Leu-Pro	LHON
	T4216C	Tyr-His	LHON
ND2	C4640A	Ile-Met	LHON
	A4917G	Asp-Asn	LHON
	G5244A	Gly-Ser	LHON
CoI	G5920A	Trp-TER	Myoglobinuria, intolerance display
	G6930A	Gly-TER	Multisystem dysfunctions
	G7444A	TER-Lys	LHON
CoII	T7587C	Met-Thr	Mitochondrial encephalomyopathy
	T7671A	Met-Lys	Mitochondrial myopathy
	G7896A	W-TER	Multisystem dysfunctions
ATP6	T8993G	Leu-Arg	NARP
	T8993C	Leu-Pro	NARP/Leigh syndrome

	T9101C	Ile-Thr	LHON
	T9176G	Leu-Arg	Leigh syndrome
CoIII	G9438A	Gly-Ser	LHON
	G9738T	Ala-Ser	LHON
	G9804A	Ala-Thr	LHON
	G9952A	Trp-TER	Mitochondrial encephalopathy
	T9957C	Phe-Leu	Progressive encephalopathy, MELAS
ND3	T10191C	Ser-Pro	Epilepsy, impact, optical atrophy, reduced cognitive ability
ND4L	T10663C	Val-Ala	LHON
ND4	A11084G	Thr-Ala	MELAS
	G11778A	Arg-His	LHON
	G11832A	Trp-TER	Intolerance display
	A12026G	Ile-Val	Diabetes mellitus
ND5	G13513A	Asp-Asn	MELAS
	A13514G	Asp-Gly	MELAS
	A13528G	Thr-Ala	LHON-like
	G13708A	Ala-Thr	LHON
	G13730A	Gly-Glu	LHON
ND6	G14453A	Ala-Val	MELAS
	G14459A	Ala-Val	LHON + dystonia
	T14484C	Met-Val	LHON
	C14568T	Gly-Ser	LHON
Cyt b	G15059A	Gly-TER	Mitochondrial myopathy
	G15150A	Trp-TER	Intolerance display
	T15197C	Ser-Pro	Intolerance display
	G15242A	Gly-TER	Mitochondrial encephalomyopathy
	G15257A	Asp-Asn	LHON
	G15615A	Gly- Asp	Intolerance display
	G15762A	Gly- Glu	Mitochondrial myopathy
	G15812A	Val-Met	LHON

Though nuclear DNA mutations may cause mitochondrial dysfunction, a great part of hereditarily determined mitochondrial diseases is induced by mtDNA mutations [49, 69, 99]. It was reported that mtDNA point mutations and deletions were related to (or responsible for) mitochondrial myopathies and/or encephalomyopathies [49, 69, 99]. Commonly, patients with such diseases manifest clinical signs in the young age, and additional multisystem

dysfunctions such as encephalopathy and/or peripheral neuropathy may develop in them with the age. The majority of mitochondrial myopathies proceeds sporadically and is frequently induced by large-scale mtDNA deletions [49]. Usually, these patients possess specific mtDNA mutations, and they frequently demonstrate defects in NADH–CQ reductase and/or cytochrome c oxidase.

Human mtDNA mutations are manifested as a rearrangement, at which mtDNA genes are removed or duplicated, point mutations in tRNA or rRNA genes as well as in genes programming polypeptides that changes the genetic meaning, which disturbs functions of acting polypeptides forming the oxidative phosphorylation system (Table 2).

Molecular-biological, biochemical and pathological consequences of each type of these mtDNA mutations are described in the following sections.

3.1. mtDNA deletions.

Large-scale deletions were the primary mutations in mtDNA, which were associated with human diseases [100]. These mtDNA deletions, usually heteroplasmic, as a rule, eliminate several genes which encode respiratory enzyme subunits and tRNA genes [69]. It has been found that mtDNA with deletions, usually being sporadic, are infrequently inherited by children after mothers [69]. In some cases, the mtDNA duplication product is more than the wild type of mtDNA and contains two tandem molecules, which consist of full length and deleted mtDNA [101].

The highest variability in amount of 5 KB deletions is observed in various brain zones (substance nigra, caudate nucleus, and putamen [102]) and the lowest – in cerebellum [102, 103].

Large deletion of 7.4 KB was determined in old individuals without pathologies in myocardial tissue [104], cerebral cortex and putamen [103]. The same deletion increase to 5 and 6 KB with age was observed in liver mtDNA [105] and other human tissues [106]. Biochemical defects and clinical signs were ambiguous until the number of genomes possessing mtDNA deletion reaches some critical value, 50% according to some data [108].

Interesting results were obtained in the study of oxidative damages of skeletal muscle in patients with chronic uremia [109]. In these patients, large-scale mtDNA deletions frequently occurred between nucleotide positions 7900 and 16300. Among them, deletion of 4977 nucleotide pairs (np) was the most frequent and scaling in skeletal muscle with uremia [113]. The mtDNA

deletion level of 4977 np satisfactorily correlated with 8-OH-dG level in total DNA of the muscle. The use of PCR-amplification and sequencing of mtDNA allowed for identification and characterization of multiple deletions in 16 of 19 patients with uremia. The largest deletion of 8041 np is localized between 8035 and 16035 nucleotide pairs (Figure 3). It was flanked by 5-np direct repeats 5 - CCCAT-3 [109]. It should be noted that accompanying this large deletion, genes of 5 subunits of NADH-dehydrogenase, 2 subunits of ATP-synthase, subunit of cytochrome c oxidase and cytochrome b, and genes of several tRNA are eliminated from genome (Figure 3).

Large-scale mtDNA deletions are usually related to CPEO, KSS, and Pearson's syndrome [69, 99]. KSS is characterized by an early attack of ophthalmoplegia and pigmented retinitis and may be related to cerebellar ataxia or cardial conduction block [69, 100]. However, a single or a set of large-scale deletions and duplications were identified for other mitochondrial dysfunctions, including diabetes mellitus and deafness, Wolfram's syndrome, MELAS, KSS–MELAS overlapping syndrome, and Leigh's disease [69, 70].

3.2. Point mutations in tRNA genes of mtDNA.

Several particular syndromes were found related to particular point mutations of mtDNA [69, 99, 101]. It is reported that several point mutations happen in tRNA genes. For example, **A8344G** mutation is present in patients with MERRF syndrome [92], which was the first mitochondrial disease with protein synthesis defect in mitochondria identified as the result of point mutation in tRNA gene [92]. Over 80% of patients with MERRF possess **A8344G** mutation in tRNA*Lys* gene of human mtDNA. A small part of patients with MERRF possess T → C mutations in nucleotide pair 8356 of mtDNA [69]. For the first time, mtDNA mutation **A3243G** was identified in a subgroup of patients with MELAS syndrome [87]. Point mutations in 3271 np and 3291 np in tRNA*Leu* (UUR) gene also relate to MELAS [87, 88]. About 80% of patients with MELAS have **A3243G** mutations of mtDNA in damaged tissues [49, 87]. Other mutations in tRNA*Leu* (UUR) gene such as T → C transition in 3271 np is also connected to MELAS syndrome, but the action range is much shorter [88]. It has been definitely noted that there are no direct relations between mtDNA genotype and clinical phenotype. Mutation **A3243G** in tRNA*Leu* (UUR) gene is preferably related to MELAS syndrome, but may also be expressed as CPEO, chromosomal myopathy, diabetes mellitus and

deafness [49, 69]. Since diabetes induces a strong oxidative stress, it has been suggested that mutation **A3243G** accumulation in mtDNA can be accelerated in case of this disease. Actually, the level of this mutation in peripheral blood of newborns was low. Patients with diabetes displayed the frequency increase with the age and diabetes duration indicating somatic origin of the mutation. For example, in the middle age group (41 – 60) accumulation level of **A3243G** mutation was 4 times higher in patients with type II diabetes rather than in healthy indivuduals [173].

For patients with diabetes, an increase of point somatic *transversions* in mtDNA was also observed. This was considered as a new biomarker of mtDNA damages related to hyperglycemia and, possibly, induced by the oxidative stress [176].

3.3. Point mutations in rRNA genes of mtDNA.

Homoplasmic **A1555G** mutation in 12S ribosomal RNA gene was first observed in patients with neurosensor dysfunctions of the hearing apparatus [91]. However, not all individuals with this mutation were deaf. Meanwhile, a new point mutation **C3093G** in mitochondrial 16S rRNA gene was detected in patients with MELAS syndrome, diabetes mellitus, hyperthyroidism and cardiomyopathy [75]. This mutation in high ratio coexists with **A3243G** mutation of mtDNAin skeletal muscle of the foremother. Interestingly, **C3093G** mutation is very infrequent in blood cells and follicles of the foremother and has high level in her skeletal muscle only. No symptoms of **C3093G** mutation were found in her three sons. Basing on clinical signs and molecular genetics of the foremother and her family members, Wei and Lee [44] have suggested that these two point mutations in mtDNA are synergic in case of a disease.

3.4. Point mutations in proteins programmable by mtDNA genes.

Several point mutations in mitochondrial genome affect genes which program proteins (Figure 3, Table 2). Mutations T → G or T → C in ATPase 6 gene in 8993 np of mtDNA is one of pathogenic mutations in case of Leigh's disease [49, 69, 73]. Mutations **G11778A**, **G3460A**, and **T14484C** were related to LHON [49, 69, 73].

Besides Leber's disease, NARP is another disease induced by **T8993G** mutation in ATPase 6 gene of mtDNA [69, 73]. It is characterized by neurogenic muscle weakness, sensor neuropathy, ataxia and pigmented retinitis, and in some cases – even dementia. Leber's disease is characterized by symmetrical necrosis zones, which include midbrain, basal ganglion, thalamus, a part of brainstem and optic nerves [69]. The individuals with **T8993G** mutation level below 70% are usually symptom-free. Leber's disease is displayed only at mutant mtDNA ratio equal 90% or higher. Patients with NARP and mutant mtDNA level in muscles or blood between 70 and 90% show different development delays [69].

Mutations of mtDNA which induce a defect in the complex I [92] are conjugated with LHON. Primary mutation related to LHON is G → A in ND4 gene in 11778 np [69, 92]. Transition **G11778A** is the most frequent mtDNA mutation at LHON and defines over 50% of all cases of the disease. Mutations **T3460C** in ND1 gene and **T14484C** in ND6 gene were primary mutations in about 10% of patients with LHON [69]. Three primary LHON mutations (Table 2), each of which is associated with acute or sub-acute beginning of bilateral loss of sight, was usually observed for young men. This observation and the fact that the majority of women with homoplasmic LHON mutation never obtain sight loss development indicate that mtDNA mutation is not the unique determinant of the morbid phenotype [69].

Though the number of diseases induces by mtDNA mutations increases, the whole sequence of molecular mechanisms leading to development of these pathologies are now just partly understood. However, the main chain in this sequence is already known. It is oxidative damage and mtDNA gene mutations which encode respiratory enzyme subunit structure and, therefore, cause improper assembly of the respiratory chain. In turn, at the turnover, this induces production of a great quantity of ROS. Repeating of this cycle with always increasing dysfunctions of respiratory chains induces both more intensive ROS generation and deterioration of ATP synthesis that cause further damaging of mitochondrion and its genome, and the whole cell. This is the essence of the so-called ***vicious circle theory*** of the oxidative damage of mtDNA [179]. Apparently, this vicious circle makes a significant contribution into development of mitochondrial diseases, aging degenerative diseases, aging itself [1], and apoptosis. The knowledge about other pathogenetic factors and mechanisms of mitochondrial diseases are rather scanty. This is testified about, in particular, by the interest rousing observation that not only the same mutation in the same gene, for example, in tRNA*Leu* (UUR) gene can induce

different diseases (for instance, MELAS syndrome or diabetes mellitus), but also different mtDNA mutations may induce the same disease [49].

4. mtDNA MUTATIONS AND AGING

4.1. mtDNA deletions.

The first exciting proof of damages in mtDNA at aging issued from the discovery of its large deletions in various tissues of adult humans and rats, and their progressive increase with aging. Thus deletion sized 5 KB typical of Kearns–Sayre syndrome of chronic progressive external ophthalmoplegia (CPEO) was detected in myocardium [110, 111], brain [102, 103], skeletal musculature and other tissues of aged individuals [110]. As indicated, a large deletion of 7.4 KB was observed in old individuals in myocardium tissue [104], cerebral cortex and putamen [103], and the quantity of such deletions increased with the age.

Such age-dependent increase of deletions to 5 and 6 KB was determined in mtDNA of liver [105] and other human tissues [106]. With respect to observations of this kind a hypothesis has been suggested, according to which accumulation of point mutations 8-OH-dG and other oxidative adducts may be the reason for occurrence mtDNA deletions [104]. Actually, a clear correlation between accumulation of large deletions and age-dependent increase of 8-OH-dG quantity was observed in mtDNA of myocardium [104] and diaphragm muscles [104, 107].

The study of extraocular muscular fiber of a subject older 70 carried out by histochemical and immunocytochemical analysis of cytochrome c–oxidase (COX) activity and *in situ* hybridization on frozen slices indicate a clear correlation between age-dependent COX deficiency display in muscular fiber and the change in hybridization character which, in turn, indicated mtDNA exhaustion or occurrence of the general 5 KB deletion [56]. It was also reported about focused accumulation of large deletions in COX-deficient isolated muscular fibril [112].

Recently, it has been demonstrated that (by long PRC-amplification and sequencing of full mtDNA lengthof a single cardiac hystiocyte) the cells of each of four tested long-livers possess large mtDNA deletions (from 11 to 64%), and the cells of three persons aged 31 – 72 have no these deletions

[113]. However, of the higher value in the cited work was the fact that each cell carried only one type of deleted mtDNA, whereas different cells carried different types of deletions that strongly supported the idea about clonal expansion of the primary single mutational event. However, another group of investigators found that the majority of fibrils contained many deletions, in some of which no wild mtDNA was detected [114]. It should be mentioned that in other investigations of skeletal muscle mtDNA no significant accumulation of large age-dependent deletions was observed [115, 116]. Inter- or intracellular mosaic structure in distribution of deletions allows an assumption that tissue dysfunction may happen even when the total level of deletions is relatively low.

The inconsistency of the present data on the level of age-dependent deletions currently makes low-probable a possibility of their consideration as the main reason for mitochondrial function loss, which takes place in old individual cells. In addition, they may hardly be sufficient in neurodegenerative diseases, such as Parkinson's and Alzheimer's disease [117]. However, it should be mentioned that in the most of works the quantitative level of specific mtDNA deletions on a current piece of tissue or total cellular population taken from normal aged individuals was not studied, as well as possible intercellular or intracellular mosaic structure in distribution of deletions was not investigated.

4.2. Point mutations of mtDNA.

In early investigations of age-induced mtDNA point mutations, it has been shown that A → G transition increase to 2% in mitochondrial tRNA*lys* gene in 8344 np position in extraocular muscle of normal old subjects is associated with MERRF encephalomyopathy, whereas A → G transition increase to 1% in tRNA*leu* (UUR) gene in 3243 np position in the same muscles is associated with MELAS encephalomyopathy [118]. Moreover, it was informed about focused accumulation of MERRF tRNA*lys* and MELAS tRNA*leu* (UUR) mutations and three more tRNA mutations in isolated muscular fibril of aged subjects [112]. The data on low frequency of the above-mentioned mutations and some conflicting results have brought up a question about their connection to aging processes [51].

In 1999, a significant age-dependent accumulation of point mutations in human mtDNA was primarily reported [119]. In this work, mtDNA segment selected for analysis was the main control region containing two areas, which

initiate transcription of heavy (H) mtDNA strand, the promoter for light (L) strand transcription, which is also the promoter for RNA-primer of H-strand synthesis and primary (O_{H1}) and secondary (O_{H2}) areas initiating H-strand synthesis [120]. It is of interest that the prerequisite for this investigation were ideas, according to which this main control region is evolutionally the most variable segment of mitochondrial genome of a man [121], and it seemed probable that the mechanisms inherent to the evolution may also operate in the aging processes. Actually, a great quantity of mutations was detected in the control region of mtRNA replication in skin fibroblasts [119]. However, this class of point mutations was present in individuals older 65 only, and they were multiple varying within the range of 20 – 50%. Surprisingly, the most frequent mutation in this class, **T414G** transversion, was detected in 8 of 14 individuals oalder 65 (57%) and was absent in 13 younger individuals. Two samples of skin fibroblasts taken from the same individual with the interval of 15 – 19 years were analyzed and clearly showed non-hereditary type of these mutations. As already mentioned, these specific point mutations were localized in the areas critical for mtDNA replication. For example, **T414G** mutation occurs in the middle of the promoter of RNA-primer for H-strand synthesis (LSP), whereas mutations in positions 189, 195, 146, and 152 fall very close to the primary origin (position 191) or secondary origin (positions 147 – 151) of mtDNA H-strand synthesis.

Screening of the distribution of the mentioned mutations in several tissues in the same old individuals showed their presence in skin and the absence in skeletal musculature, myocardium, lymphatic glands and spleen [122]. Moreover, more comprehensive analysis has shown that these mutations have tissue-dependent specificity, being absent or just insignificantly presenting in the skeletal muscles [122], brain [123, 124] and leukocytes [125].

The analysis of **T414G** mutation distribution in origins of fibroblast populations displayed its amazing clonal nature [126].

Interestingly, analysis of the control region of mtDNA in fibroblasts of patients with Down syndrome (DS) which, as is known, are subject to quicker aging, determined the expected accumulation of **T414G** transversions. In particular, five of nine patients with DS 45 or older possessed mutations in proportion 5 – 70%, whereas in all 12 patients with DS below 45 this transversion was not observed [127].

Large-scale analysis of the main control region of mtDNA in 26 individuals aged 53 – 92, having no neuromuscular diseases, showed accumulation (in the same replication control area) of two new point mutations

– **A189G** and **T408A**, which were absent or present in insignificant amounts in 19 individuals younger than 34 [51].

Recently, sequencing analysis of the main control region of mtDNA in each of 36 cardiac hystiocytes in three individuals aged 51 – 109 indicated clonal expansion (from 25 to 100% of cellular mtDNA) of the point mutation in one or another nucleotide in the area of 16025 – 16055 pairs of mtDNA nucleotide sequence and its absence in similar number (only one mutation was observed) of cells from young individuals [58]. High conservatism of these nucleotide positions in human population and subhuman primates and their distribution in extremely narrow area (similarly to the above-described **T414G** mutation of fibroblast mtDNA) definitely indicate their participation in any important mitochondrial function.

4.3. mtDNA mutations and life span.

The above-described tissue-specific, extraordinary and aging dependent accumulation of mutations in the main control region of human mtDNA, as well as the role of this area, critical for replication, clearly indicates the functional validity of these mtDNA mutations. Moreover, the occurrence of **T414G** transversions, specific for fibroblasts, within the RNA-primer promoter of mtDNA H-strand synthesis in four of six long-livers [119, 128] allowed G. Attardi and colleagues from Biology Department of California Institute of Technology for the first time bring up a question, if these mutations and other aging dependent mutations may have a positive effect on the life span of the organism [51]. This possibility was strongly supported by recent report describing results of theanalysis of main control region of leukocyte mtDNA from a b ig group of Italian long-livers and younger individuals [125]. In particular, in this investigation homoplasmic **C150T**-transition was observed near the origin of mtDNA H-strand synthesis for 17% of the group of 52 subjects aged 99 – 106 and, vice versa, only in 3.4% of 117 younger individuals. The proofs of soma contribution into the mentioned phenomena are supported by discovery of **C150T**-transition in human fibroblasts and the observation of somatic expansion of this age-dependent mutation (up to homoplasmia) in some long-term studies of fibroblasts. Of the greatest interest in this work is the fact that **C150T**–transition is the reason for occurrence of a new replication origin in position 149, which replaces normal origin in position 151 in fibroblasts and immortalized lymphocytes [125]. It should also be

mentioned that the analysis of leukocytes did not detect **A189G** and **T408G** transversions specific for mtDNA of skeletal muscles and, vice versa, mtDNA of the muscle had no **C150T**–transition. Another unexpected observation, presented in the discussed paper, is the presence of homoplasmic **C150T**–transition in mtDNA of leukocytes taken from 30% o 20 monozygotic and 22% of 18 dizygotic twins (both groups were from the same Italian population, aged 60 – 75).

This is the real picture of the hypothesis, according to which selection of remodeled replication origin, hereditarily or somatically obtained, give some advantage in the life span. This advantage is based on the observable correlation between high **C150T**–transition concentration and longevity of the tested individuals.

We must also mention that limitation of caloric content of food – the most important method of experimental prolongation of life of laboratory animals – decreased 8–OH–dG content in mtDNA, but not in nuclear DNA of myocardium in rats [129].

The age-dependent decrease of mitochondrial transcription and translation – the part of mitochondrial turnover which rate clearly defines sensitivity of mitochondria to oxidative damages and mutations – thus controls the cellular aging rate. The age-induced mtDNA deletions are accumulated in brain, myocardium and skeletal muscles, i.e. in organs with high energy metabolism, strictly promoting preferable aging of these postmitotic tissues.

To conclude this Section, we would be pay special attention to recently published experiment by Larsson *et al.*, who observed premature aging of mice expressing defective mitochondrial DNA-polymerase [178]. Using the transgene approach, they have created a strain of homozygous mice, which expressed defective version of mtDNA-polymerase catalytic subunit, encoded by the nucleus, which in norm acts as a corrector in mtDNA replication. It has been clearly shown that all mice display development of the mutant mtDNA phenotype with three-five-fold increase of both pint mutations and mtDNA deletions. This increase of somatic mtDNA mutations related then lafe duration decrease for animals and premature initiation of aging-induced phenotypes, such as weight loss, decrease of hypodermic fat, baldness, spinal curvature, osteoporosis, anemia, reduction of reproduction ability, and heart expansion. Therefore, these are the initial results indicating ***the direct reasoning relation*** between mtDNA mutations and aging phenotypes in mammals [178].

5. MOLECULAR AND BIOCHEMICAL ASPECTS OF mtDNA MUTATION INFLUENCE ON DYSFUNCTION OF MITOCHONDRIA

Recently, consequences of some mtDNA mutations were extensively studied on molecular and cellular levels.

5.1. Molecular aspects

5.1.1. Mutation in transcription termination area. Mutation **A3243G** happens in mtDNA terminating sequence (TAS) which links mitochondrial transcription termination factor (mTERF) [130, 131]. Linking of mTERF in this area usually selectively increases transcription rate of 12S and 16S rRNA genes rather than heavy H-strand genes. This guarantees sufficient synthesis of 12S and 16S rRNA for mRNA transcriptions encoding mitochondrion polypeptides. Experiments *in vitro* show that this mutation really decreases the affine linking of mTERF to mtDNA [131] and rRNA gene transcription termination rate [132]. Moreover, it has been suggested that incompletely processed 19S pre-RNA transcript containing 16S rRNA + tRNA*Leu* (UUR) + ND1 is accumulated in cells with **A3243G** mutation [133] and thus promotes a decrease of synthesis rate and stable polypeptide level, encoded by mtDNA. It has also been suggested that the defects of RNA processing [134] or RNA synthesis termination in the TAS zone near tRNA*Leu* (UUR) gene [132] are responsible for suppression of the respiratory function in tissues of patients with MELAS syndrome.

Some investigations show that **A3243G** mutation is associated with protein synthesis suppression in damaged cells [81, 133]. When the ratio between mutant and total mtDNA exceeded 95%, the protein synthesis activity suddenly decreased [81, 134], apparently, due to reduction of aminoacyl–tRNA level.

5.1.2. Mutations in tRNA genes may effect on stability of tRNA molecule resulting in low stationary level of aminoacyl–tRNA [81, 135]. Yasukawa *et al.* [136] reported that tRNA*Leu* (UUR) mutations in positions 3243 (A → G) and 3271 (T → C) abruptly decreased the stationary level of tRNA. The sum of

leucyl–tRNA*Leu* (UUR) having **A3243G** or **T3271C** mutations was by 30% smaller than the sum of intact tRNA of the wild type [136]. The studies of **A8344G** mutation of tRNA*Lys* at MERRF syndrome indicate that ~50% reduction of aminoacyl–tRNA*Lys* causes a serious suppression of the protein synthesis and production of not yet formed polypeptides [137]. Moreover, half lifetime of tRNA*Ile* transcribed from tRNA*Ile* gene with the point mutation **A4296G** can be reduced both *in vitro* and *in vivo* [138].

Apparently, tRNA mutations may change the codon–anticodon recognition. It is shown that tRNA*Leu* (UUR) with **A3243G** or **T3271C** mutations [136] and tRNA*Lys* with **A8344G** point mutation [139] are deficient by post-transcript modification in the *wobble* position of anticodon that may cause production of abortive polypeptides and decrease of mitochondrial protein synthesis [139]. Defects of the *wobble* modification significant for decoding their semantic partners may, apparently, be interpreted as a general mechanism in pathogenesis of mitochondrial diseases, induced by mutations in tRNA genes of mtDNA. However, tRNA*Ile* with **A4296G** mutations presented normal post-translational modification [138]. For example, seemingly, the defects of this modification are not universal for mutations of all tRNA genes.

5.1.3. Mutations in mtDNA genes encoding proteins. Mutation **T8993G** transforms highly conserved leucine to arginine in subunit 6 of ATPase, which is the component of proton conductivity channel (Fo) of the complex V [69, 79]. Though **T8993G** mutation will preferably induce weakening of FoF1 ATPase complex, this effect is not quite clear yet. It is shown that replacement of Leu (position 156) by Arg of FoF1 ATPase α-subunit (as a result of **T8993G** mutation) will reduce the rate of ATP synthesis in submitochondrial thrombocyte particles [140]. In human cells, mutant subunit 6 of ATPase is related to a break in complex V assembly [141].

The cells carrying mtDNA deletion in 4977 np possess reduced respiratory function, and this decrease varies as the increase of deleted mtDNA level [142], whereas cells possessing over 60% of deleted mtDNA show a significant decrease of respiratory enzyme activity and synthesis of proteins encodes by mtDNA [80]. In mice with predominant quantity of 4696 np deletions in mtDNA, simultaneous decrease of cytochrome c oxidase and mitochondrial translation activities was observed [143]. It is suggested that large-scale deletions disturb mitochondrial translation due to loss of tRNA genes encoded in eliminated mtDNA fragment. As soon as proportion of

deleted mtDNA molecules reaches the critical level, mitochondrial translation may become the limiting factor due to the absence of enough amount of tRNA.

5.2. Biochemical aspects

Deficient function of the respiratory chain induced by mtDNA mutations may effect on various biochemical functions of mitochondria, such as potential of mitochondrial membrane, ATP synthesis, the ATP/ADP ratio, ROS production, mitochondrial turnover, and potassium homeostasis [101].

5.2.1. mtDNA deletions. In cells containing less than 55% of 4977 np deletions, the rate of ATP synthesis and ATP/ADP ratio are preserved at the level similar to cells with undamaged mtDNA molecules [144]. As soon as the contribution of deletions reaches this sensitivity threshold, mitochondrial membrane potential, ATP synthesis rate and ATP/ADP ratio decrease abruptly [144]. These results allow for a supposition that bioenergy deficiency induced by mtDNA deletion accumulation promotes functional defects in cells in tissues-targets of patients with mitochondrial diseases.

Recently, Inoue *et al.* [151] have described a generation of mice with mitochondrial dysfunction, obtained by injection of mitochondria with mtDNA 4696 np deletion to zygotes of intact mice. It has been found that accumulation of mtDNA deletions induces mitochondrial dysfunctions in various tissues. The studies of skeletal muscle slices displayed good correlation between COX deficiency and deletion predominance [151]. All muscle fibers, including cardiac hystiocyte, containing over 85% of deleted mtDNA, were COX deficient, whereas below 85% they equaled to norm. In mutant cardiac mitochondria, an anomalous morphology of mitochondrial cristae was observed [152]. The authors of the latter work have suggested that high level (> 91.6%) of deleted mtDNA accumulation stimulates reduction of the mitochondrial translation due to the absence of six tRNA genes in mitochondrial genome, which results 4696 np deletion. Moreover, it has been found that the deficiency of all translation products encoded in mtDNA leads to incorrect assembly of respiratory enzymes in complexes on internal mitochondrial membranes. Hence, this leads to an anomalous structure and function of mitochondria of damaged cells [152].

5.2.2. Point mtDNA mutations. Studying a culture of skin fibroblasts with **A3243G** or **A8344G** mutations, James *et al.* [145] indicated significantly

decreased potential of the mitochondrial membrane and respiration intensity in mutant cells. These two mutations in tRNA genes induce production of energetically degenerative mitochondria and, therefore, cause functional defects in complexes I and IV [145]. Moreover, a significant increase of the cellular volume filled with secondary lysosomes and residual bodies indicates that the cells of patients with MELAS or MERRF syndrome display increased degradation of mitochondria [145]. The ability of mitochondria to synthesize ATP and support the optimal ATP/ADP ratio indicated a tendency to decrease in skin fibroblasts of patients with MELAS or MERRF syndrome compared with healthy individuals [146]. Alongside with this, it has been found out that the cells containing **A3243G** or **A8344G** mutations suffer with the lack of ATP [146]. Recently, Pang *et al.* [77] have demonstrated that the cells possessing over 90% **A3243G** mutations in mtDNA show much lower respiration intensity and reduced electron transport activity, and, consequently, lower ATP/ADP ratio and reduced energy consumption. Moreover, cells having high fraction of **A3243G** mutations also show quite high sensitivity to exogenic oxidants [77]. These observations allowed for a suggestion that these mutations in tRNA genes disturb the mitochondrial protein synthesis and suppress the electron transport activity and the energy metabolism of mitochondria, as well as suppress the cell respiration, the membrane potential and the proton gradient via internal mitochondrial membrane that reduces the maximal rate of ATP synthesis. Moreover, these mutations strictly affect the mitochondrial membrane potential, which is the motive force of Ca^{2+} absorption, Ca^{2+} inflow buffering, and modulation of cytoplasmic Ca^{2+} modulation level [147]. It is shown that the decrease of mitochondrial membrane potential in fibroblasts of patients with MELAS syndrome increases the intracellular calcium concentration and disturbs the cell ability to control the excessive calcium supply [148]. Therefore, these results imply an important role of Ca^{2+} homeostasis perturbation in pathogenesis of various mitochondrial diseases, induced by mtDNA mutations.

Alongside with the latter, defect respiratory function induced by mtDNA mutations promotes increased ROS and free radical production. In this relation, it produces higher level of the oxidative stress and deep oxidative damages in cells [77, 142]. Since the increased oxidative stress or disturbed calcium homeostasis are the important factors for initiation of the cell death process, the dysfunction of mitochondria in MELAS and MERRF patient cells may significantly promote pathogenesis of these diseases.

In four patients with the family mitochondrial myopathy, serious defects in complex I and complexes I + III of the respiratory chain were observed [149].

Meanwhile, direct correlation between **A3243G** mutation level and defect COX function was also observed in separate muscle fibers [150].

6. OXIDATIVE STRESS, mtDNA MUTATIONS AND APOPTOSIS

Apoptosis or programmable cell death is the evolution-proved mechanism, sufficient for morphogenesis, development and homeostasis of tissues [153]. The molecular mechanisms of apoptosis were studied in lots of investigations and discussed multiple reviews [154 – 157, etc.].

Apoptosis is the extremely complicated, multistage, cascade process, which invokes expression on many tens of genes and biochemical processes, controlled by cellular p53 protein, which is conditionally called the *tumor suppressor*. However, we should mostly pay attention to relation between programmable cell death and oxidative stress and mtDNA mutations, because it has been found that mitochondria subject to oxidative stress play the key role as apoptosis trigger and mediator [158 – 160]. Proapoptotic protein transfer to external mitochondrial membranes changing the permeability vector of their pores (Figure 4), and stimulates diffusion of dissolved lowmolecular substances through the internal mitochondrial membrane [167]. This causes swelling of mitochondria and may disrupt the external mitochondrial membrane, induce extraction of intermembrane localize dproteins such as cytochrome c, apoptosis inducing factor (AIF) and other proapoptotic proteins (Figure 4). All this precedes nuclear DNA fragmentation and apoptosis [159, 160]. It seems highly probable that apoptosis provides for pathogenesis of mitochondrial diseases [162].

6.1. Apoptosis and mitochondrial diseases.

Recently, Mirabella et al. [163] have reported about DNA fragmentation and expression of proteins related to apoptosis (Fas, p75, and caspase-3) in muscle biopsies of patients with mtDNA point mutations and deletions. It has been found that patients carrying a single mtDNA deletion or point mutations in tRNA genes (tRNA*Lys*, tRNA*Ile*, and tRNA*Trp*), display

the apoptosis level in muscle biopsies corresponded to the number of mutant mtDNA and the depth of both mitochondrial myopathy and neurological phenotype of the patients. However, despite the presence of genomes modified in high propotion (97 – 100%), only modest or unexpressed signs of apoptosis were observed in the muscle of patients with mitochondrial diseases, induced by point mutations in structural genes [163].

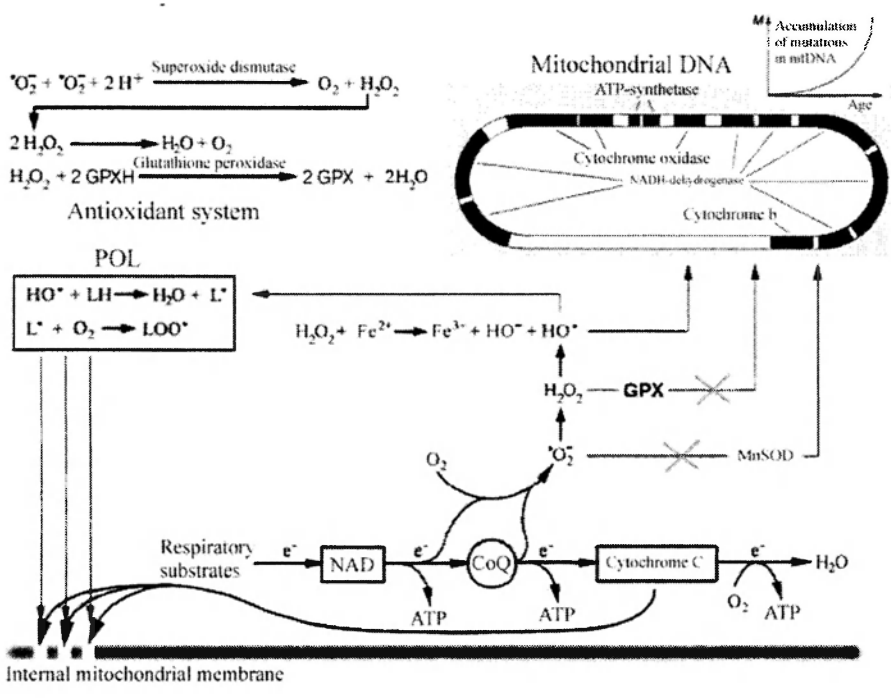


Figure 4. Mitochondrial metabolism. The scheme of oxidative phosphorylation, ROS production ($^{\bullet}O_2^-$, H_2O_2, $HO^{\bullet}$), enzymatic antioxidant protection (MnSOD, GPX), peroxide oxidation of lipids (POL), mtDNA mutagenesis (its exponential increase with the age), some elements of apoptosis mediated by mitochondria (adapted from [1])

The deficiency of cytochrome c oxidase is one of the most general enzymatic defects in patients with mitochondrial diseases, independently of the presence or the absence of one or another mutation in mitochondrial or nuclear

genomes [69]. Recently, Di Giovanni et al. [164] have shown that in patients with cytochrome c oxidase deficiency the varying number of muscle fibers has apoptotic nuclei, the amount of which corresponds to the reduction level of respiratory enzymes and roughly correlated with the muscle weakness. However, no signs of apoptosis were observed in patients with deficient complexes I and II, which had no muscle weakness [164]. These observations indicate that apoptosis is of potential pathological importance for mitochondrial diseases associated with COX deficiency.

The massive apoptosis was observed in the culture of skin fibroblasts possessing over 90% concentration of **T8993G** mtDNA mutations [165]. Moreover, at the oxidative stress induces by superoxide anions, **T8993G** mutation was also detected in fibroblasts. As is known, this mutation is localized in ATPase subunit 6 gene. Interestingly, the apoptosis can be cancelled by treatment with molecules carrying an antioxidant spin-trap [165]. These observations indicate that ***superproduction of superoxide anions related to T8993G mutation of mtDNA is enough for initiation of apoptosis*** in brain of a patient with NARP or Leigh's disease. Both these diseases are characterized by symmetrical double-sided necrotic damages on the cerebral stem, basal ganglion, thalamus, and spinal marrow. Recently, Wang *et al.* [166] have managed to prove that the respiratory chain deficiency *in vivo* predisposes cells to apoptosis. They observed massive apoptosis in Tfam-knockout embryo at the 9.5 embryonic stage. The apoptosis was also increased in myocardium of mice with tissue-specific Tfam-knockout [166]. Cells taken from the patients with MELAS, MERRF, and LHON were to a higher extent sensitive to the cell death at exposure to hydrogen peroxide compared with normal cells, and this cell death might be eliminated by Ca^{2+} exhaustion [172].

All these results indicate that ***intensification of the cell apoptosis is a pathogenic event related to development of mitochondrial diseases, induced by mtDNA mutations.*** However, only future systematic investigations can present an adequate assessment of the role of mtDNA and mtDNA mutations in apoptosis, related to mitochondrial diseases. While the cell death is the decisive development factor of some mitochondrial diseases, apoptosis may not be pathologically significant for all tissues.

Cellular death may be initiated by several mechanisms, including ROS production increase, oxidation of mitochondrial pool of glutathione, chronic rise of free Ca^{2+}, ATP exhaustion or intracellular pH change, i.e. any factor which may influence on mtDNA mutation leading to a mitochondrial dysfunction. ROS is the important proapototic signal in the armory of biological systems responding to disturbance of the vital activity of cells. In

some cases, it may be included to a signal stimulating the cell death [168]. Nevertheless, there are no unambiguous direct proofs of relation between oxidative stress and mtDNA diseases, though there is an indirect confirmation that some mtDNA mutations increase expression of enzymes–detoxicants (MnSOD and glutathione peroxidase) in a culture of cells and muscle biopsies of patients with mitochondrial diseases [169 – 171], showing their natural response to the oxidative stress.

If ***apoptosis development*** is a pathogenic factor of mitochondrial diseases then, in the contrary, ***disturbance of the apoptosis mechanism*** may be important for pathogenesis of absolutely different group of human diseases, such as cancer, autoimmune and neurodegenerative diseases [161].

6.2. Apoptosis and aging.

Tumor suppressor – protein p53 responding to the oxidative stress of cells, DNA damage and changed expression of some oncogenes is capable of inducing termination of the cellular cycle and apoptosis development [180 – 182]. Primarily, the relation between protein p53 activation and aging was observed in cells. For instance, injection of viral oncoproteins, mutant p53 forms or antibodies inactivating the "*wild*" p53 protein type decelerated aging of primary human cells [183 – 185]. Vice versa, when cultivated diploid human fibroblasts have aged, the activity of p53 protein increased in them [186 – 188]. Injection of an oncogene *Ras* to primary fibroblasts of mice initiated multiple mitosis, but then stimulated early, "*normal*" p53–dependent aging [189]. All these data have supported the hypothesis that the cellular *replicating* aging operates as the tumor suppressing mechanism [190].

The attempts were also made to determine the influence of p53 protein on the life span of the entire organism. A generation of mice p53 protein deficiency was obtained. Unfortunately, the studies of aging in these experiments were hindered by early cancer development [191].

Among works devoted to relations between apoptosis and aging [51], to our point of view, the work by Tyner *et al.* [192] is of the greatest interest and gives the most unexpected results. They managed to show that p53-mutant mice maintain resistance to cancer, but on the other hand, show signs of accelerated aging. Transgene-encoded mice selected by the authors carried deletions of the six (among eleven) initial exons of p53 gene, which led to "*truncate*" mRNA expression. Though the authors failed to detect a protein

promoting this "m"-mutation in p53+/m mouse tissues, they have determined and increased level of controllable p53 activity in these heterozygotes. Apparently, "m"-allelic gene increased stability of the wild p53 protein by activating NADH quinone oxidoreductases I, which regulated stability of this protein [193]. However, the most intriguing result has indicated that heterozygous p53+/m mice obtained lesser quantity of spontaneous tumors developed, than mice with the wild p53+/+ homozygote, and by several signs displayed faster aging. The frequency of cancer occurrence was < 6% for animals with p53+/m, which is much lower compared with > 45% in animals with p53+/+, whereas the average life duration in these two groups of animals equaled 96 and 118 weeks, respectively.

Concerning the observable signs of aging, it should be noted that p53+/m mice possessed quicker age-dependent loss in the weight of muscles, spleen, liver, kidneys and testicles (Figure 5). Contraction of the mass of organs was always stipulated by total decrease of the cellular structure. Intensified age-induced losses were also observed in the skin thickness, at hair growth, small wound healing, and resistance to stress. Nevertheless, no accelerated aging by other aging markers, including liver pathology, hair canities and alopecia, villous atrophy, skin ulceration, cerebral atrophy, amyloid depositing, cataracts, and general disorganization of the organism functions. In this relation, animals with p53+/m resemble some human syndromes of accelerated aging, for example, of Werner's syndrome type, which may be manifest themselves partly, and incompletely imitate quick human aging [194]. Such manifestations are correspondingly called the "segmental aging syndromes".

The main conclusion made by the authors of these very interesting work is that tumor p53-suppressor plays a significant role in the control of aging and life span of mice. Moreover, the authors suggest that these results support the idea that aging is a mechanism of tumor suppression. Discussing these unexpected results, they conclude that "some of aging signs allow for a suggestion of suppressed stem cell proliferation. During the aging process, this proliferative reserve may decrease quicker in p53+/m mice, because their stem cells are subject to replication aging quicker than in their p53+/+ partners" [192].

However, the suggested explanation seems ambiguous to us, for example, because the confirmations of the idea that stem cells are simply subject to replication aging similar to observations, made for terminally differentiated cells, such as fibroblasts, are rather scanty [195]. We would like

to suggest absolutely different interpretation of these outstanding results related to *the role of the oxidative stress in apoptosis implementation.*

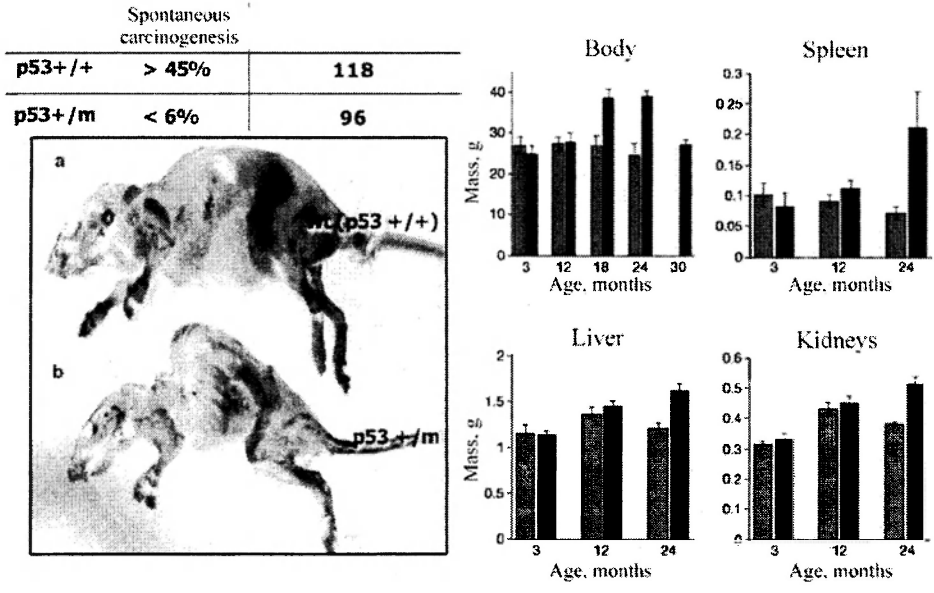


Figure 5. Signs of wild p53+/+ and mutant p53+/m induced aging in mice. Photographs show fully scalped 20 month old mice. Normal mice (a) above, mutant (b) mice below. p53 +/m Mice typically show senile rachiocampsis (lordosis), body mass loss, muscular atrophy, and fat tissue loss. Comparison of body, spleen, liver and kidneys mass in p53+/+ animals (black columns) and p53+/m (light columns) (adapted from [192]).

It is well known that ROS are strong indicators of p53 activity and, moreover, they are of importance for realization of apoptosis dependent on p53. A group of Italian scientists [196] has determined that transformed, p53-deficient mouse fibroblasts are much more resistant to cytotoxic effect of a sequence of prooxidants than the wild type control.

The activity of MnSOD was also increased in the liver tissue of mice with p53 deficiency compared with the wild type tissue. Vice versa, a short-term p53 (wild type) transfaction into HeLa cells led to a significant reduction of the stationary MnSOD mRNA level and its enzymatic activity showing that

the expression of this antioxidant enzyme is negatively regulated by p53. These data identify MnSOD as a potential addressee for the tumor suppressor p53 and outlines relevancy of MnSOD modulation, because, apparently, it is required with respect to a multiplicity of facts about abnormally increased MnSOD expression in human cancers. Actually, inhibition of MnSOD activity makes cancer cells, for example, rectum cancer cells with wild type p53, more sensitive to apoptosis, induced by radiation, hyperthermia and specific antitumor preparations [197].

Beside p53 and MnSOD, a complex reaction chain leading to apoptosis includes expression products of more than 20 genes, in particular, caspase cascade, mitochondrial cytochrome c and poly(ADP-riboso)polymerase I (PARP). Protein p53 is the addressee of the PARP nuclear fragment, activated with DNA damaging by genotoxic agents [198]. Increased MnSOD expression inhibited PARP activity that activated protection against apoptosis [199].

The results of these investigations can be considered as an evidence of ***negative p53 regulation by MnSOD.***

Direct proofs of the existence of mutual negative (reciprocal) regulation of MnSOD and p53 gene expression from the side of their proteins, respectively, were obtained by Pascal Drane et al. [200]. It has been shown that the wild type p53 suppresses MnSOD gene expression at the *promoter* level. In its turn, increased MnSOD expression reduces transcription of p53 gene at the *promoter* level and inhibits mediated p53 induction of apoptosis. This influence of MnSOD limits p53 ability to stimulate apoptosis by a multistage reaction, which includes induction of transcription of several genes related to the redox potential and important for ROS synthesis, and prevents oxidative degradation of mitochondrial components and, therefore, apoptosis.

Turning back to accelerated aging induced by nonforecasing influence of increased p53 activity [192], we can suggest the following explanation for these unexpected effects. As mentioned above, p53 protein specifically (at the promoter level) inhibits MnSOD mitochondrion expression. As a consequence, increased p53 activity will lead to a significant decrease of antioxidant potential of mitochondria and the entire cell that must increase the degradative effect of ROS which, finally, will be expressed in *mitoptosis* development (see below for deatils) and the following apoptosis. Further on, as is shown [201, 202], nonspecific cytolysis of primary apoptotic cells will induce their *clusterization* that, obviously, must reduce masses and functions of organs and tissues. The value of this reduction will depend on both p53 activation level and total antioxidant potential in one organ or tissue or another. This very decrease of the *cellularity* and mass of many organs and tissues was observed

[192] in mutant mice with increased p53 concentration (Figure 5). It is the authors' opinion that all these factors predetermine a significant shortening of life for p53+/m heterozygous mice. Of course, permanent oxidative stress is able to intensify replicative aging [203, 204], to which the authors relate the observed phenomena. However, in the current experiment this factor seems to be of the secondary importance.

In a broader biological sense, at the cellular level these reciprocal relations between p53 and MnSOD look like a competition between systematic protection mechanisms of the entire organism (p53 protects the organism from tumor development) and intracellular protection mechanisms against oxidative stress. It is noted that cancer cells show extremely high MnSOD activity and, as a rule, low p53 activity that provides them with protection against various antitumor therapeutic treatments [199].

Hence, high p53 activity suppresses MnSOD and leads [192] to early aging. Moreover, transgenic mice with fully absent MnSOD gene die a week after birth due to cardiomyopathy development [211]. Apparently, the rates of carcinogenesis and aging processes are defined by complex relations between systemic protection mechanisms (the immune system, p53 and apoptosis) and subcellular antioxidant and DNA-repairing enzymatic systems.

6.3. Mitoptosis – the functional precursor of apoptosis.

V.P. Skulachev [205, 206] was the first who suggested an interesting hypothesis about the mechanism of apoptosis initiation under the influence of mitochondrial oxidative stress. The well-known function of mitochondrial protein of heat shock (mHsp70) consists in both correction (*the chaperon function*) of mitochondrial denatured protein structure and stimulation of transport of encoded nuclear DNA of proteins from cytoplasm to these organelles. In particular, this stimulated transport is displayed in the activation of oxidative phosphorylation (and, consequently, in the increase of ROS production). Besides, mHsp70 has one more property: it participates in the composition of mitochondrial endonuclease (Sce I), the enzyme *breaking mtDNA in strictly defined places*. To avoid mtDNA degradation, activity of this endonuclease in norm must be suppressed. Apparently, mHsp70 plays the role of regulatory Sce I subunit, because the active site of this enzyme is localized on another subunit of it. It is suggested that mitochondrial denatured proteins link ROS stoichiometrically. Further increase of denatured protein quantity

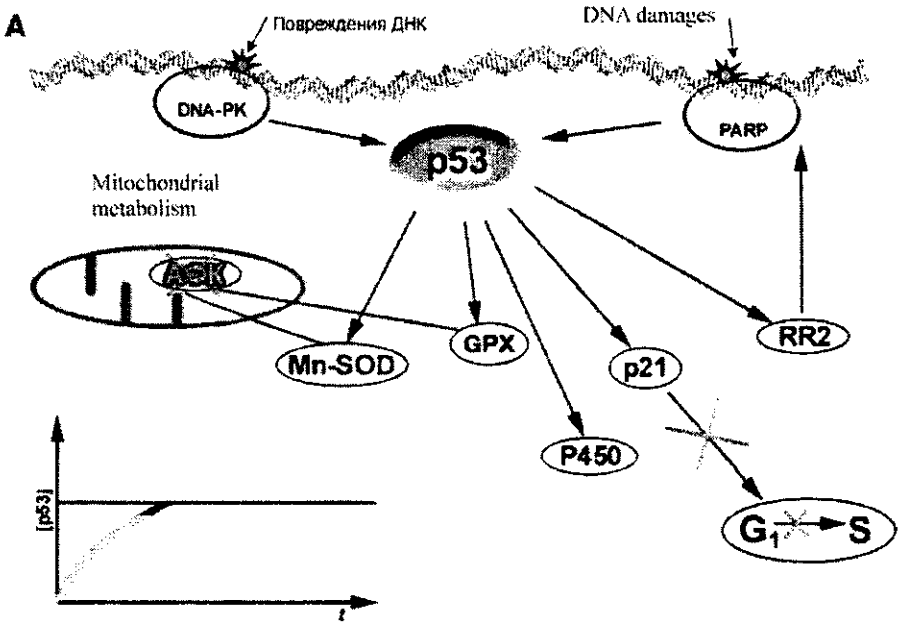
affected by ROS will cause stronger bonding of mHsp70 and, finally, removal of endonuclease regression, and then to mtDNA degradation. As mentioned above, we suppose that this mechanism can provide the foundation for formation of multiple mtDNA deletions. All this will induce degradation of the current mitochondrion. This way might be apparent for purifying the mitochondrion population from those possessing too much quantity of denatured proteins. V.P. Skulachev has called similar phenomenon the ***mitoptosis*** [205, 206] and proved that it may also induce the cellular apoptosis, if mitoptosis is of the massive type, because degradation of mitochondria must be accompanied by release of mitochondrial proteins – the apoptosis initiators – to cytoplasm: *cytochrome c, apoptosis inducing factor (AIF), and caspase 3* (Figure 4).

6.4. Possible inversion of p53 functions

In recent 10 – 15 years, intensive studies of subcellular and molecular mechanisms of apoptosis allowed for determination of expression sequence of a group of genes (and intracellular processes) and a complex system of their interaction which, finally, leads to apoptosis of a cell, if damages of its DNA have become irreversible [154 – 157]. Among these genes and their expression products united by the general control system, first of all, ***poly(ADP-riboso)polymerase-1 and DNA-dependent protein kinases*** should be mentioned [29, 30], which inform ***p53*** about DNA damages and stimulate its activity by increasing the corresponding gene expression or increasing (or reducing) the life time of p53 protein. This key event includes the mechanism of cellular cycle termination and/or a complex cascade degradation mechanism of subcellular structure, which leads the cell to apoptosis.

Recently, besides the above-mentioned large group of genes invoked in apoptosis, a direct regulatory connection between p53 and functionally different group of genes playing an important role in the intracellular protection system. First of all, these are enzymatic antioxidation, xenobiotic neutralization and DNA repair systems.

The *inhibiting* effect of p53 on transcription of gene encoding mitochondrial MnSOD [200] have been thoroughly discussed above in the context of cellular mechanisms, which include a reaction cascade leading to apoptosis.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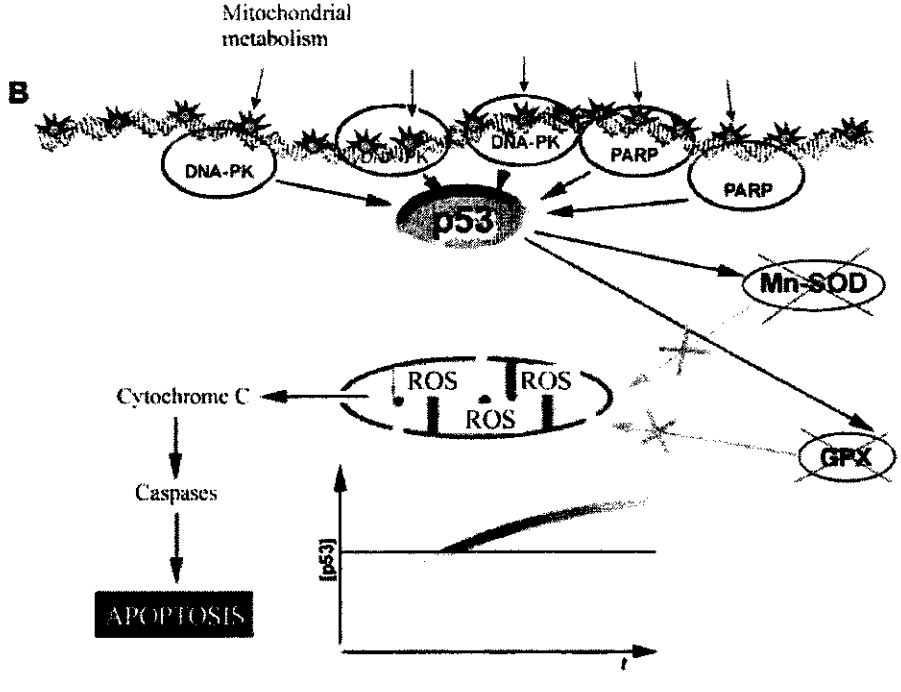
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Figure 6. The relation of p53 protein with protective cellular systems

A. At low DNA damage level, low activity of poly(ADP-riboso)polymerase I (PARP) and DNA-dependent protein kinase (DNA-PK)induces a low p53 activity level. It is suggested that under these condition p53 optimizes superoxide dismutase (MnSOD) and glutathione peroxidase (GPX), protection against xenobiotics – P-450 isoenzymes, the repair system – ribonucleotide reductase (RR2) subunit isoform. The activity of all these enzymes is directly regulated by p53 (see the text) that provides for stationary mode of the cell functioning.

B. At high level of DNA damages, PARP and DNA-PK responses significantly stimulate p53 content and activity that inhibits protective systems and, especially, MnSOD and GPX. This will removes protection and induce a sharp increase of ROS production, POL activation and future breaks of the mitochondrial membrane structure with the output of proapototic factors to the cytoplasm: cytochrome c, apoptosis inducing factor (AIF), and caspase 3

Vice versa, recently, it has been shown [207] that p53 activates glutathione peroxidase (GPX) gene transcription at the promoter level. This glutathione peroxidase is the route antioxidant enzyme, which neutralizes hydrogen peroxide or organic hydroperoxides. Endogenic GPX expression was significantly activated at both mRNA and enzymatic activity levels. As a consequence, GPX is a new target gene for p53.

One more apparent target of p53 protein is presented by isoenzymes P-450 which neutralize xenobiotics appearing in the organism. An indirect proof of this is presented by data, according to which mutant p53 +/– mice have displayed no induction of P-450 isoenzyme synthesis (compared with the synthesis level in the wild type mice) in response to introduction of a strong xenobiotic *nitrosamine* [208].

To conclude the discussion, Tanaka *et al.* [209] have observed that in case of serious DNA damages, p53 specifically induces expression of previously unknown isoform of a small subunit of ribonucleotide reductase (R2), which is included in repair only and localizes in the cell nucleus, and intensifies ribonucleozide diphosphate transformation to deoxyribonucleozide triphosphates nearby the DNA damage zones. After that the cell continues to

vitally exist. It should be mentioned that ribonucleotide reductase (RR) is the key enzyme in the whole enzymatic chain of DNA repair [210].

A definite discrepancy in results of p53 effect on antioxidant enzyme genes (MnSOD inhibition and GPX activation), p53 action as a stimulant on small PP–R2 and P-450 subuinit isoforms, the absence of data on relatively concentrational relation in p53 influence on these targets, as well as yet far from disclosing a complex unity of p53 functions and its interrelations with various cellular systems, allowed us for putting forward a hypothesis about p53 inverse action on protective systems of the cell.

In the case of normal cellular cycle and at rather low damaging of DNA, relatively low p53 concentration will continuously stimulate intracellular enzymatic protection systems: RR – the key factor of DNA repair; MnSOD – the first barrier on the way of ROS flow; GPX – the second barrier on the way of ROS flow; P-450 isoforms – the barrier for xenobiotics already penetrated into the cell. Similar optimal "tonus" of the protective system functioning forms conditions for long-term stationary mode in the vital activity of the cell (Figure 6A).

If under different conditions of metabolism, under the effect of exogenic or endogenic damaging factors, the level of DNA damages will be significantly increased and, therefore, automatically activate poly(ADP-riboso)polymerase–I and DNA-dependent protein kinases, p53 concentration will steadily increase. This raise of p53 concentration will now inhibit RR activity, DNA repair, P-450 activity, and mostly activity of antioxidant enzymes – MnSOD and GPX (Figure 6B). This will increase the flow of ROS and free radicals activating all mitochondrial ways of apoptosis, which were numerously mentioned above (Figure 4).

Actually, the working hypothesis cannot give full description of cellular stability and programmable cellular death mechanisms. However, it discloses possible ways for its experimental confirmation.

7. WHY SOMETIMES THREE PARENTS ARE MORE USEFUL (FOR THE SAKE OF CONCLUSION)

Yes, three parents are more useful: one father and two mothers – the main mother and a secondary mother. This was the true topic for discussion in many papers and mass media all over the world, reflecting the novel ideas and

preliminary experiments of British scientists. Being highly affected by the success in studying the role of mitochondria and their genome in pathogenesis of some diseases and progression of aging, they have suggested an interesting procedure, concluding in the following. Any normal ovum is fertilized by normal spermatozoon. Therefore, normal zygote is formed, from which a normal organism will develop. But if ovum mitochondria (and we know that they are present in the ovum only) are loaded by hereditary mutations (the quantity and character of which can be simply determined by modern methods of molecular genetics), especially in respiratory enzyme subunit genes, the future descendant is doomed to some level of the energy deficiency in cells and tissues which, as is known, may induce mitochondrial diseases, accelerated aging, and inadequate apoptosis of cells. Could we avoid this? Definitely, yes. We have just to find a woman without any hereditary mutations (or with a low level of the later), remove haploid nucleus from her ovum and inject diploid nucleus from the above-mentioned zygote. The technique of growing up an organism from fertilized ovum is well developed and widely introduced into practice. Hence, we will get an organism carrying nuclear heredity of father and the main mother, and healthy mitochondrial heredity of the secondary mother, which guarantees normal energy basis for the future organism. Smart, ain't it?

Of course, practical application of the current approach is rather ambiguous. However, the extraordinary break-through in the investigations of mitochondrial oxidative stress, mtDNA mutations and their role in pathogenesis of mitochondrial diseases, aging acceleration, and apoptosis activation significantly extends our understanding of the living nature and provides for broad perspectives in searching for possible therapeutic treatments of diseases, reduction of aging and limitation of programmable cellular death.

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THE STUDY OF BIOLOGICAL ACTIVITY OF ANTIOXIDANTS ON HERBAL OBJECTS

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Studying kinetic regularities of animal and human cancer development, N.M. Emanuel has also noticed tumor processes in plants. In 1975, he established the laboratory for physicochemical fundamentals of herbal system stability. One of the tasks of this laboratory was the study of regularities of cancer disease development in plants and possibilities of their inhibition using bioantioxidants. Investigations were carried out both in the laboratory and in the field on the classical type of plant tumors, induced by phytopathogen *Agrobacterium tumefaciens* (Smith and Town) Conn. The study of tumor growth, induced by this phytopathogen in tomatoes and greens indicated description of this process by an S-shaped kinetic curve (Figure 1), i.e. they obey one of the laws defining development of tumors in animals [1, 2].

The important physicochemical characteristic of tumor growth and development is the change in the concentration of free radicals in tumoral tissues [3]. Kinetic regularities of changes in concentrations of free radicals were studied on the example bacterial cancer in greens plantules, induced by *Agrobacterium tumefaciens*. It has been shown that the processes of tumor growth and changes in free radical concentration possess several interrelated stages. Hyperbolic dependence of variations of free radical concentration characterizes the induction period of tumor growth, which includes bacterial transformation (1 – 4 days) and tumor formation (5 – 9 days) processes. The lag-phase of tumor development is accompanied by sharp increase of free radical concentration, whereas the terminal phase is characterized by abrupt decrease of this parameter. The free radical concentration maximum is reached at the moment of reaching the highest rate of tumor growth. Generally, this phenomenon is similar to the previously determined regular biophysical shift at development of regrafted animal tumors [4]. Concentrations of free radicals were measured by ESR-spectroscopy method. This work was implemented by L.P. Kayushin and G.N. Bogdanov.

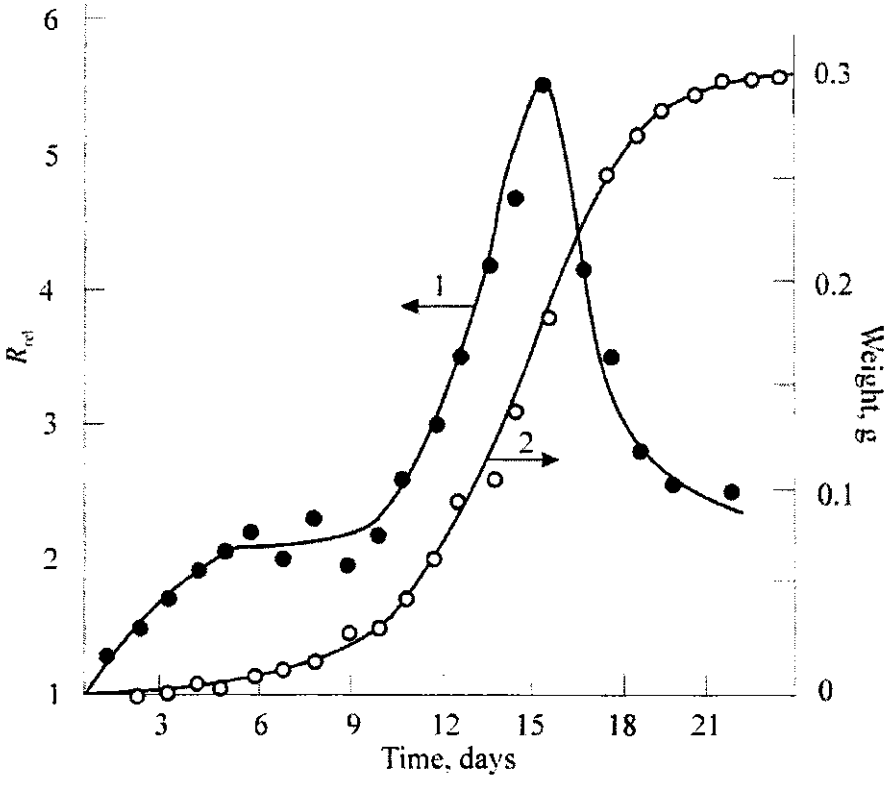

Figure 1. Changes in free radical concentration in tumor tissue of greens with respect to the tumor development stage
1 – free radical concentration; 2 – tumor weight

Also, N.M. Emanuel has suggested the study of influence of free radical inhibitors and antitumor compounds, already studied in animals, on free radical concentration in tumor tissues of plants. For this purpose, propylgallate (PG) and N-nitroso-N-methylurea (NMU) were tested. The treatment of ten-day tumors by 0.25% aqueous solutions of these compounds inhibited their growth and abruptly decreased free radical concentration in the tumor tissue. In this case, PG demonstrated the highest effectiveness (Figure 2).

Tumor diseases in plants, induced by phytopathogenic bacteria, are widely spread in the nature. Bacterial cancer can damage about 40 families of plants. Fruit trees and grape are mostly subject to such damages. Cancer in plants, induced by *Agrobacterium tumefaciens*, represents a hyperplastic disease, at which, as a result of uncontrollable cell fission, tumors are formed

in the damaged tissue. Genetic mechanisms of tumor formation in plant are similar to viral cancer in animals. For argobacteria, the role of its plasmids in tumor formation process is typical of [5].

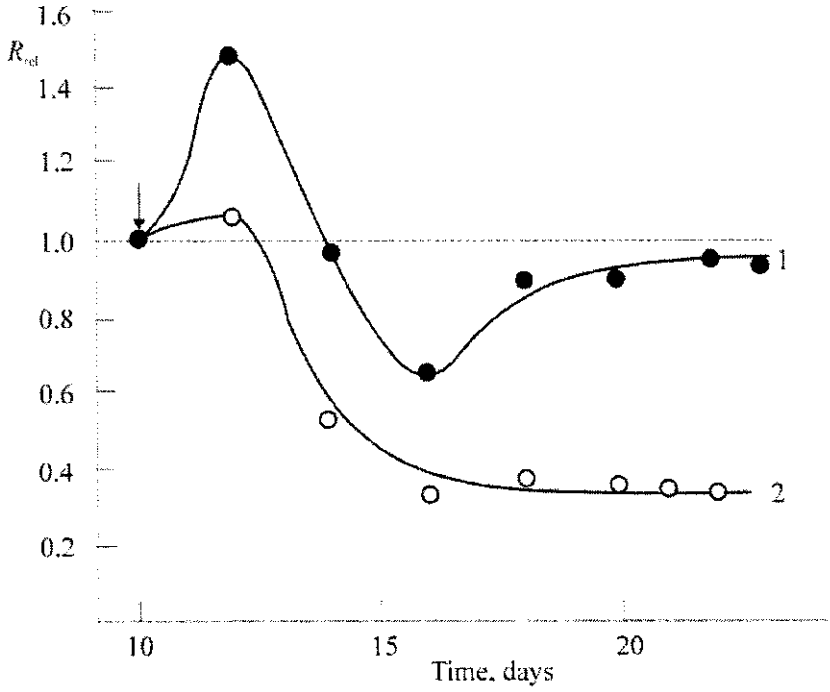

Figure 2. Changes in free radical concentration in tumor tissue of greens after treatment by N-nitroso-N-methylurea (1) and propylgallate (2)

Bacterial cancer is one of the main diseases, which causes thinning out and premature death of grape trees. Meanwhile, our laboratory in cooperation with Moldova Institute of Viniculture and Wine Making have carried out investigations on the grape willows how antioxidants behave as effective compounds suppressing the disease. The field investigations were supervised by N.B. Lemanova [6]. The results have shown that one-time treatment by antioxidants of bacterial cancer damaged grape willows in the early spring decreases frequency of new tumor occurrence for healthy plants by 4 – 8 times. Therefore, the disease spreading is practically stopped. For damaged plants, the frequency of new tumor formation was decreased by 3 – 4 times [7, 8]. Antioxidants hydroquinone and phenosan, synthesized in the division headed

by N.M. Emanuel, were found the most effective antioxidants. According to experimental results, these compounds possess antitumor activity enough for their recommendation for practical use.

In the course of investigations of antioxidant mechanism in plants, we have studied their effect on ethylene-forming enzyme in plants, which is potential target for antioxidant action. This enzyme synthesizes ethylene-forming phytohormone responsible for plant development. For the first time, it is shown that enzyme sensitivity to antioxidants is different *in vivo* and *in vitro*: it is inhibited by antioxidants *in vivo* and not inhibited *in vitro*. Several hypotheses reasoning this phenomenon and describing its meaning for the plant development are put forward. New inhibitors of ethylene-forming enzyme were discovered. ESR data indicated formation of inhibitor radical at the enzyme inhibition *in vitro*. This testifies about radical mechanism of 1-aminocyclopropan-1-carboxylic acid (ethylene precursor *in vivo*) oxidation to ethylene by this enzyme [9].

Since free-radical mechanisms play the decisive role in radiation damaging of animals and plants, N.M. Emanuel suggested a series of experiments on plants, studying radioprotector and antimutagenic properties of synthetic and natural antioxidants. Rutin, quercetin, dihydroquercetin, hydroquinone, and some compounds synthesized in the division, headed by N.M. Emanuel (phenosan, stable nitroxyl radicals, ambiol and its homologues), were studied. It has been shown that all studied compounds reduce the frequency of radiation-induced chromosome aberrations in meristem of primary roots of plants by 3 – 7 times [10, 11]. N.M. Emanuel was the co-author of three patents for the most active compounds.

Consequently, these works were continued in studies of the plant genome response to low-dose radiation impact. It is found that antioxidant treatment of seeds, both before and after impact of adaptive and damaging radiation, allows increasing the adaptive response or detecting it, if without treatment it is absent [12 – 14].

As shown in special works [15, 16], preliminary treatment of seeds by antioxidants reduces mutagenic effect of subsequent action of alkylating agents. The detailed study of antioxidant mechanisms increasing the adaptive response and antimutagenic effect shows that these two properties are based on the single inducible process, which is called stimulated repopulation. The specific feature of this system is that it protects genome by total substitution of damaged cells by undamaged ones in the population rather than intracellular repair of DNA damages. This happens due to involving of additional, not

dividing in norm, cells into the mitotic cycle. Actually, contrary to enzymatic DNA repair, which is not always full and exact, repopulation is really exact process and, if it is complete, eliminates all premutational damages. Contrary to simple repopulation, stimulated repopulation requires participation of some additional push, which starts up the system and forms conditions for its operation. The antioxidant effect plays the role of this stimulus.

In this article, we described just the most important works studying antioxidant effect on plants. Completing the investigations, initiated by N.M. Emanuel, we have proved experimentally a possibility of antioxidant application to solving the following urgent practical tasks:

1) protection of cultured plants against cancer and other diseases;
2) plant genome protection from damaging and mutagenic action of physical and chemical factors;
3) growth stimulation and protection of agricultural plants and productivity increase;
4) the increase of agricultural product protection during storage.

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PRINCIPLES OF GENETIC ACTIVITY OF NITRIC OXIDE (NO) AND NO-DONATING AGENTS IN MODELING OF THE CELL STRESS RESPONSE AND NEW DRUG DEVELOPMENT

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> Combined chemotherapy becomes the basic method for therapeutic treatment of tumors Drugs for combinations are usually selected basing on the specificity of each of them.
>
> N.M. Emanuel, 1977

In recent decades, the progress in biological science was marked with the discovery of the fundamental role of nitric oxide radical – a simple chemical compound, formed enzymatically in the organisms and being one of the universal metabolism controllers – in the vital activity of the cell.

Nitric oxide (NO) is adapted well for implementation of signaling functions in biological systems. This is confirmed well by the wide spread of NO-synthases in fauna representatives [1], higher plants, fungi and bacteria [2]. Reduction of nitrates by bacteria, fungi and plants is the alternative source of NO for them [3].

In mammals, NO is generated by L-arginine, O_2 and NADPH NO-synthase [4, 5]. Being extremely reactive, NO implements vascular relaxation, neurotransmission, is included in organism protection against pathogens and tumor cells [6, 7].

Clearly in the biological environment, NO exists and is transported in the form of more stable complexes, such as dinitrosyl iron complexes with thiol-containing ligands (dinitrosyl iron complexes – DNIC), nitrosogluthatione (GSNO), nitrosocysteine, S-nitrosopenicillamine (SNAP), etc. [8].

In line with the same functions in various physiological and biochemical processes, NO participates directly in genetic transcription and translation processes. Low NO stability in cells and tissues, generally stipulated by the lethal effect of superoxide O_2^- anion, is overcome by including of NO molecules into S-nitrosothiols (RS-NO) and DNIC with natural thiol-containing ligands $(RS^-)_2Fe^+(NO^+)_2$, acting as the NO deposit and transporters of the molecule in cells and tissues [8]. RS-NO and DNIC may act both as donors of neutral NO molecules as well as nitrosonium (NO^+) ion and $Fe^+(NO^+)_2$ group donors, and provide for RSNO and DNIC functioning as an original signaling molecules. The bonding of NO^+ and $Fe^+(NO^+)_2$ with thiol groups of proteins and enzymes induces S-nitrogenation of these biomolecules or formation of DNIC bound to them, respectively, and as a consequence, the change of the functional activity of the proteins and enzymes.

An increase in functional activity of proteins-chemosensors – the transcription regulators for corresponding regulons, results in increasing of the gene expression. To study NO functional activity, we have selected proteins-regulators of DNA repair systems and expression of corresponding genes in E.coli as the models: SoxR [2Fe-2S] protein – SoxRS DNA repair system of oxidative DNA damages (gene reporter SoxS), Ada protein – Ada-regulon and addaptive response to alkylating DNA damages (alkA, alkB and aidB genes-reporters), and RecA controlling SOS DNA repair system, induced by single- and double-strand DNA breaks (gene reporter sfiA).

BACTERIAL STRAINS

The sfiA gene expression was studied on *E.coli* isogenic strains: PQ65 uvr+ (F^-, thr, his, pyrD, galE, galY, rpoB, ΔlacU169, trp::MuC$^+$, Pho^c, sfiA::Mud(Aplac)C^{ts}) and PQ66 - uvrA mutant of PQ65. The study of soxS gene expression was performed in *E.coli* strain TN530 (F^-, Δ(lacU-argF)U169, rpsL179, soxRS$^+$, λϕ(ΔsoxS'::lacZ)). For Ada protein activation the expression of Ada-controllable genes alkA, alkB and aidB was studied in the following strains: MV 1571 alk51::Mu-d1 (Ap lac) thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 rfa-550; MV 1601 – isogenic MV 1571, but alkB52::Mu-d1 (ApR lac); MV 2176 – isogenic MV 1571, but aid::Mu d1 (bla lac)TR Δ(argF-lacZ)205(u169). In the mentioned strains, *β*–galactosidase structural gene lacZ

is controlled by a gene-reporter promoter, and genome possesses a deletion of chromosomal lac-operon. Therefore, the expression of genes under study is measured indirectly, basing on β–galactosidase activity in a simple colorimetric assay.

The absorption value was measured at λ = 420 nm. The activity of β–galactosidase was calculated by the following equation:

$$(\beta\text{-Gal}) = \frac{D_{420}}{t},$$

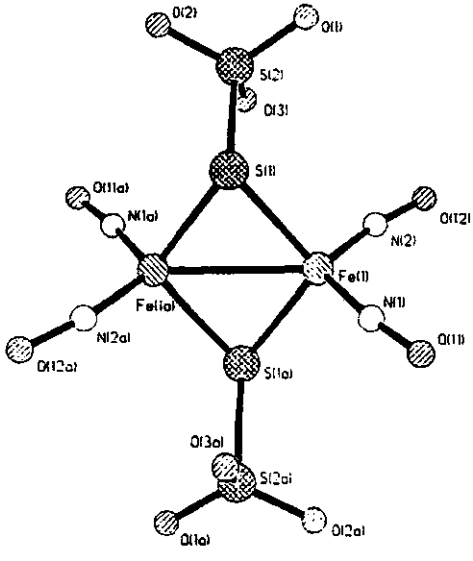
where D is the absorption value at 420 nm; t is time of incubation with chromogene. The relative activity of β–ga lactosidase was calculated by the equation:

$$(\beta\text{-Gal})_{\text{rel}} = \frac{(\beta-\text{Gal})_0 - (\beta-\text{Gal})_c}{(\beta-\text{Gal})_c},$$

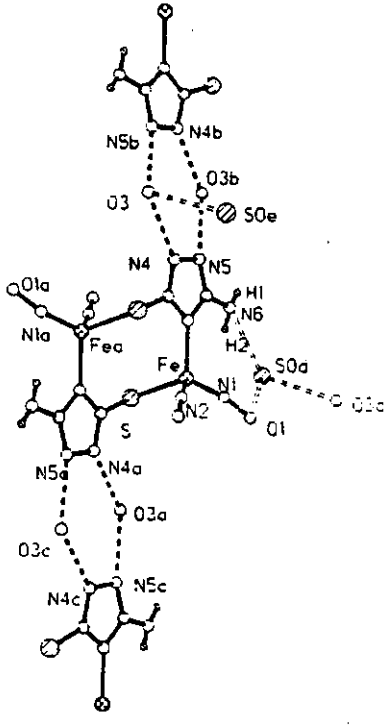
where $(\beta\text{-Gal})_0$ is β-galactosidase activity in cells treated with the mentioned agent; $(\beta\text{-Gal})_c$ is β-galactosidase activity in untreated control.

The positive control in experiments of gene expression was 4HXO in 2.63 nM concentration.

We have achieved the comparative study of DNIC genetic activity with natural ligands: cysteine (DNICcys), glutathione (DNICglu), and several polycrystalline nitrosyl complexes of iron with mononuclear $[Fe(SR)_2(NO)_2]^-$ and binuclear $[Fe_2(SR)_2(NO)_4]^-$ synthetic ligands, newly synthesized in IPCP RAS (Chernogolovka, Moscow District, Russia) [9] (Figure 1). The main attention was attracted by determination of the chemical origin of genetic signal transduction initiators in the mentioned regulons.



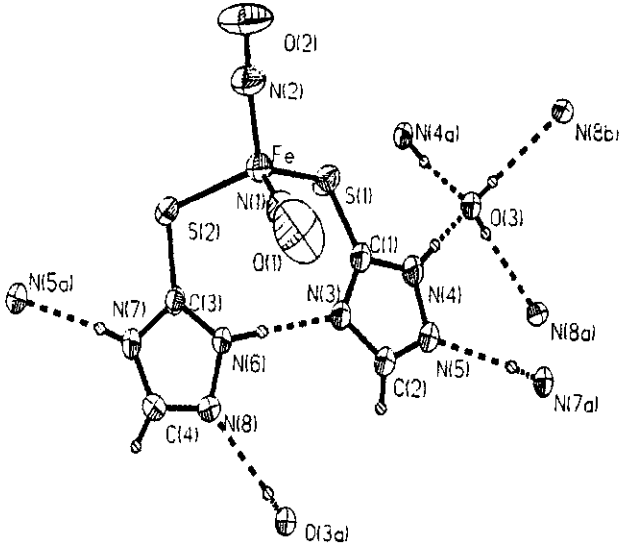
(a)



$Fe_2[(SC_2H_2N_4)_2(NO)_4]\cdot 2H_2O$

(c)

Figure 1



$Fe(SC_2H_3N_3)(SC_2H_2N_3)(NO)_2 \cdot 1/2H_2O$
(b)

Figure 1. Structures of stable NO-donating agents: *a* – thiosulfate dinitrosyl iron complex (TNICthio); *b* – triasolyl dinitrosyl iron complex (DNICtria); *c* – aminotriasolyl tetranitrosyl iron complex (TNICatria)

DNIC STABILITY IN AQUEOUS SOLUTIONS

In contrast with the dimer form of DNICglu, DNICthio crystalline preparations (including thiosulfate), DNICtria (with triazole), and TNICatria (with aminotriazole) are characterized by low stability at dissolution in water. This is confirmed by quick elimination (within 20 – 30 min) of ESR signal, typical of this complex, at g = 2.03 in the aqueous solution at neutral pH. Further corresponding injection of thiosulfate, triazole or aminotriazole did not reduce paramagnetic properties of these complexes. Dimeric shape of DNICglu in the aqueous solution gave no detectable ESR signal due to spin coupling, typical of this shape, in mononuclear DNIC forming the dimer. ESR signal in

this complex solution sharply increased with injection of thirty-time excessive glutathione quantity and solution pH increase to 10 – 11. This property of dimer DNICglu was preserved at its exposure to aqueous solution during 5 – 6 h. Decomposition of DNIC and TNIC crystalline preparation in the aqueous solution was accompanied by formation of iron hydroxide residue, precipitated from complexes at their degradation, and nitric oxide formation in the solution. The latter was detected by its vasodilating effect on isolated coronary vessels in rats. This activity was interlocked by guanylyl cyclase inhibitor – methylene blue. As is known, activation of this enzyme by nitric oxide initiates vasodilation.

INDUCTION OF sfiA AND SoxS GENE EXPRESSION IN *E.COLI* CELLS BY DNIC AND TNIC

All DNIC and TNIC studied in the current work possessed relatively low cytotoxicity (Figure 2) and were able, with respect to their concentration, to activate *E.coli* SOS and SoxRS regulons (Figures 3 and 4).

The value of sfiA gene expression was 2 – 4 time higher in the mutant strain PQ66 uvrA than in isogenic PQ65 uvr+ strain, independently of the structure studied nitrosyl iron complexes. At low concentrations, all studied compounds induced nearly identical expressing of sfiA gene in PQ66 uvrA cells, whereas in PQ65 uvr+ cells this process was only initiated by polycrystalline preparations of the complexes. However, as the inducer concentration increases to 80 (M or higher, the activity of these preparations was abruptly decreased, obviously, because of their cytotoxicity. At higher concentrations, more stable and lower cytotoxic DNICglu activated sfiA gene expression. For example, at 0.5 mM concentration this expression was two times or more higher than the maximum values typical of polycrystalline complexes in PQ66 uvrA strain, whereas in PQ65 uvr+ strain it sharply increased from almost zero values to the level typical of polycrystalline TNIC and DNIC. Analogous situation was observed at the activation of SoxS gene expression (Figure 4).

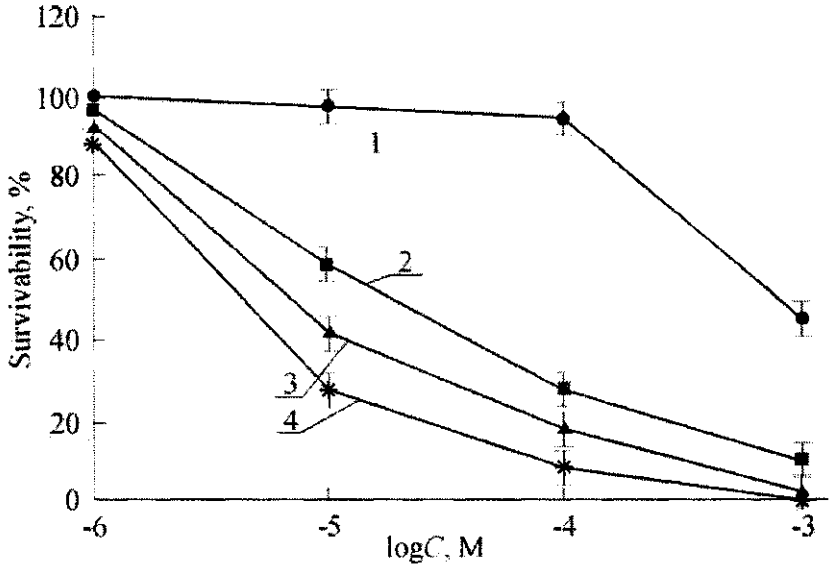


Figure 2. Comparative cytotoxicity of NO donors. Survivability of *E.coli* PQ66 treated by: DNICglu (1), TNICthio (2), DNICtria (3), TNICatria (4)

In accordance with the data, previously obtained by the authors on the interlocking action of bivalent iron chelator, which is *o*-phenanthroline, on sfiA and SoxS genes expression induced by DNICglu or DNICcys [10, 11], this chelator strongly suppressed (down to the basal level) expression of these genes, induced by polycrystalline DNIC and TNIC. As an example of this action, Figure 5 shows experimental results on SoxS gene expression induced by DNICthio in *E.coli* TN530 cells in the presence of 0.5 mM phenanthroline or in its absence. Special experiments show that in this concentration *o*-phenanthroline has not cause a noticeable toxic effect on tester *E.coli* strains.

It should be noted that incubation of bacteria with all studied DNIC and TNIC induced occurrence of paramagnetic DNIC in them, characterized by ESR signal with $g_{\perp} = 2.04$ and $g_{||} = 2.014$, when frozen. As the recording temperature increased to room level, the signal shape did not also change. This fact may testify about protein origin of these complexes. Obviously, their formation was caused by $Fe^{+}(NO^{+})_2$ group transfer from lowmolecular DNIC and TNIC to thiol groups of proteins. The addition of *o*-phenanthroline to cellular suspension induced elimination of ESR signal and coloring the

suspension in red. The latter is the result of complex formation with bivalent iron at DNIC degradation.

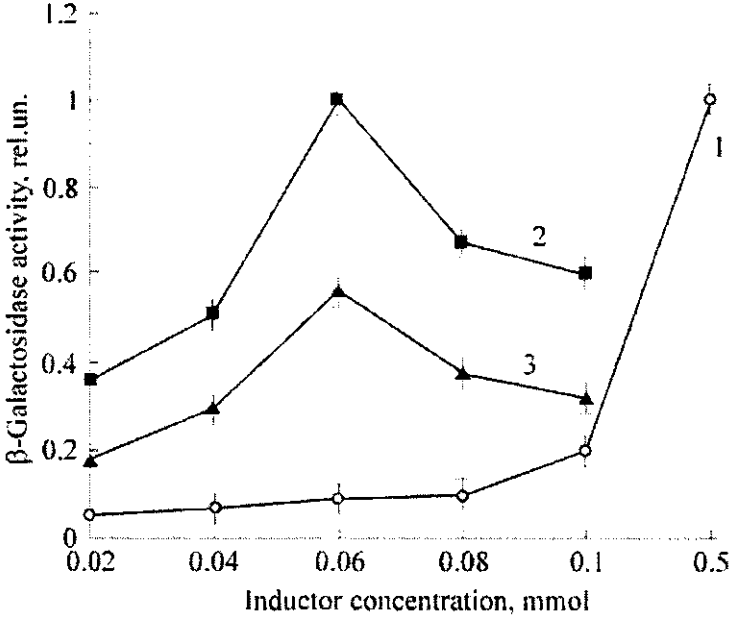


Figure 3. Induction of sfiA gene expression in *E.coli* PQ65 uvr+ by NO donors. Dependence of *β*-galactosidase activity on the concentrations of DNICglu (1), TNICthio (2), DNICtria (3)

Thus, nitrosyl iron complexes (DNIC and TNIC) themselves, but not their components – nitric oxide and bivalent iron present being chemically equilibrated in solution with the complexes – are responsible for SOS and Sox RS activation for *E.coli* regulons. Compared with more stable DNICglu preparations, low stability of polycrystalline DNIC and TNIC preparations in aqueous solution testifies about a shift of the mentioned equilibrium towards their components for the first type of complexes. Therefore, if free NO molecules and bivalent iron ions themselves induced sfiA and SoxS gene expression, then DNIC and TNIC polycrystalline preparations in the aqueous solution would be more effective inducers than DNICglu, which is not observed in the experiment. On the contrary, accumulation of these free components in solution with increasing concentration of complexes caused not

the inductive but toxic effect on the cells according to the above-described mechanism.

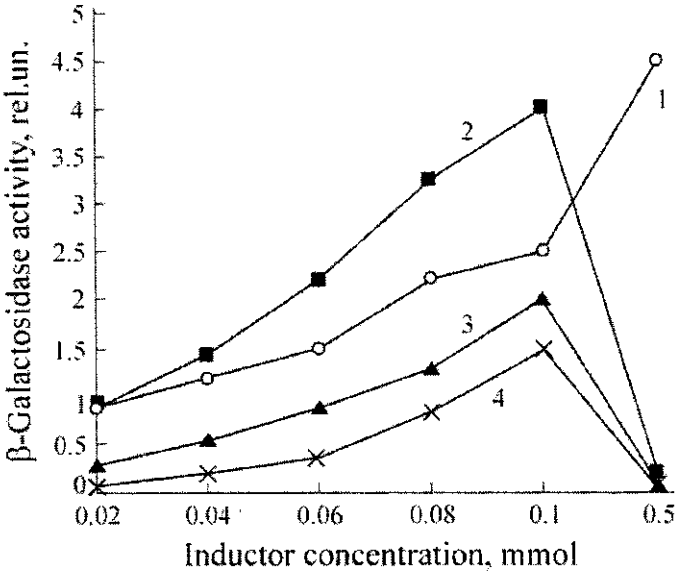


Figure 4. The induction of SoxS gene expression in *E.coli* TN530 by NO donors. Dependence of *β*-galactosidase on the concentrations of DNICglu (1), TNICthio (2), DNICtria (3), TNICatria (4)

This is also confirmed by the results of authors' experiments on the comparative SoxS gene expression by DNIC aqueous solution or a mixture of DNICthio and excessive ligand – sodium thiosulfate (1:5). The excessive ligand had no effect on SoxS gene expression and the indices of *β*-galactosidase relative activity equaled 0.8 and 1.0, respectively. Meanwhile, no changes in pH of the reaction mixture with excessive sodium thiosulfate were recorded.

A different situation was observed at incubation of cells with stable and, therefore, low-toxic DNICglu: as its concentration in the cellular increased, expression of both sfiA and SoxS genes was intensified (Figures 3 and 4). This means that the complex itself has initiated the process. Degradation of this complex as well as its polycrystalline analogues, caused by *o*-phenanthroline, led to an abrupt decrease of gene expression activation, and nitric oxide, released in this case, did not display such activity (Figure 5).

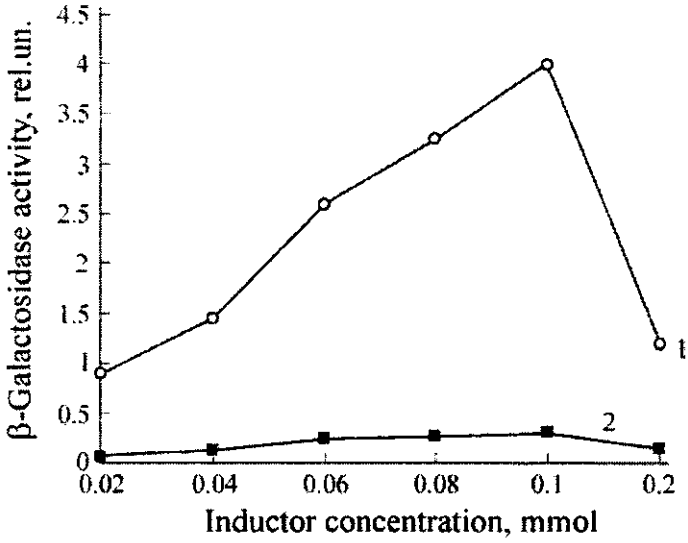


Figure 5. Suppression of soxS gene expression in *E.coli* TN530 under treated by TNICthio: 1 – without *o*-phenanthroline preprocessing; 2 – preprocessed by 0.5 mM *o*-phenanthroline

Despite the intensive efforts of the investigators undertaken during 30 years, the molecular start-up mechanisms for polyfunctional SOS regulon in *E.coli* are not completely determined yet.

It is shown that nitric oxide (NO) cytotoxic and cytogenetic effects are stipulated by active oxidant formation – peroxynitrite, in the reaction of NO interaction with superoxide anion-radical:

$$NO^{\bullet} + O_2^{\bullet} \rightarrow ONO_2^{-}$$

or protonated shape of peroxynitrite

$$^{\bullet}N{=}O + {}^{\bullet}O{-}O^{-} \rightarrow O{=}N{-}O{-}OH.$$

It is found that penetrating through membranes with the help of anion transmitters, peroxynitrite and its protonated shape have a broad spectrum of chemical activity, induce DNA strand break, oxidize its bases, nitrate guanine and various proteins, oxidize lipids of biological membranes, induce

degradation of Fe–S sites of several basic enzymes, including aconitases and the respiratory chain components. The mentioned interactions result in cytogenetic and mutagenic effects.

Taking into account the type of DNA damages produced by NO action, and as a consequence of these damages, we have suggested formation of SOS-signal and DNA repair SOS-system activation in *E.coli* cell. This is confirmed by the results of our experiments.

Many investigators associate the activating influence of NO-containing compounds on SoxRS-regulon with Fe-S site degradation in the transcription protein – SoxR [2Fe-2S] sensor and DNIC occurrence in it. It has been suggested that NO molecules themselves are able to substitute bridge atoms of inorganic sulfur in Fe–S clusters of this protein that causes a direct lead to DNIC formation, bound to apo-proteins by thiol groups [12]. However, the experiments carried out with adrenodoxine, characterized similar to E.coli SoxR protein by double-nuclear Fe–S sites, do not confirm this supposition [13]. Nitric oxide degraded Fe–S sites in this protein and formed protein DNIC only in the presence of exogenic bivalent iron. Hence, primarily, these complexes included exogenic iron and then endogenic iron, released for Fe–S sites. Similar result was obtained in case of lowmolecular DNIC injection to adrenodoxine solution with further treatment with nitric oxide solution.

Therefore, lowmolecular DNIC, both exogenic or formed in the reaction between exogenic iron and NO, initiated Fe–S site degradation according to autocatalytic mechanism. At the first stage, lowmolecular DNIC were bound to protein thiol groups participating in the composition of Fe–S sites. This freed endogenic iron, which formed DNIC with nitric oxide, which then attacked other Fe–S sites and so on. The provided for self-accelerated character of Fe–S site degradation with consecutive formation of protein DNIC. It can be suggested that this very mechanism forms the basis for DNIC action on SoxR sensor protein of SoxRS-regulon in *E.coli*. Our data allow speaking about action of NO-containing complexes in activation processes of already studied protection regulons not as NO but $Fe^{+}(NO^{+})_2$ group donors, degrading Fe–S sites of sensor and transcription proteins and, therefore, initiating gene expression.

Recently, new publications were presented, in which the ability of various RS–NO to activate a multiplicity of mammal genes was proved [14]. Meanwhile, it is suggested that these compounds induce expression by direct S–nitrosination of transcription inductors. It may not also be excluded that this

process is mediated by DNIC, because according to recent indications RS–NO easily convert to DNIC in the presence of free iron and thiols.

DISCOVERY OF A NEW PHENOMENON OF QUASI-ADAPTIVE RESPONSE TO ALKYLATING AGENTS

As the products of metabolism, potentially mutagenic alkylating agents are present in the environment and living cells. All organisms – eubacteria, archeans, and eukaryotes – possess cell protection mechanisms against toxic and genotoxic alkylation effects [15]. In E.coli these protection systems include tag and ogt genes, which are expressed constitutively, and ada, alkA, alkB, and aid genes, which expression is related to induction of the adaptive response – Ada [16]. The products of tag and AlkA genes cut off N^3-meA from DNA with apurine site formation; however, specificity of these glycosilases is somewhat different [17].

Unambiguously, special place among alkylation products of DNA bases is devoted to O^6–meG due to preferable coupling with T instead of C in DNA replication process [18]. Being the minor adduct, O^6–meG is the basic factor in mutagenesis, carcinogenesis, apoptosis and clusterogenic action of methylating and chloroethylating agents. O^6–meG is also a lethal DNA damage, which repair is absolutely dependent on the post-replication repair system – mismatch-repair. Though O^6–meG is the most dangerous cyto- and genotoxic adduct in DNA, it is completely repaired by 1 – 2 proteins only by way of the unique irreversible stoichiometric reaction [15]. For mammals, it is O^6–meG–DNA–methyl transferase – MGMT protein, the amount of which is strictly controlled and preserved at the level of 10^5 per cell [19].

Unspecialized *E.coli* cells contain about 60 Ada protein molecules, but within one hour of cultivation with a nontoxic dose of alkylating agent this protein is fully induced [20].

Since direct study of the protein–DNA complex structure is impossible yet, there are several models for alkylated Ada protein conversion to Ada-regulon gene transcription regulator [15] and O^6–meG repair.

To-date, the study is finished and the crystalline structure of the unique protein Ada – from *E.coli* is published. However, the works on complete decoding of the analogous protein of mammals are almost finished.

Basing on similarity of the general structure, homology of C–terminal domains and regulation of adaptive response proteins of mammals and *E.coli* [21], a bacterial cell can be used as a model for studying general regularities of molecular mechanisms of adaptive response protein interaction with potential inductors and searching for prospective compounds –adaptation regulators. Basing on the fact that methylation of Ada protein cysteine by SH-groups is deciding for its activation, we suggested an alternative way for activity regulation of this protein by NO–compounds with replacement of the key S–methylcysteine by S–nitrosyl cysteine in Cys-69.

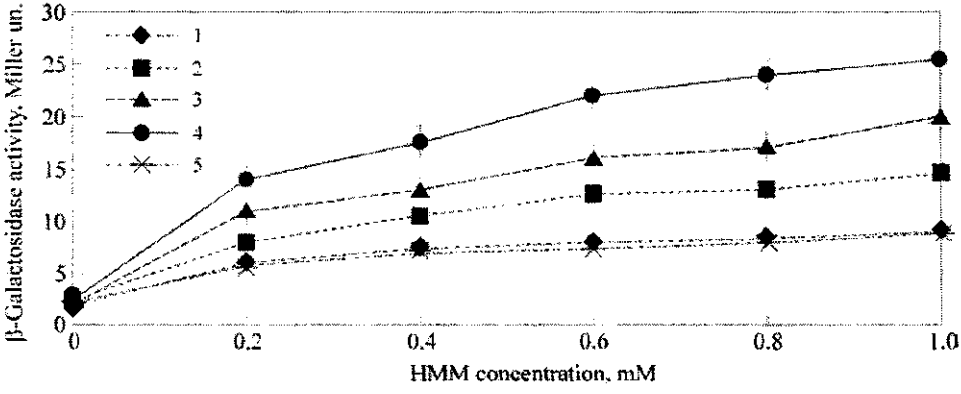


Figure 6. Induction of alkA gene expression in *E.coli* MV 1571 by HMM. Cell preprocessing alternatives: (1) without preprocessing (♦); (2) 0.01 mM HMM (■); (3) 0.1 mM DNICglu (▲); (4) 0.01 mM HMM → 0.1 mM DNICglu (●); (5) OP → alternatives 3, 4 (Iron)

The first stage of a complex of works on stipulation of the hypothesis of quasi-adaptation suggested became the comparative study of induced expression of adaptive response genes in the real and quasi-adaptive responses in HMM. The cells were pre-treated by HMM or DNICglu in sublethal concentrations, respectively. As found in preliminary experiments, DNICglu was characterized by relatively low cytotoxicity in E.coli, and for studied strains the following concentrations were sublethal for 30-min treatment: 0.1 mM for mutant alkA, 0.2 mM for aidB, and 0.5 mM for alkB. The study of dose dependencies of Ada-regulon gene expression levels in experiments with HMM indicated a significant increase of expression both in classical and quasi-adaptive responses. In adaptive *E.coli* cells, alkA::lacZ and alkB::lacZ indices

of 1.0 mM HMM gene expression 2 times, approximately, exceeded the control indices in the real Ada response, whereas at quasi-adaptation – by 3.5 times (Figure 6). However, the display of quasi-adaptation in the cells with high concentration of Ada protein was maximal after preliminary treatment by HMM sublethal dose. Hence, the level of alkB gene expression exceeded the control by 5 times or more and expression at the real HMM adaptation by 2 times or more (Figure 7).

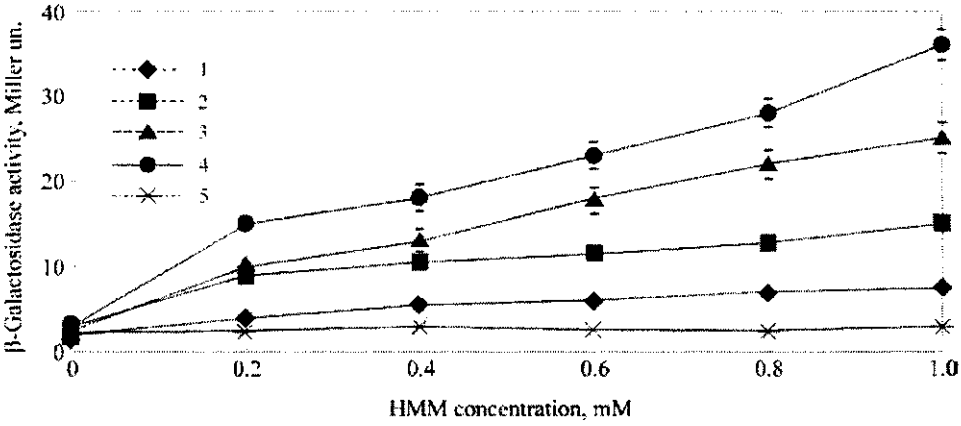


Figure 7. Induction of alkB gene expression in *E.coli* MV 1601. Cell pretreatment alternatives: (1) without pretreatment (♦); (2) 0.01 mM HMM (■); (3) 0.5 mM DNICglu (▲); (4) 0.01 mM HMM → 0.5 mM DNICglu (●); (5) OP → alternatives 1, 2, 3, 4 (iron)

Table 1

The increase of induced Ada–response gene expression in E.coli at adaptive and quasi-adaptive response development

Ada-response genes	Maximal excess of induced gene expression above spontaneous background (times): alternatives of cell pretreatment with sublethal doses: HMM (II), DNICglu (III) and HMM → DNICglu (IV)*			
	I*	II	III	IV
alkA	3	5.6	8	10
alkB	4	7.5	12.5	17.5
aidB	1.8	2	2.15	2.6

Note: * The basic cell treatment with 1.0 mM HMM.

Table 2

The increase of *E.coli* alkB::lacZ cell resistance to HMM at adaptation

HMM concent-ration at basic treatment	Cell survivability, %			Arg^+ revertant frequency per 10^6 survived cells		
	Control	Pretreatment alternatives		Control	Pretreatment alternatives	
		HMM	DNICglu		HMM	DNICglu
0.01	1	15.5	19.5	4.6	1.8	1.3
0.02	6	12.0	16.75	40	9.5	5.0
0.04	0.16	1.3	1.5	380	21	16.5

Commonly, in experiments with HMM aidB gene expression is much lower compared with alkA and alkB independently of adaptation [22]. Figure 3 shows that the maximum of aidB expression in intact cells was observed at 0.2 mM HMM, whereas in adapted cells at five-fold higher concentration (1.0 mM), but all alternatives with pre-adaptation indicate an increase of aidB expression.

Phenotypical indications of Ada-response to alkylating agents in E.coli is, by definition, the resistance increase of adapted cells to toxic and mutagenic effect of alkylating agents. The experiments on quasi-adaptation carried out on E.coli strain alkB::lacZ also indicate increased resistance of cells to the mentioned actions (Table 2), much higher than in case of the real adaptation.

THE INFLUENCE OF DNICGLU ON THE ACTIVITY OF *β*-GALACTOSIDASE ENZYME *IN VITRO*

Cytotoxic action of NO and its compounds is basically related to inactivation of cellular enzymes. In this work we judge about gene expression by indicet signs, for example, by activity of inducible *β*-galactosidase enzyme, then we carried out control tests on HMM and DNICglu effect on activity of this enzyme in a cell-free system. The inductor concentrations giving the maximal gene expression were used. This experiment has not discovered any HMM and DNICglu influence on *β*-galactosidase enzyme activity.

In the majority of experiments, to hit the target, we provided for a complex effect of genotoxicants HMM and DNICglu on the cell. In this connection, we also analyzed their possible interaction with one another, by

absorption UV-spectrum characteristics of their aqueous solutions and the mixture. Addition of DNICglu to HMM solution caused no changes in absorption UV-spectrum characteristics. It is of interest that HMM and DNICglu possess similar absorption maxima in the area between 200 and 250 nm.

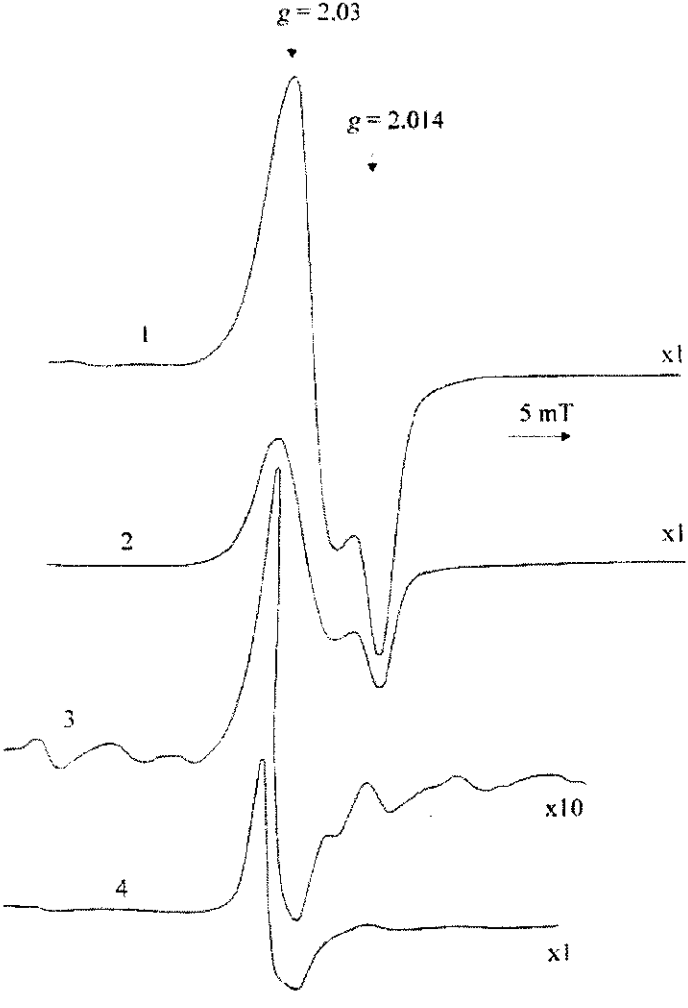


Figure 8. ESR spectra of *E.coli* cells: treated with DNICglu (1), after washing (2), treated with *o*-phenanthroline + DNICglu (3); the same as 3, but after washing (4)

The ESR-spectroscopy method was applied to control permeability of *E.coli* cells for DNICglu and changes of its intracellular concentration and structure. It transits from dimer to monomer and display paramagnetic properties in solution containing free thiols or as bonded to SH–groups of proteins. Incubation of cells with DNICglu demonstrated ESR spectrum, typical of this complex, with anisotropic *g*-factor possessing $g_{\perp}$= 2.03 and $g_{||}$ = 2.014 (Figure 8). After one-time cell rinsing with water (alternative 2) the signal intensity decreased, on average, by two times that may indicate sorption of a significant quantity of donor by the cell surface and its loss during culture rinsing. Preliminary incubation of cells with 0.1 mM OP (during 30 min) reduced ESR signal intensity ($g_{\perp}$= 2.03) in cells processed with DNICglu by an order of magnitude compared with the initial level. The spectrum shape also changes, apparently, because of its degradation. As shown on the spectrum (alternative 4), ESR signal intensity ($g_{\perp}$= 2.03) in OP-preprocessed cells, one time rinsed with water, two-fold increased and corresponded to the signal level in alternative 2. Compound OP reduced alkA gene expression down to the control level in all experiments with DNICglu (Figure 6), whereas in tests with alkB gene OP inhibited expression in all cases (Figure 7).

In a complex sequence of processes representing DNA repair in *E.coli* adaptive response to alkylating genes is the unique phenomenon. Ada protein is a chemosensor in transduction of genetic signal, because residues of cysteines in its structure play the role of alkyl group acceptors from alkylated DNA. On the other hand, this protein is invoked in direct removal of alkyl group from O^6–alkG and native DNA structure repair.

Creation of molecular-genetic models of O^6–metG repair by MGMT (Ada) protein and confirmation of this process persistence in different organisms [23] have initiated investigations on searching for compounds – MGMT activity controllers, including the use of bacterial cells. Theoretical calculations were laid to the foundation of designing the most active human MGMT protein inhibitor, which is O^6–benzylG, the pseudosubstrate for MGMT [24, 25]. It is absorbed well by mammal cells; moreover, during 10 min 0.2 mM O^6–benzylG inactivated 90% human MGMT in a cultural medium [26]. Despite high toxicity in bone marrow cells, this compound is applied in clinics in chemotherapy by alkylating carcinolytics [23]. MGMT inhibitors are partly species-specific: for example, Ada protein of *E.coli* is much less sensitive to O^6–benzylG than human MGMT. However, other guanine derivative – O^6–allylG – is highly effective in relation to both MGMT and Ada protein [26].

Usually, the interaction with NO induces inactivation of cell proteins and enzymes such as protease cysteine residue, alcohol dehydrogenase, protein kinase, and human MGMT [27]. In *E.coli*, similar to superoxide-anion O_2^-, NO induces activation of SoxRS- and OxyR-regulons, however, the mechanisms of these processes are different [28]. The initial stage of SoxR protein activation is a two-step process, which includes formation of lowmolecular DNIC-primers. The latter interacting with thiol groups of protein in the composition of Fe–S complex promotes disintegration pf [2Fe–2S] clusters in SoxR protein [29]. Thiol-disulfide mass-spectroscopy and titration data show that intramolecular disulfide S=S bond is formed between Cys_{199} and Cys_{208} residues of protein – OxyR tetramer at its oxidation [30]. Therefore, this direct oxidation of OxyR protein is the mechanism of OxyR-regulon control of the cell sensitivity to the oxidative stress.

In addition to SoxRS- and OxyR-regulon activation, in *E.coli* DNICglu initiates development of quasi-adaptive response to HMM, substituting it during the adaptive processing. This means, on the one hand, broadening of nitric oxide functions in genetic signal transduction, and on the other hand, discovery of a new activation mechanism for Ada protein-chemosensor function in regulation of Ada-regulon gene expression. There no proofs for the fact yet that in quasi-adaptation the function of Ada-protein is activated, which is associated with O^6–alkG repair, though a decrease of Arg^+ revertant frequency at quasi-adaptation has been determined (Table 1). The formation of Arg^+ revertants in *E.coli* AB1157 argE3-oc, which derivative is *E.coli* MV1601 alkB::lacZ [9], is stipulated, at least, by four alternative mechanisms. Hence, only low-quantity suppressor conversions of supE44-am to supB-oc are resulted by HC → AT transition typical of the adaptive response [31]. It should be noted that generally, preferable induction of HC → AT transitions so typical of other alkylating agents, methyl methane sulfonate, in particular, is not confirmed for HMM-induced mutagenesis.

Gene aidB, selectively and strongly activated by chlorine-derivatives of nitrosoureas [11], was weakly expressed in experiments with HMM, independently of the cell adaptation method (Figure 3). This is, apparently, associated with the requirement of higher Ada protein concentration for its bonding with aidB promoter rather than with alkA and alkB promoters and indication of low Ada protein affinity to aidB-Ada bonding site [20].

Iron chelators modulate toxic effect of free radicals in biological systems: they weaken or intensify it, testifying about the important role of iron

ions in O_2^- toxicity [15, 16]. In accordance with the authors' data, even low OP concentration suppressed development of quasi-adaptation in alkA strain, apparently, due to destruction of the complex. The results of alkB expression inhibition by OP are, apparently, explained by Fe^{2+}-dependence of AlkB protein. Actually, the intensity of ESR signal $g = 2.03$ in the alternative with OP was 7 – 10-fold reduced, and the shape of the signal changed.

Thus, in the present work a new mechanism of Ada protein activity regulation as a sensor in quasi-adaptive response to NO-containing iron complexes $[Fe^2(NO^{2+})_2]$ with glutathione was theoretically stipulated and for the first time experimentally confirmed. In contrast with the real adaptive response, in which S–methylCys–69 formation in the acceptor site of Ada protein initiates adaptation, in the quasi-adaptive response the key structure is, appagently, S–nitrosylCys–69. This means that Ada-response in E.coli is induced not only by alkylating agents and testifies about expansion of NO function as the signal molecule in transduction of genetic signal in alkylated DNA repair.

The results of our experiments indicate that Ada adaptation protein displays selective functional activity at nitrosination with NO donors, and with respect to the ligand structure this activity may increase (in the presence of DNICglu) or decrease (in the case of TNICthio). In the latter case, cell sensibilization to alkylating agents will be suppressed. Taking into account the known similarity in topology, functions and regulation of *E.coli* Ada adaptation proteins and mammal MGMT, it is suggested that the newly discovered quasi-adaptation phenomenon will also be developed in mammal cells. This method will be applied to combined chemotherapy, being the most effective treatment technique. In 1978, Academician N.M. Emanuel has described the advantages of this method [32].

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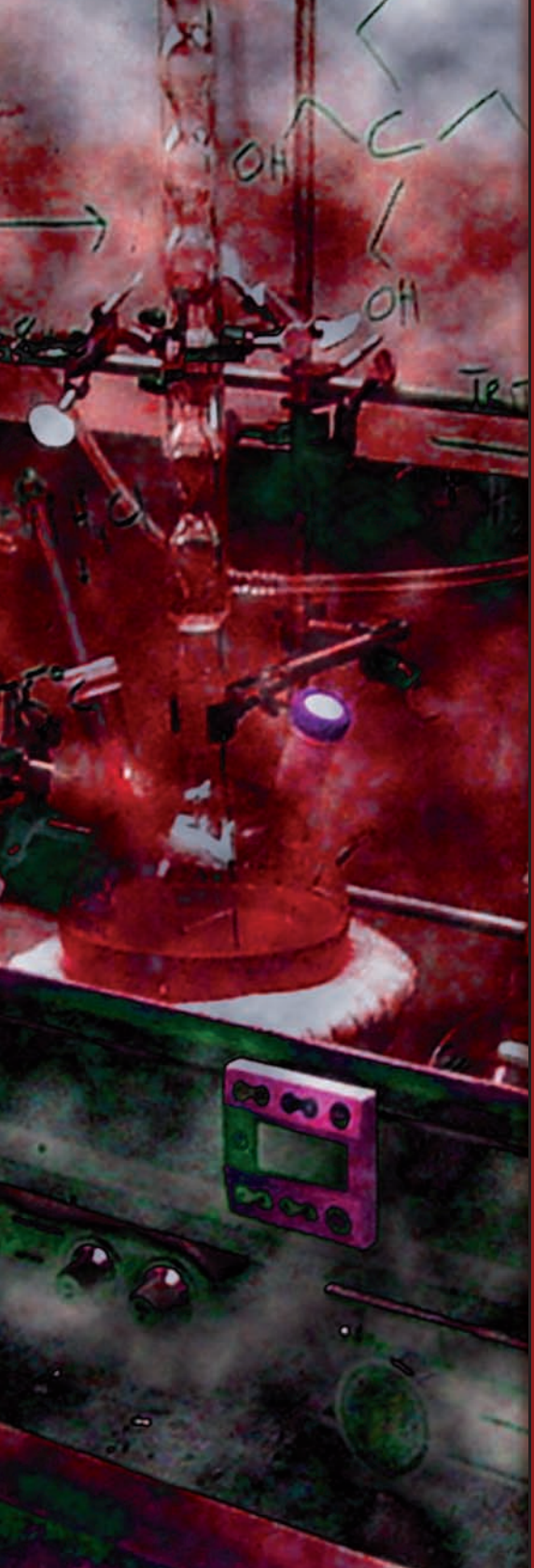

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