Chapter 7

Kinetics and Mechanism of Inhibition of Oxidation Enzymes by Herbicides

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Additional information is available at the end of the chapter

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1. Introduction

"A lack of knowledge in the area of biology of grown plants and specific features of the medium of their dwelling in each specific field cannot be compensated by an excess of pesticides, fertilizers, or melioration."

Academician D.N. Pryanishnikov, 1934

Pesticides as a whole and herbicides in particular are substances with high biological activity. They can exert a toxic effect on many components of cells: enzymes, structural and functional proteins, lipoproteids, polysaccharides, nucleic acids, and others. The elucidation of the mechanism of toxic effect is an important challenge, the solution of which would allow one to establish the real and potential danger of application of these or other compounds for human and non-targent organisms. Despite the enormous scale of production and use of chemical means for cultivated plant protection, there is still much unknown on the mechanism of their action. It is considered that, probably, each pesticide acts through a unique mechanism. For example, the acting components of pesticides, namely, zenkor, lontrel, roundup, kusagard, setoxidim, basagran, tilt, and tachigaren, belong to different classes of chemical compounds. According to available literature data (Table 1), they interact with various enzymatic systems, have their own specific binding sites, and are characterized by different mechanisms of action.

Much data concerning the influence of herbicides and fungicides on various components of the living cell (Fedtke, 1982; Kadyshev, 1970; Fudel-Osipova, 1981), in particular, on some enzymes (Mathew et al., 1998; Forthoffer et al., 2001; Banas et al., 2000; Knecht & Löffler, 1998; Du, 2000a; Gruys et al., 1993; Nosanchuk et al., 2001; Kiyomiya et al., 2000) have been reported. For instance, anticholinesterase compounds, organophosphorus pesticides, carbamates and triazines (Grin & Goldberger 1968), are structurally similar to substrates and

competitively inhibit their activity. The effect was evaluated for fries of Mediterranean fishes *Dicentrarchus labrax* (Varo et al., 2003) and for rats (*Maple amber, M. arrow*) fed with soybean after treatment with zenkor and atrazine (Mathew et al., 1998). It was shown that herbicide basagran suppresses the antiphosphatecholinesterase activity and results in an increase in the hydroxylase activity (Al-Mendofi & Ashton, 1984; Forthoffer et al., 2001).

The growth of fungi *Cryptococcus neoformans* was suppressed by glyphosate due to the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (Nosanchuk et al., 2001). A non-productive four-membered complex is formed between the enzyme, pesticide, and phosphate (Du, 2000b). Octahedral coordination is performed by the metal ion: Co glyphosate enzyme as in 3-deoxy-D-arabiheptulosonate-7-phosphate synthase localized in cytosols (Ganson & Jensen, 1988).

Oxidative phosphorylation is performed by Zn-containing enzymes. Dinoseb, pentachlorophenol, dichlorodiphenyltrichloroethane, and Sevin separate oxidative phosphorylation in mitochondria of Palma Christi (Kuz`minskaya, 1975) and decrease the ATP content in glycols of soybean (Gruenhagen & Moreland, 1971). Chloro-containing organic pesticide endosulfan reacts with glutathione (cofactor of glutathione peroxidase), considerably decreasing the activity of the enzyme. The loss of secretory reactions in thylakoids of adrenocortical steroidogenic cells and changes in the enzyme activity indicate that the pesticide was involved in the oxidative reactions (Dorval et al., 2003).

The formation of complexes of vegetable peroxidase with various substrate-inhibitors was established (Ugarova & Lebedeva, 1978). Both the direct participation of the metal in the substrate addition to the protein part of the molecule and providing of a relationship between the flavine group and apoenzyme under the action of the metal are assumed. The neighborhood of the pyridine nitrogen atom to the carboxyl group in picolinic acid (picloram) is manifested in the ability to complexation and metal removal from enzymes (Shcheglov et al., 1967).

The tests on human and rat tissues showed that tachigaren and its metabolites (four enzymes synthesizing pyrimidine) inhibit mitochondrial [EC 1.3.99.11]. This results in the changes in the pyridine–nucleotide pool that provides the work of immune cells. The reaction is reversible and its mechanism is uncompetitive with respect to the substrate and cofactor ubiquinone (Knecht & Löffler, 1998).

On the other hand, diverse xenobiotics, both pesticides and metals, are abundant in considerable amounts in the nature, namely, in air, soil, and water (Banas et al., 2000; Knecht & Löffler, 1998; Du, 2000a). If these xenobiotics get into the human organism, they may cause various diseases (Gruys et al., 1993; Nosanchuk et al., 2001). In the presence of pesticides with ligand properties, their combined effect on living organisms can be enhanced or weakened.

The ability of the environment to self-purification, *i.e.*, decomposition of contaminants, is determined, to a great extent, by the occurrence of enzymatic redox processes in cells of plants and microorganisms. One of the enzymes performing the redox processes in biological systems is NADH-oxidoreductase (NADH-OR) (Tukhvatullin et al., 2001; Sommerhalter et al., 2004;

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Common name	Range of application	Mechanism action	Reference
Zenkor, Metribuzin	selective to dicotyledons and solanaceous	complexes with membrane lipids	Ziegler et al., 1982
Lontrel, Clopyralid	a wide spectrum of action	similar to auxin	Hall et al., 1985
Kusagard	selective to dicotyledons, beet, cotton	lesion meristem tissues	Iwataki & Hirono, 1978
Roundup, Glyphosate	for struggle against perennial weeds	inhibitor of enolpyruvateshekemate-phosphate synthase	Amrhein et al., 1980
Setoxidim	selective to dicotyledons, beet, solanaceous		
Basagran, Bentazon	selective to grains	inhibitor of photo- ; protein- ; lipids- ; RNA - synthesis	Trebst & Wietoska, 1975 Osama & Ashton, 1984
Tachigaren, Hymexazol	selective to the grass, sugar beet	inhibitor of dehydrogenase (mitochondrial)	Knecht & Löffler, 1998
Titl, Propiconazole		7-etoxyrezofurine O-diethylase; inductor glutathione S-transferase	Levine & Oris, 1999; Egaas et al., 1999
Lontrel metal complexes	a wide spectrum of action	inhibitor of NADH-oxidoreductase	Saratovskikh, 2005; Saratovskikh, 2007

Table 1. Pesticides mechanism action from literary

Bagirov et al., 1989; Lycholat & Bilchuk, 1998) possessing a broad substrate specificity. This enzyme is in the composition of the monooxygenase system that utilizes substrates and transforms xenobiotics into the lowly toxic state. NADH-OR, [EC 1.6.99.25] from the methyl-otroph *Methylococcus capsulatus* (strain M) (Burbaev et al., 1990) transfers electrons for the mixed reduction of oxygen to water, methane transformation to give methanol in the active center of methane hydroxylase, and the reduction of dioxygen to water in the active center of cytochrome oxidase. The enzyme studied consists of four subunits, each including FAD and the iron-sulfur cluster, 2Fe-2S (Tsuprun et al., 1987; Bagirov et al., 1989). NADH-OR functions according to the following scheme:

$$NADH + A_{ox} \xrightarrow{\text{NADH-}OR} \text{NAD}^+ + A_{\text{red}}$$

where A is acceptor.

The most part of biological oxidation processes is performed by an array of carriers, which are grouped in the electron transfer chain and the respiratory chain, one end of which contains the active metabolite and the $1/2O_2$–H_2O system is localized on another end. Among the main components of the chain electron transfer are nicotinamide (pyridine) coenzymes NADH and NADFH.

The sequence of electron transfer from NADH to an electron acceptor is still unknown. However, by analogy with other reductases, one can suggest that the electrons are transferred

from NADH to FAD and then to the iron sulfur 2Fe-2S cluster and to the electron acceptor. Neotetrazolium chloride (NT) was used in this work as the artificial electron acceptor.

Enzymes of this type are present in the cells of almost all organisms. Therefore, the general features of the interaction of this enzyme with pesticides can also be applied to NADH-OR from other organisms.

It is well known that nucleotides play an important role in organisms: energy and regulatory processes and biosynthesis. Nicotinamide adenine dinucleotide functions together with several vitally important enzymes. Therefore, it was of interest to perform a kinetic study of some widely used pesticides on the activity of the enzyme acting together with NADH of one of the enzymes performing oxidation and on nucleotide NADH.

Here we present the data showing the formation of complexes of a series of pesticides with dinucleotide NADH and data on the kinetics of NADH-OR inhibition by commercial herbicides and fungicides of various structures and several complexes of the herbicide lontrel with doubly charged metal ions.

2. Materials and methods

2.1. Compounds, concentrations and replicates

2.1.1. Pesticides

The active substances of the herbicides and fungicides (their formulas are shown below) were isolated from commercial preparations by extraction (Saratovskikh et al., 1988). After isolation the purification was as follows: glyphosate (roundup), tachigaren, and basagran were purified by double recrystallization from water (Mel`nikov, 1987); kusagard and setoxidim were subjected to chromatography on a column with SiO_2; tilt was obtained as nitrate followed by the isolation of the base; lontrel was recrystallized from benzene and then twice recrystallized from hexane; zenkor was recrystallized from hexane and then from a hexane–benzene mixture. The chemical and structural formulas of the used pesticides and lontrel metal complexes are presented in Table 2.

2.1.2. Metal salts

Co, Mn, Ni, and Cu acetates and Fe lactate for the synthesis of metal complexes (pure grade, Reakhim, USSR) were purified by double recrystallization from water.

2.1.3. Reagents

Commercial NADH (nicotinamide adenine dinucleotide) and NT (Sigma no. 2251, Reanal, Hungary) as an artificial electron acceptor were used.

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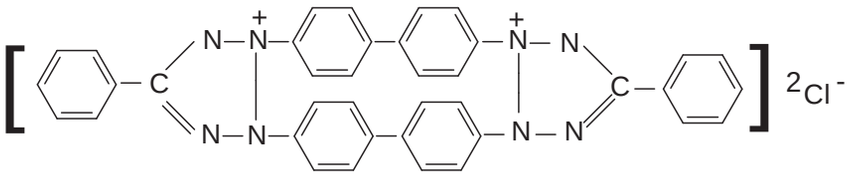


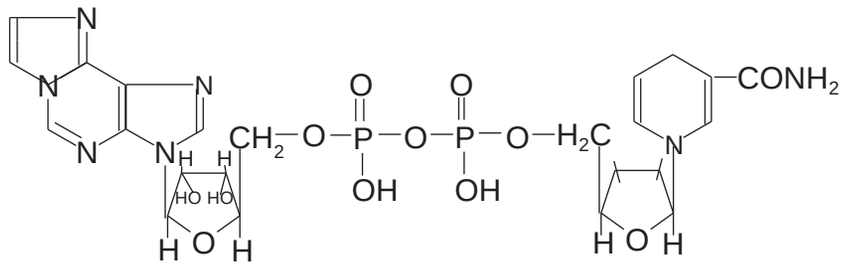
2.2. Compound synthesis

2.2.1. The metal complexes of lontrel (ML_2)

ML_2 where L is lontrel, were synthesized by refluxing ethanolic solutions of lontrel with the corresponding divalent metal salts (Aliev et al., 1988; Saratovskikh, 1989).

2.2.2. The synthesis of ε-NADH

The synthesis of ε-NADH for the study of complexation with pesticides was carried out according to (Lichina et al., 1978; Lichina et al., 1979). Ethenonicotinamide adenine dinucleotide (ε-NADH) has the structure



The degree of modification of adenine ε-NADH is 100%.

2.3. Enzyme inhibition

2.3.1. Bacterial culture preparation

Methane-oxidizing bacteria *M. capsulatus* (strain M) were grown in a 20-L flow-type fermenter in a 10-L salt medium at 42°C. The rate of supply of air mixed with the gas-main natural gas was 300+100 L min^{-1} (Burbaev et al., 1990). The flow rate was 0.24 m^3 h^{-1}.

The cell suspension was collected, concentrated by separation, and washed twice with a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. The cells were destroyed in a DKM-5 semiatomated disintegrator (produced at the Institute of Problems of Chemical Physics of the RAS, Chernogolovka). The cell-free preparation was centrifuged for 30 min at 3000g, the supernatant was centrifuged for 1 h at 65000g, and the precipitated membrane structures and the supernatant fraction ($SF_{65\text{-}1}$) were collected separately, frozen, and stored in liquid nitrogen until used.

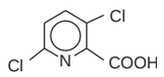
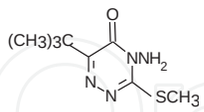
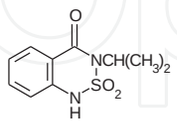
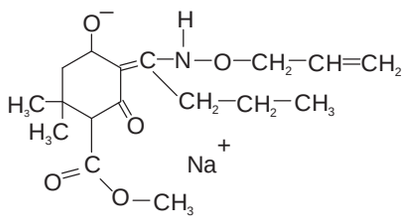
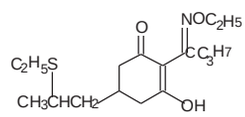
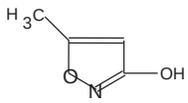
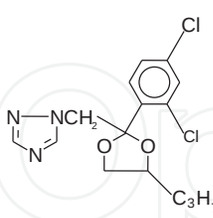
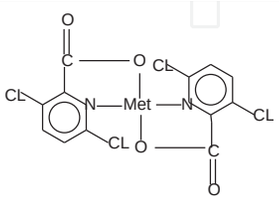
№	Common names of pesticides	Chemical formula	*K*, 10^{-3} M with ε-NADH
1.	Lontrel, Clopyralid 3,6-Dichloropicolinic acid		11.7 ± 0.4
2.	Zenkor, Metribuzin 4-amino-6-tert-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one		22.0 ± 2.0
3.	Basagran, Bentazon 3-isopropyl-1*H*-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide		not determined
4.	Roundup, Glyphosate N-(phosphonomethyl) glycine	$(OH)_2POCH_2NHCH_2COOH$	2.2 ± 0.4
5.	Kusagard Sodium salt of 2-(1-allyl-oxyamino butylidene)-5,5-dimethyl-4-methoxycarbonylcyclohexane-1,3-dione		2.5 ± 0.1
6.	Setoxydim 2-[1-(ethoxyimino)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one		2.8 ± 0.7
7.	Tachigaren, Hymexazol 5-methyl-1,2-oxazol-3-ol		1.8 ± 0.4
8.	Titl, Propiconazole 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole		0.46 ± 0.06
9.	Lontrel metal complexes $M(L)_2$:		
	$Cu(L)_2$		4.6 ± 0.2
	$Co(L)_2$		3.1 ± 0.1
	$Ni(L)_2$		4.7 ± 0.3
	$Fe(L)_2$		0.55 ± 0.06
	$Mo(L)_2$		2.2 ± 0.1

Table 2. Industrial and nomenclature names and chemical formula of the investigated substances and the complexation constants of pesticides with ε-NADH

2.3.2. NADH-OR isolation and purification

The fraction $SF_{65\text{-}1}$ (500 mL, 60 mg of protein mL^{-1}) was passed through a column (30x7 cm) with DEAE-cellulose 52 (Whatman, UK), and the column was washed with 1 L of $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. NADH-OR was eluted using a linear gradient of 0-0.35 *M* NaCl in the same phosphate buffer. The protein fraction with the maximum NADH-OR-activity was eluted with 0.2 *M* NaCl. The eluate was collected, concentrated under argon by ultrafiltration through the Vladipor porous membranes under a 5 atm pressure to 60 mg mL^{-1} of the protein, and fractionated successively on a column with Sephadex G-75 (4x70 cm) and a column with Sepharose 2B (4x80 cm) (Pharmacia, Sweden) in a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 7.0. The enzyme preparation with a specific activity (with respect to NT) of 1.3 μmol L^{-1} min^{-1} (mg protein)$^{-1}$ (20 °C) was collected, concentrated by ultrafiltration to 21 mg mL^{-1} of the protein, frozen, and stored in liquid nitrogen until used.

2.3.3. Determining Enzyme activity and inhibition constant (K_i)

The activity of NADH-OR was determined from the rate of reduction of NT to formazan in a $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 8.0. The rate of formazan formation was estimated (Burbaev et al., 1990) from the change in the absorbance at 550 nm using a Specord M-40 (GDR) spectrophotometer. The reaction was carried out in 3-mL cells (10x10 mm). The reaction mixture contained 0.1 mL of NADH-OR (1 mg of the protein), 0.3 mL of the test compound, 0.1 mL of NADH ($1.0 \cdot 10^{-3}$ mol L^{-1}), and $2.0 \cdot 10^{-2}$ *M* phosphate buffer, pH 8.0, added up to 3 mL. The reaction was initiated by adding 0.2 mL of a solution of NT ($1.5 \cdot 10^{-3}$ mol L^{-1}).

The study was carried out by the traditional Michaelis–Menten procedure. The first task was to elucidate the dependence of the rate constant for the enzymatic formation of formazan on the pesticide concentration and to determine I_{50}, *i.e.*, the concentration of the pesticide inhibitor, resulting in a twofold decrease in the maximum rate of the enzymatic reaction. The second stage included two series of experiments: (1) at a constant NT concentration and variable NADH concentrations, and (2) at a constant NADH concentration and variable NT concentrations.

The K_i values were calculated from the equation (Dixon & Webb, 1979; Emanuel & Knorre, 1969)

$$K_i = (I_{50} \cdot K_m) / (SV / v - K_m),$$

where K_i is the inhibition constant; I_{50} is the concentration of the pesticide inhibitor; K_m is the determined Michaelis constant for NT or NADH; v is the rate; S is the concentration of NT or NADH; V is the maximum rate determined from the Lineweaver–Burk plot.

The Michaelis constant in the presence of the inhibitor (K_m^1) is the following:

$$K_m^1 = [(1/v)/(1/S)] \cdot V$$

The K_m^1 and V values are dictated by the inhibition type.

The Hill coefficients were determined by the Hill formula (Dixon & Webb, 1979; Cornish–Bowden, 1976)

$$Y = \left(K_h I^h\right) / \left(1 + K_h I^h\right),$$

where Y is the degree of protein saturation with the ligand and is equal to the ratio of the number of occupied binding sites to the total number of binding sites; K_h is the association constant in the case where the concentration of the complex is as follows:

$$[E_h \cdot I_h] = K_h [E_h] \cdot [I]^h$$

where h is the Hill coefficient describing the degree of allostericity and equal to the number of molecules of the ligand, in this case, the pesticide inhibitor; I is the concentration of the pesticide inhibitor.

2.3.4. Studies on bacteria Beneckea harveyi

Lyophilized preparation of marin "luminescent" bacteria *Benechea harveyi* (strain B 1 7 – 667F) and *Photobacterium phosphoreum* (Zhmur & Orlova, 2007; Kuz`mich et al., 2002) was stored in a freezing box and used prior to use. The lyophilic preparation of bacteria was suspended in a 0.85% solution of NaCl.

To determine toxicity, 0.3–0.5 mL of suspended bacteria was added to 0.5 mL of the studied water. A 0.85% solution of NaCl or water from an aquarium was used as a control. The measurements were carried out by the instrumental method with a BLM-8801 luminometer (SKTB "Nauka," USSR) with detection on a voltmeter by a decrease in the bioluminescence intensity in the presence of a sample of analyzed water compared to the control.

The 50% (and more) decrease in the luminescence intensity indicates that the aqueous medium is toxic. The bioluminescence intensity of bacteria is determined by the activity of intracellular metabolic processes involving the luciferase enzyme. The decrease in luminescence can be due to the inhibition of the enzyme itself and to the influence of toxicants to other units of the metabolic chain.

The toxicity coefficient was calculated by the formula

$$T = [(I_c - I_t) / I_t] \times 100\%,$$

where I_c is the bioluminescence intensity in the control, and I_t is the luminescence intensity in the tested sample.

At $T \leq 19\%$ the tested sample is not toxic. At $19 < T \leq 50\%$ the tested sample is considered toxic, whereas at $T > 50\%$ the sample is strongly toxic.

Each toxicological experiment was carried out at least three times and then the results obtained were statistically processed.

2.4. Instrumental analysis

2.4.1. Fluorimetric measurements

The fluorescence spectra of the etheno-modified compounds were recorded on an Aminco–Bowman spectrofluorimenter (US) in 3.5-mL quartz cells. The fluorescence excitation wave-

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length for ε-NADH is 312 nm, and the fluorescence emission maximum is 420 nm. The fluorescence intensity of ε- NADH was measured in a 0.025 M tris-HCI buffer (pH = 6.8) at 20^0C. The concentration of ε-NADH equal to $1 \cdot 10^{-4}$ M was used in experiments. The fluorescence spectrum of ε-NADH was accepted to be 1, and then a solution of nucleotide was titrated in the cell with an aqueous solution of the studied pesticide in the concentration from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-2}$ M.

The complexation constants of pesticides with nucleotides were calculated from the experimental titration curve for each point. The obtained values were averaged. Theoretical titration curves were calculated from the values of complexation constants obtained by the experimental data (Saratovskikh et al., 1988).

2.4.2. Electron spin resonance analyses

ESR spectra were recorded at 77 K on an SE/X2544 Radiopan radiospectrometer (Poland) at a 10 mW microwave radiation and a magnetic field modulation of 0.4 mT. The samples were prepared in a $2.0 \cdot 10^{-2}$ *M* tris-HCl buffer, pH 7.0. ESR spectra were recorded in 50% glycerol.

3. Results and discussion

The study of the inhibition of NADH-OR by pesticides and metal complexes of herbicide lontrel (see Table 2) was started from the consideration of their interaction with coenzyme NADH.

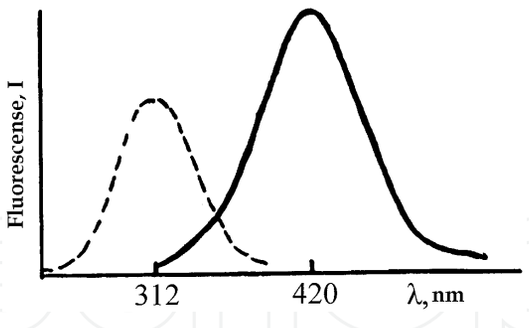
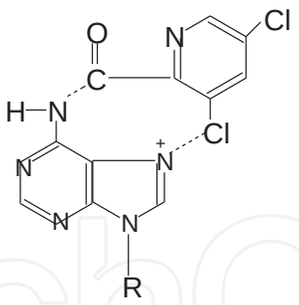


Figure 1. Excitation and fluorescence spectra of ε-NADH.

It is known (Blagoyi et al., 1991) that polynucleotides, particularly, pyridinenucleotides, form complexes of various types, including charge-transfer complexes, and are highly reactive towards a series of metals. However, the introduction of the etheno group does not almost change the electronic structure of the nucleotide fragment of a NADH molecule. Therefore, the complexation of pesticides with NADH was judged about on the basis of the value of fluorescence quenching of its chemical analog, modified dinucleotide ε-NADH in which the adenine fragment is subjected to etheno-modification. Figure 1 illustrates the excitation and fluorescence spectra of ε-NADH.



Figures 2 and 3 represent the obtained dependences of the change in the fluorescence intensity of ε-NADH on the concentration of various quenchers. When the concentration of pesticide (or metal complex) increases, the fluorescence quenching of compound ε-NADH is observed, which is not accompanied by a shift of the position of the excitation maximum and fluorescence emission. The absence of spectral changes in all cases considered indicates the absence of changes in the ground and excited levels of the modified based upon the interaction with pesticides. Fluorescence quenching was observed at the pesticide and lontrel metal complexes concentrations ranging from 10^{-6} to 10^{-3} M. Such low concentrations of the quencher exclude the assumption that the quenching proceeds via the Stern–Volmer mechanism due to random collisions. Therefore, the result of quenching is the formation of a covalent bond with the adenine fragment, as it is shown in the scheme of the [NADH–lontrel] complex

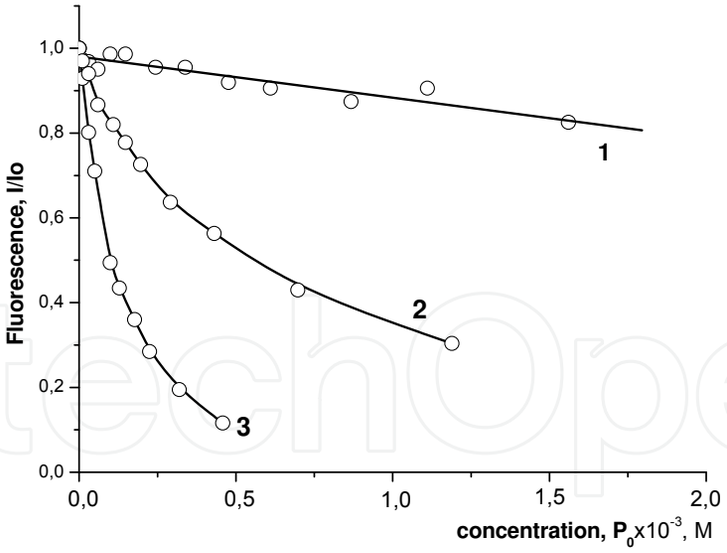


Figure 2. Dependences of the fluorescence intensity of ε-NADH on the pesticide concentration: (1) tilt; (2) kusagard; (3) zenkor. The concentration of ε-NADH is $1 \cdot 10^{-5}$ M. Solid lines are theoretical curves, and points are experimental data.

The mathematical model of the process was considered to refine the mechanism of formation of complexes [ε-NADH–pesticide] and to estimate their stability constants. It was assumed that the pesticides interact with ε-NADH according to the scheme

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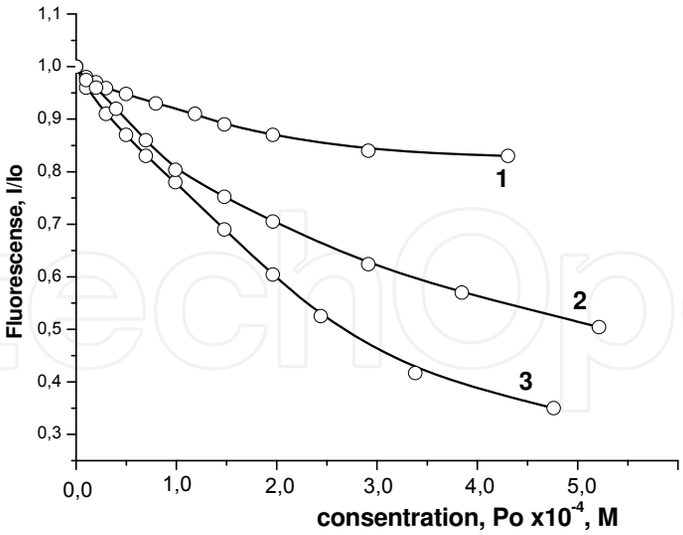

Figure 3. Dependences of the fluorescence intensity of ε-NADH on the concentration of the lontrel metal complexes: (1) Fe(L)2; (2) Ni(L)2; (3) Cu(L)2. The concentration of ε-NADH is $1 \cdot 10^{-5}$ M. Solid lines are theoretical curves, and points are experimental data.

$$[A] + n[P] \leftarrow \frac{\kappa^{+1}}{\kappa^{-1}} \rightarrow [\Pi] \tag{1}$$

where A is the concentration of etheno-modified units of the nucleotide (adenine) in ε-NADH, P is the pesticide concentration, Π is the concentration of the reaction product = complex, $K = \kappa^{+1} / \kappa^{-1}$ is the complexation constant, and n is the stoichiometric coefficient equal to the number of equivalent binding sites.

At equilibrium the process is described by the following system of equations:

$$K[A] \times [P]^n = [\Pi] \tag{2}$$

$$[A] + [\Pi] = [A_0] \tag{3}$$

$$[P] + n[\Pi] = [P_0] \tag{4}$$

Equations 2–4 make it possible to determine the values of the complexation constant from the experimentally determined concentration

$$[A] = \frac{I - I_\kappa}{I_0 - I_\kappa} A_0,$$

where I_o is the fluorescence intensity of free ε-NADH, I_κ is the limiting value of fluorescence intensity of ε-NADH at the maximum concentration of the quencher, and I is the fluorescence intensity of ε-NADH at the given concentration of the quencher.

$$K = \frac{[A_0] - [A]}{[A]\{[P_0] - n([A_0] - [A])\}^n} \tag{5}$$

The value of stoichiometric coefficient n should preliminarily be determined from the data obtained at a rather high pesticide concentration at which the following equation is fulfilled:

$$[P_o] >> n([A_o] - [A]). \tag{6}$$

After condition (6) is fulfilled, equation (5) can be rewritten in the form

$$\ln\left(\frac{[A_0] - [A]}{[A]}\right) = \ln K + n\ln[P_0], \tag{7}$$

from which it follows that the dependence of ln $\ln\frac{[A_0]-[A]}{[A]}$ on ln[P_o] is a straight line with the angular coefficient equal to n.

As can be seen from Fig. 4, stoichiometric coefficient n is equal to 1±0.2 for all pesticides and metal complexes evaluated. Therefore, we may conclude that only one pesticide molecule interact with one molecule of ε-NADH. Having determined the value of n at high P_o, one can find the value of K at other pesticide concentrations. The use values of P_o should not be too low, since at very low [P_o] relative errors of the values of ([A_o] – [A]) and ([P_o] – n[A_o] – [A]) can be too high and the errors of complexation constant determination will be unacceptably high.

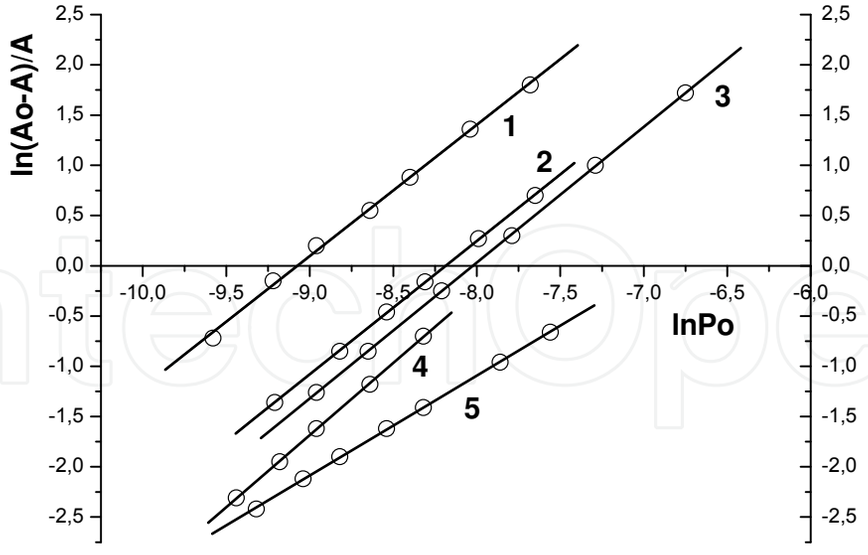

Figure 4. Dependences of the ln $\frac{Ao - A}{A}$ on the lnPo. (1) zenkor; (2) $Cu(L)_2$; (3) setoxidim; (4) $Mo(L)_2$; (5) $Co(L)_2$.

Figures 2 and 3 show the experimental dependences of I/I_o on [P_o] and the corresponding theoretical curves for the pesticides and $Cu(lontrel)_2$ complexes ($Cu(L)_2$) calculated by the

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values found for n and K. Satisfactory coincidence of the experimental and theoretical data indicates that the developed model is valid.

As can be seen from Table 2, of the synthesized pesticides, zenkor has the lowest complexation constant (K) with ε-NADH ($K = 2.1 \cdot 10^4$ M^{-1}) and tilt has the highest one ($K = 4.6 \cdot 10^2$ M^{-1}). It is noteworthy that the complexation constant of the lontrel metal complexes with ε-NADH is substantially lower than the corresponding constant for lontrel. It is known (Luisi et al., 1975) than in solution NADH exists predominantly in a folded conformation in which the adenine part of the molecule is localized near the nicotine amide part of the nucleotide. About 90% dinucleotide exists in this conformation in solution. The rest 10% exist in solution in the "open" conformation when the nicotine amide part is remote from the adenine structure. Therefore, it can be assumed that the decrease in the complexation constants with ε-NADH for the metal complexes compared to lontrel indicates steric hindrances appeared upon the formation of the [NADH–$M(L)_2$] complex.

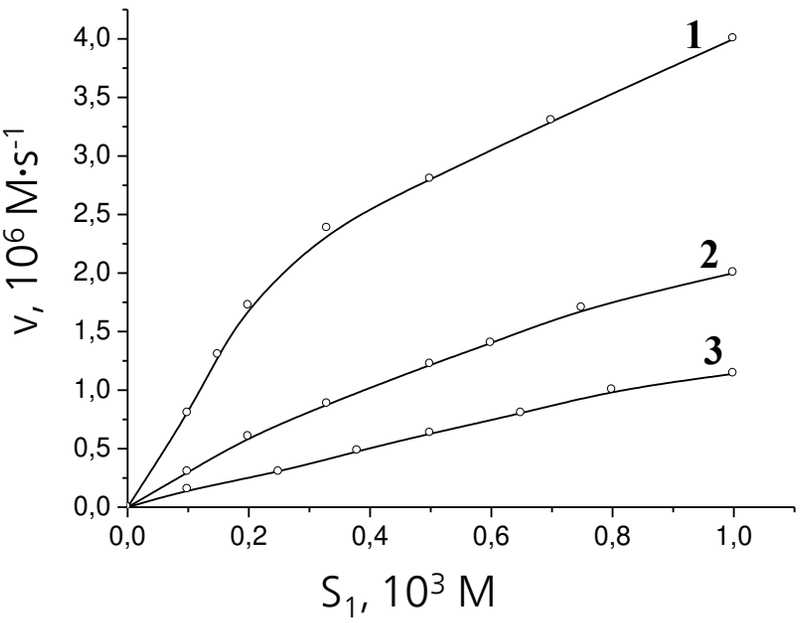

Figure 5. Kinetic curves for the oxidation rate of NADH-OR *vs*. the concentration of NADH at a constant concentration of NT in the absence of an inhibitor (1) and in the presence of $0.33 \cdot 10^{-4}$ (2) and $1.00 \cdot 10^{-4}$ mol L^{-1} (3) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 1.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

The effect of pesticides on the activity of NADH-OR is illustrated by Figs 5–8. The experimental kinetic curves for the rate of NADH-OR oxidation *vs.* concentration of the NADH (S_1) substrate at an invariable NT concentration are presented in Fig. 5. The plots converted to the Lineweaver–Burk coordinates are shown in Figs 6–8. Figure 6 shows the pattern of OR inhibition by lontrel as a function of the concentration of NADH (at a constant NT concentration). The intersection of these straight lines in one point on the ordinate (see Fig. 6) indicates that the herbicide lontrel inhibits NADH-OR and competes with NADH for the region of binding with the enzyme. The $1/S_1$ intercept on the abscissa was used to calculate the inhibition constant (S_1 is the NADH concentration, S_2 is the NT concentration).

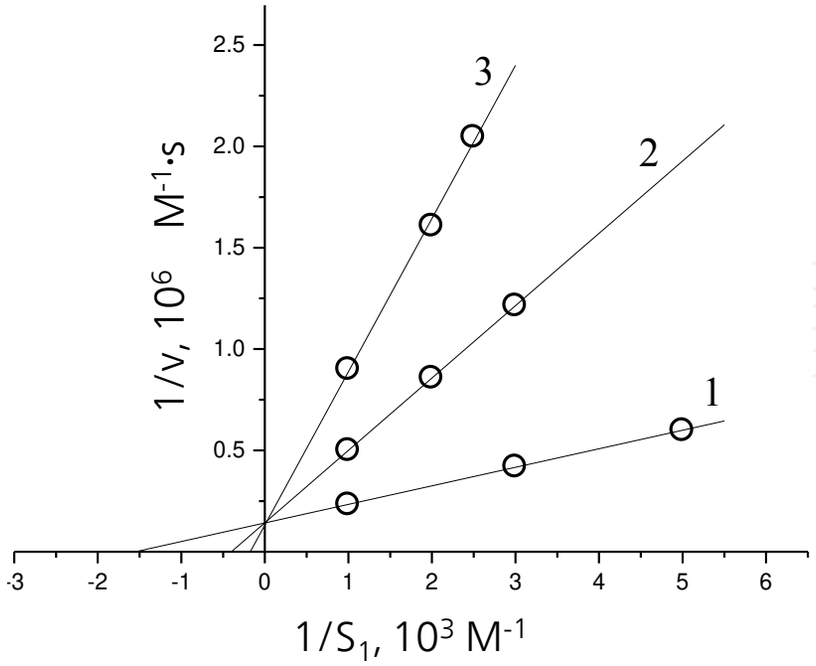

Figure 6. Inhibition of the NADH-oxidoreductase by lontrel (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $0.33 \cdot 10^{-4}$ (2) and $1.00 \cdot 10^{-4}$ mol L^{-1} (3) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 1.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

It can be seen from Tables 3 and 5 that the lontrel complex with the copper ion, although follows a competitive mechanism of inhibition with respect to NADH, still inhibits the oxidation of NADH almost 30 times stronger than the parent lontrel. The I_{50} values are equal to $1.1 \cdot 10^{-3}$ and $3.3 \cdot 10^{-4}$ mol L^{-1} (K_i are $1.0 \cdot 10^{-4}$ and $6 \cdot 10^{-6}$ mol L^{-1}).

Pesticide	*I*50 mol L^{-1}	*h*	for NADH				for NT			
			V_{max} mol/ L s	S_1 mol L^{-1}	$K_i \cdot 10^4$	Type**	V_{max} mol/ L s	S_2 mol L^{-1}	$K_i \cdot 10^4$	Type**
Zencor	$5.00 \cdot 10^{-4}$	1.952	-	$4.93 \cdot 10^{-3}$	0.25	A	$0.23 \cdot 10^{-6}$	$3.39 \cdot 10^{-4}$	8.94	B
Lontrel (L)	$1.10 \cdot 10^{-3}$	1.726	-	$1.23 \cdot 10^{-3}$	1.00	A	$1.88 \cdot 10^{-6}$	$6.98 \cdot 10^{-4}$	7.42	B
Bazagran	$6.00 \cdot 10^{-4}$	2.086	$1.82 \cdot 10^{-6}$	$1.83 \cdot 10^{-4}$	12.80	B	$0.26 \cdot 10^{-6}$	$2.55 \cdot 10^{-4}$	8.40	B
Kuzagard	$27.0 \cdot 10^{-2}$	1.575	-	$9.86 \cdot 10^{-3}$	14.00	A	-	$5.72 \cdot 10^{-3}$	158.9	A
Tachigaren	$2.70 \cdot 10^{-3}$	1.920	-	$2.47 \cdot 10^{-3}$	21.00	A	-	$5.30 \cdot 10^{-3}$	4.55	A
Roundup	$1.70 \cdot 10^{-3}$	1.328	$3.33 \cdot 10^{-6}$	$6.17 \cdot 10^{-4}$	22.0	C	$0.21 \cdot 10^{-6}$	$2.00 \cdot 10^{-4}$	42.90	B
Tilt	$2.20 \cdot 10^{-3}$	2.483	$1.25 \cdot 10^{-4}$	$5.98 \cdot 10^{-4}$	23.00	C	-	$13.00 \cdot 10^{-3}$	1.52	A
Setoxidim	$17.0 \cdot 10^{-2}$	1.832	$2.00 \cdot 10^{-6}$	$7.59 \cdot 10^{-4}$	397.50	C	-	$11.00 \cdot 10^{-3}$	8.04	A

* In the absence of an inhibitor *V*max = $7.40 \cdot 10^{-6}$ mol L^{-1} s^{-1}; $S_1 = 6.58 \cdot 10^{-3}$ mol L^{-1}; $S_2 = 2.65 \cdot 10^{-3}$ mol L^{-1}.

** The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 3. Effect of inhibitors on NADH-oxidoreductase*

It can be seen from Fig. 7 that the herbicide roundup does not compete with NADH for the enzyme binding site.

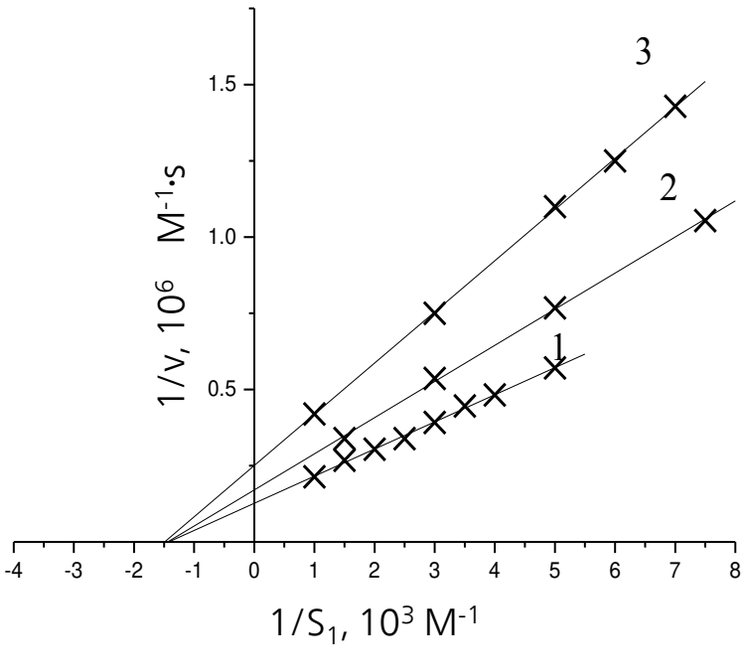

Figure 7. Inhibition of the NADH-oxidoreductase with roundup (in the Lineweaver-Burk coordinates) in the absence of the inhibitor (1) and in the presence of $1.17 \cdot 10^{-3}$ (2) and $2.50 \cdot 10^{-3}$ mol L^{-1} (*3*) roundup; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NADH} = 2.0 \cdot 10^{-4}$ - $1.5 \cdot 10^{-3}$ mol L^{-1}.

The dependences of the reciprocal reaction rate on the reciprocal concentration of the NT electron acceptor (with the NADH concentration remaining constant) are shown in Figs 8 and 9. The herbicides lontrel and zenkor (see Fig. 8, Table 3) also inhibit the rate of electron transfer from the NADH-OR active center to NT. The inhibition pattern is uncompetitive (K_i are equal to $7.42 \cdot 10^{-4}$ and $8.94 \cdot 10^{-4}$ mol L^{-1}, respectively). The lontrel complex with the copper ion exhibits noncompetitive inhibition, while the complex with cobalt exerts mixed inhibition (see Fig. 9, Table 4).

The Michaelis constants (K_m) calculated without inhibitors are $6.6 \cdot 10^{-4}$ and $2.47 \cdot 10^{-3}$ mol L^{-1} for NADH and NT, respectively. It was determined in the preliminary experiment that all compounds under study reversibly inhibit NADH-OR.

The data on the effect of other herbicides, fungicides, and lontrel metal complexes on the rate of NADH oxidation and the rate of NT reduction with NADH-oxidoreductase are presented in Table 3.

Of all the compounds studied, the highest inhibitory activities were found for zenkor and basagran (I_{50} are $5.0 \cdot 10^{-4}$ and $6.0 \cdot 10^{-4}$ mol L^{-1}, respectively). Lontrel, roundup, tachigaren, and tilt inhibit NADH-OR somewhat less efficient (I_{50} are $1.1 \cdot 10^{-3}$, $1.7 \cdot 10^{-3}$, $2.7 \cdot 10^{-3}$, and $2.2 \cdot 10^{-3}$ mol L^{-1}, respectively, see Table 3). Kusagard and setoxidim exhibit weak antireductase activities; they depress the enzyme activity when are present in higher concentrations: $2.7 \cdot 10^{-2}$ and 1.7·

10^{-2} mol L^{-1}, respectively. In terms of the K_i values with respect to NADH, the herbicides and fungicides can be arranged in the following activity sequence: zenkor > lontrel > basagran > > kusagard > tachigaren > roundup > tilt > setoxidim. This sequence is similar to the sequence of complexation constants of these compounds with NADH given in Table 2.

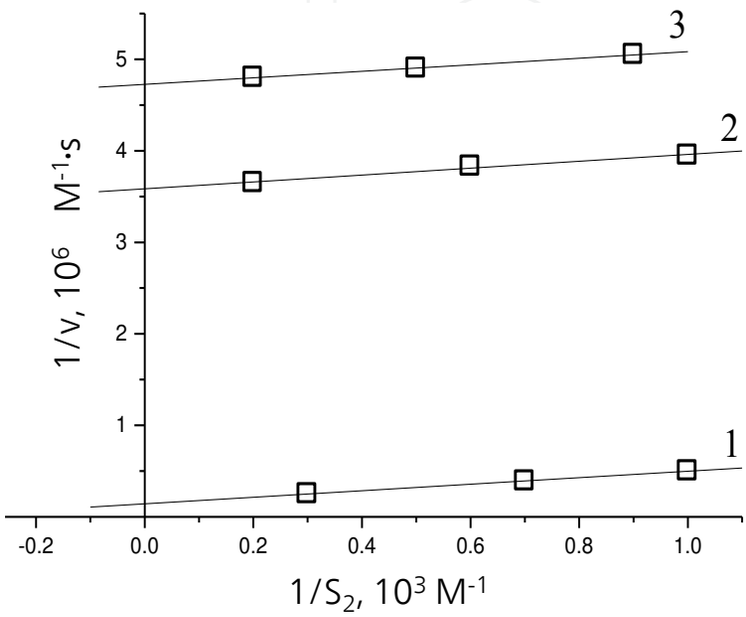

Figure 8. Inhibition of the NADH-oxidoreductase with zenkor (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $3.33 \cdot 10^{-4}$ (2) and $5.00 \cdot 10^{-4}$ mol L^{-1} (3) of zenkor; $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NT} = 7.2 \cdot 10^{-5}$ - $6.4 \cdot 10^{-3}$ mol L^{-1}.

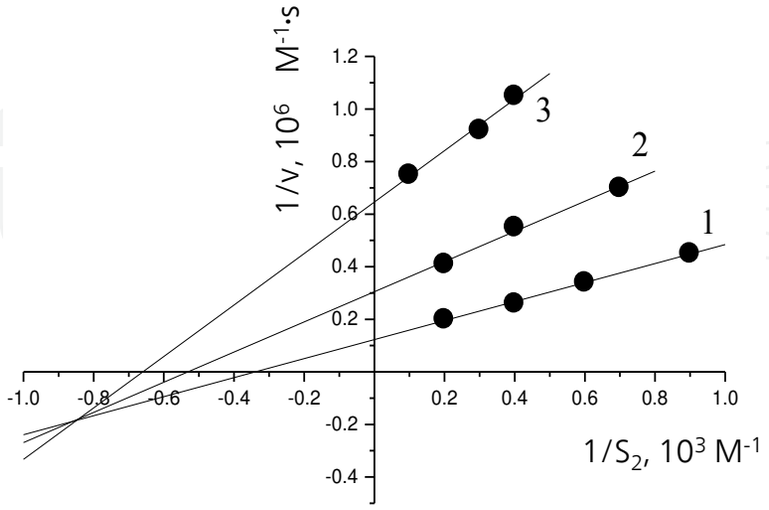

Figure 9. Inhibition of the NADH-oxidoreductase with zenkor (in the Lineweaver-Burk coordinates) in the absence of an inhibitor (1) and in the presence of $3.33 \cdot 10^{-4}$ (2) and $5.00 \cdot 10^{-4}$ mol L^{-1} (3) of zenkor; $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NT} = 7.2 \cdot 10^{-5}$ - $6.4 \cdot 10^{-3}$ mol L^{-1}.

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Lontrel, zenkor, basagran, and roundup inhibit the reduction of NT in a uncompetitive manner, apparently, due to nonspecific interaction with the protein matrix outside the enzyme active center. This interaction could induce conformational changes around the electron transfer site, which result in the inhibition of enzymatic activity. Meanwhile, kusagard, setoxidim, tilt, and tachigaren compete with NT for the binding region on the enzyme. These differences can be due to different structures of the pesticides examined.

Inhibitor – solt	S_2, 10^{-4} M	K_i, 10^{-4} M^{-1}	Type *	h	Inhibitor – $M(L)_2$	
					K_i, 10^{-4} M^{-1}	Type *
$Cu(CH_3COO)_2$	13.2	0.7	A	1.0	4.0	C
$(NH_4)_6Mo_7{\cdot}O_{24}$	6.6	4.4	A	1.0	0.4	A
$Co(CH_3COO)_2$	8.8	0.02	A	1.0	13.1	D
$Fe(C_3H_9COO)_2$	4.4	4.1	A	1.0	11.7	C
$Ni(CH_3COO)_2$	14.7	0.7	A	1.0	11.7	C
$Mg(CH_3COO)_2$		no inhibition			3.6	A
$MgSO_4$		no inhibition				
$Zn(CH_3COO)_2$		no inhibition			2.5	D
$ZnSO_4$		no inhibition				
$Mn(CH_3COO)_2$		no inhibition			22.3	D
$MnSO_4$		no inhibition				

In the absence of an inhibitor $V_{max} = 2.8{\cdot}10^{-6}$ mol L^{-1} s^{-1}; $S_2 = 3.3{\cdot}10^{-4}$ mol L^{-1}.

* The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 4. Kinetic parameters of inhibition of NADH-oxidoreductase at the artifical electron acceptor NT.

The metal complexes of lontrel are known (Saratovskikh et al., 1988; Saratovskikh et al., 1990) to exhibit herbicide activities *in vivo*. In addition, as noted above, the complex formed by the herbicide lontrel with the copper ion exhibits a much higher inhibitory activity than the starting lontrel. Therefore, we carried out an additional study of a series of complexes of these pesticides with different doubly charged metal ions, $M(L)_2$, and the salts of these metals.

As can be seen from the experimental kinetic curves of the dependence of the oxidation rate of NADH-OR on the NADH concentration at a constant NT concentration presented in Fig. 10, the addition of $Ni(ac)_2$ in the concentrations from $3.3{\cdot}10^{-6}$ to $5{\cdot}10^{-4}$ M inhibits oxidoreductase functioning and decreases the rate of formation of the reaction product formazan, and at a considerable inhibitor concentration the reaction rate can decrease to zero. Similar studies were carried out with other salts. The dependences of the rate of the enzymatic reaction on the concentration of salt of the metal-inhibitor can be determined from the obtained curves (Fig. 11). It is seen that Mg(II) does not inhibit NADH-OR even at the highest of the concentrations studied, namely, 10^{-2} M. The Zn(II) and Mn(II) salts also exerted no inhibitory effect, and the replacement of the SO_4^{2-} anion by $(CH_3COO)_2^{2-}$ does not change the character of the process.

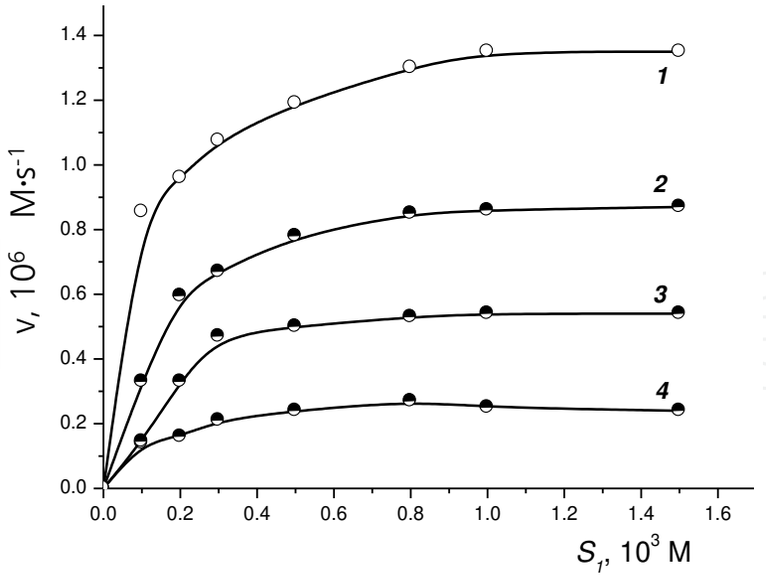

Figure 10. Kinetic curves of the dependence of the oxidation rate of NADH-oxidoreductase on the NADH concentration at a constant concentration of NT. C_{NT} = 6.68·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.0·10^{-4} – 1.5·10^{-3} M; (1) entry without inhibitors; in the presence of Ni(ac)$_2$ in the concentration (2) 3.3·10^{-6} M; (3) 3.0·10^{-5} M, and (4) 1.7·10^{-4} M.

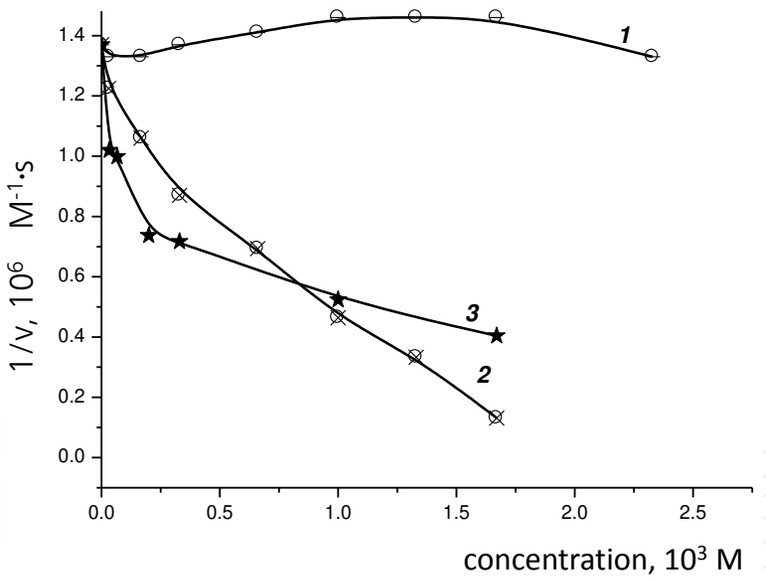

Figure 11. Dependences of the enzymatic reaction rate on the concentration of the metal salts: (1) Mg(ac)$_2$; (2) Mo(am)$_6$; (3) Fe(acac)$_2$. C_{NT} = 6.68·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.0·10^{-4}–1.5·10^{-3} M.

The dependences of the reciprocal rate of the reductase reaction on the inverse concentration of the artificial electron accetor (NT) at a fixed NADH concentration for Fe(acac)$_2$ are presented in Fig. 12. The considered metal salts inhibit the reduction of NT, competing with the artificial electron acceptor for the binding region with NADH-OR. As follows from Table 4, the corresponding complexes, except for Mo(L)$_2$, do not compete with the electron acceptor.

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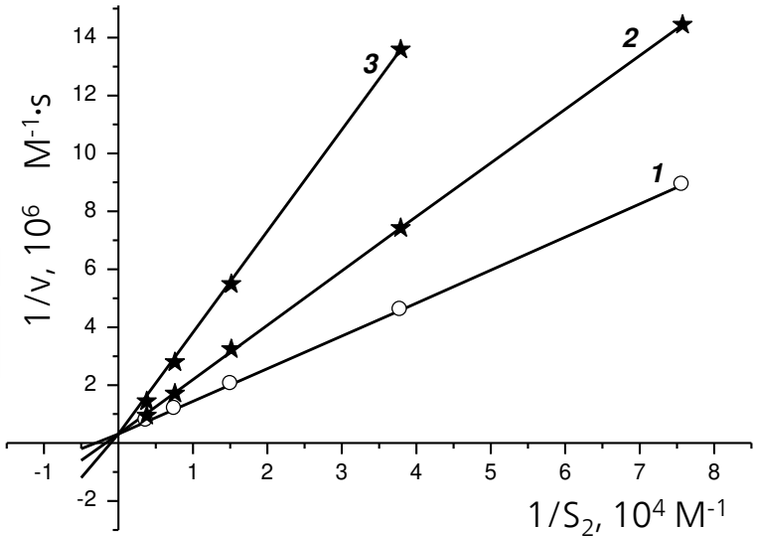

Figure 12. Dependences of the reciprocal inhibition reaction rate of NADH-oxidoreductase by Fe(acac)$_2$ on the inverse NT concentration at a constant concentration of NADH (in the Lineweaver–Burk coordinates); C_{NT} = 1.0·10^{-5}–7.0·10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.43·10^{-3} M; (1) without an inhibitor; in the presence of Fe(acac)$_2$ in the concentration (2) 3.0·10^{-4} M and (3) 5.0·10^{-4} M.

When determining the influence of the metal salts on the reduction of NT, the maximum values for the inhibition constants were found for Mo(VI) and Fe(II): K_i = 4.4·10^{-4} M^{-1} and 4.1·10^{-4} M^{-1}, respectively. The minimum value K_i = 2.0·10^{-6} M^{-1} was calculated for Co(II). The inhibition constants of Cu(L)$_2$, Co(L)$_2$, Fe(L)$_2$, and Ni(L)$_2$ are one to three orders of magnitude higher than those for the corresponding salts. On the contrary, Mo(L)$_2$ showed K_i an order of magnitude lower than that of Mo(II). The phenomena of the appearance of or increase in the inhibitory effect were also observed for the metal complexes with other organic ligands (Tatjanenko et al., 1985).

The following series can be arranged for an increase in the values of K_i with the change in the NT concentration for the metal salts: Co(II) < Cu(II) ~ Ni(II) < Fe(II) ~ Mo(VI). A similar regularity is observed for the lontrel complexes in the activity sequence by the values of K_i: Mo(L)$_2$ < Zn(L)$_2$ < Mg(L)$_2$ < Cu(L)$_2$ < Ni(L)$_2$ = Fe(L)$_2$ < Co(L)$_2$ < Mn(L)$_2$; the electron-donor properties of the metal exert a strong effect on the strength of the bond between the inhibitor and enzyme.

The results of the influence of (ML$_2$) on the enzymatic activity of NADH-OR are presented in Table 4. On going from the salt to complexes, the type of inhibition changes completely: from the same competitive type for all metal salts to several different variants, namely, mixed for Co(L)$_2$ and uncompetitive for Ni(L)$_2$, Fe(L)$_2$, and Cu(L)$_2$. Only Mo(VI) and Mo(L)$_2$ retain one, competitive, type of inhibition. The Mg(II), Zn(II), and Mn(II) ions do not inhibit NADH-OR, whereas Mg(L)$_2$ compete with an electron acceptor for the binding site on the enzyme and Zn(L)$_2$ and Mn(L)$_2$ have the mixed type of inhibition with respect to both NT and NADH. Interestingly, as shown above, Mg(L)$_2$, Zn(L)$_2$, and Mn(L)$_2$, in turn, do not interact with NADH. The formation constants of the M(L)$_2$–NADH complexes ($K_{c/f}$: Fe < Mo < (Co) < Cu < Ni) show a direct correlation with the inhibition constants of NADH-OR by an electron donor for both the complexes and metal salts K_{iM+}: Co < Ni < Cu < Mo < Fe (Table 5).

Inhibitor – solt	I_{50}, M	V_{max}, Mc^{-1}	S_1, 10^{-4} M	K_i, 10^4 M^{-1}	Type*	Inhibitor – $M(L)_2$		
						I_{50}, M	K_i, 10^{-4} M^{-1}	Type*
$Cu(CH_3COO)_2$	$3.3 \cdot 10^{-5}$	$1.1 \cdot 10^{-6}$	3.9	1.2	D	$3.3 \cdot 10^{-4}$	0.06	A
$(NH_4)_6Mo_7 \cdot O_{24}$	$3.3 \cdot 10^{-4}$	$1.4 \cdot 10^{-6}$	3.0	8.8	D	$8.5 \cdot 10^{-4}$	0.1	A
$Co(CH_3COO)_2$	$1.3 \cdot 10^{-6}$	$9.7 \cdot 10^{-7}$	1.2	0.01	B	$1.5 \cdot 10^{-3}$	13.7	C
$Fe(C_3H_9COO)_2$	$3.3 \cdot 10^{-4}$		4.6	14.2	A	$1.1 \cdot 10^{-3}$	1.1	A
$Ni(CH_3COO)_2$	$3.3 \cdot 10^{-5}$	$7.7 \cdot 10^{-7}$	3.2	0.9	D	$2.0 \cdot 10^{-3}$	12.4	C
$Mg(CH_3COO)_2$	no inhibition					$2.0 \cdot 10^{-3}$	12.7	C
$MgSO_4$	no inhibition							
$Zn(CH_3COO)_2$	no inhibition					$1.0 \cdot 10^{-3}$	10.2	C
$ZnSO_4$	no inhibition							
$Mn(CH_3COO)_2$	no inhibition					$3.0 \cdot 10^{-3}$	3.8	A
$MnSO_4$	no inhibition							

In the absence of an inhibitor $V_{max} = 2.8 \cdot 10^{-6}$ mol L^{-1} s^{-1}; $S_2 = 3.3 \cdot 10^{-4}$ mol L^{-1}.

* The type of inhibition: A - competitive, B - uncompetitive, C - noncompetitive, D - mixed.

Table 5. Kinetic parameters of inhibition of NADH-oxidoreductase at the substrate NADH.

Among the lontrel complexes with metal ions, only the complexes with Mg and Mo proved to be competitive reductase inhibitors with respect to NT. The lontrel complexes with Cu, Ni, and Fe ions inhibit the enzyme noncompetitively, while the lontrel complexes with Mn, Zn, and Co display a mixed type of inhibition.

Table 5 demonstrates the kinetic parameters of inhibition of NADH-oxidoreductase with respect to the substrate NADH. The highest inhibition ability is shown by Co(II): $I_{50} = 1.33 \cdot 10^{-6}$ M. The copper and nickel salts exhibits the equal values: $I_{50} = 3.3 \cdot 10^{-5}$ M. The weak antireductase activity was demonstrated by Fe(II) and Mo(VI), namely, $I_{50} = 3.3 \cdot 10^{-4}$ M. The metal salts can be arranged in the following series by the calculated values of I_{50}: $Co(ac)_2 < Ni(ac)_2 = Cu(ac)_2 < Fe(acac)_2 = Mo(am)_6$.

The inhibitory effect of the complexes ZnL_2, CoL_2, FeL_2, and MoL_2 is similar to that of the parent lontrel (I_{50} are $1.0 \cdot 10^{-3}$, $1.5 \cdot 10^{-3}$, $1.1 \cdot 10^{-3}$, and $0.85 \cdot 10^{-3}$ mol L^{-1}, respectively), while the inhibitory effects of MgL_2, MnL_2, and NiL_2 are much lower.

The Mg, Zn, Co, and Ni complexes with lontrel exhibit noncompetitive inhibition with respect to NADH, whereas Mn, Cu, Fe, and Mo complexes inhibit the enzyme competitively. The difference between the inhibition patterns may be related to the difference between the acceptor abilities of the metal ions.

A comparison of the data presented in Tables 4 and 5 shows that the metal salts inhibit the enzyme in much lower concentrations. The value of I_{50} of a salt is one to three orders of magnitude lower than that of the complexes of the corresponding metal. The maximum difference in the values of I_{50} is observed for Co(II) and $Co(L)_2$: $1.33 \cdot 10^{-6}$ and $1.5 \cdot 10^{-3}$ M, respectively. The minimum difference is characteristic of molybdenum: Mo(VI) $I_{50} = 3.3 \cdot 10^{-4}$

M, $Mo(L)_2$ I_{50} = 8.5·10^{-4} M; *i.e.*, the antireductase activity of the metal ion is threefold higher than that of the corresponding metal complex with lontrel. The order of increasing I_{50} (*i.e.*, decreasing the antireductase activity) in the case of the complexes $Cu(L)_2 < Mo(L)_2 < Zn(L)_2 < Fe(L)_2 = L < Co(L)_2 < Ni(L)_2 = Mg(L)_2 < Mn(L)_2$ is reciprocal to that presented above for the salts of the same metals.

The highest of the calculated inhibition constants with respect to the electron donor (NADH) belongs to Fe(II) and Mo(VI), and the values of K_i are14.2·10^{-4} M^{-1} and 8.8·10^{-4} M^{-1}, respectively. The lowest value (K_i = 1.4·10^{-6} M^{-1}) was determined for Co(II).

The character of inhibition of NADH-OR with copper acetate at different substrate (NADH) concentrations at a fixed concentration of NT is shown in Fig. 13. The intersection of the straight lines in one point but not in the axis indicates that the inhibition follows the so-called mixed type. The same character of inhibition was demonstrated by $Mo(am)_6$ and $Ni(ac)_2$. The $Cu(L)_2$, $Mo(L)_2$, and $Fe(L)_2$ complexes compete with NADH for the binding site on NADH-OR (Table 5). The strength of the bond between $Cu(L)_2$ and NADH-OR is nearly 20 times higher than that of Cu(II); K_i = 6.0·10^{-6} M^{-1} and 1.2·10^{-4} M^{-1}, respectively. A similar situation was observed for molybdenum and iron and their complexes.

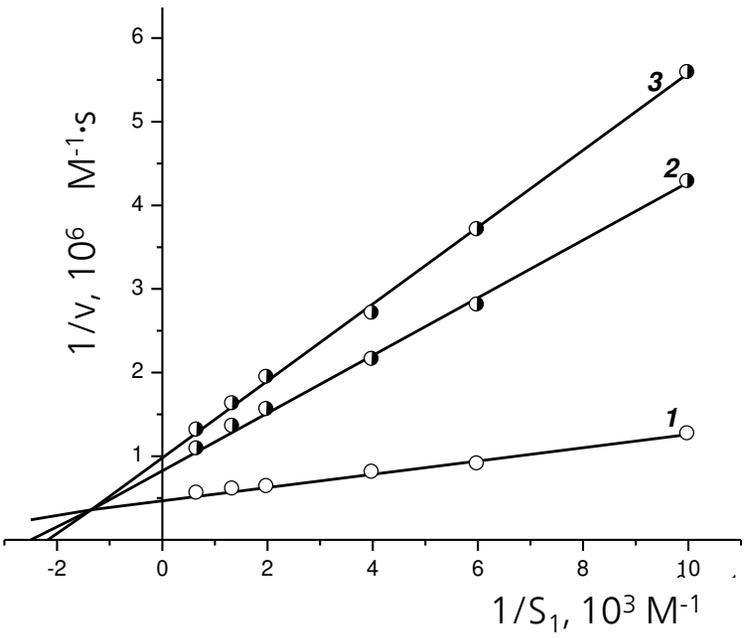

Figure 13. Dependences of the reciprocal inhibition reaction rate of NADH-oxidoreductase by $Cu(ac)_2$ on the inverse NT concentration at a constant concentration of NT (in the Lineweaver–Burk coordinates). C_{NT} = 6.68 10^{-3} M; C_{enzyme} = 2.83·10^{-7} M; C_{NADH} = 1.0·10^{-4}–1.5·10^{-3} M; (1) without an inhibitor; in the presence of $Cu(ac)_2$ in the concentration (2) 3.0·10^{-5} M and (3) 1.7·10^{-4}M.

On the contrary, the strength of the bond between $Ni(L)_2$ and NADH-OR decreases: K_i = 0.9·10^{-4} M^{-1} for Ni(II) and K_i = 12.4·10^{-4} M^{-1} for $Ni(L)_2$. The inhibition constant increases by two orders of magnitude on going from the salt to the cobalt complex: 0.014·10^{-4} and 3.8·10^{-4} M^{-1}, respectively. However, $Co(ac)_2$ inhibits oxidoreductase manifesting the noncompetitive

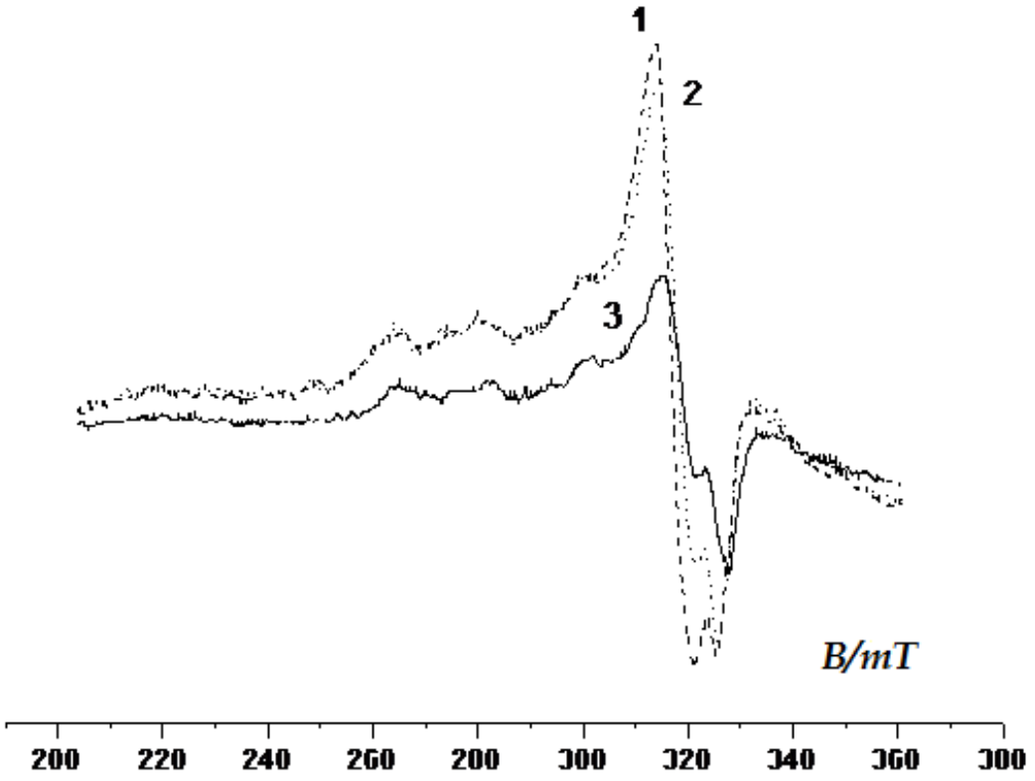

Figure 14. EPR of the metal complex (1) CuL_2 = 5.0·10^{-4} mol L^{-1}; (2) CuL_2 =5.0·10^{-4} mol L^{-1} in the presence of NADH = 6.0·10^{-4} mol L^{-1}; (3) CuL_2 = 5.0·10^{-4} mol L^{-1} in the presence of NADH = 6.0·10^{-4} mol L^{-1} and enzyme. L is lontrel. The conditions of measurements: 77 K, microwave 10 mW, magnetic field modulation 0.4 mT.

character of inhibition for NADH. At the same time, the $Co(L)_2$ complex does not compete with NADH for the binding site on the enzyme.

Of the compounds considered, only $Fe(acac)_2$ competes with an electron acceptor for the binding site in the active center of the enzyme. The same character of inhibition is retained in the iron complex with respect to NADH, but K_i for $Fe(L)_2$ is 14 times lower than that for Fe(II): 1.1·10^{-4} and 14.2·10^{-4} M^{-1}, respectively.

The change in the pattern of inhibition by the lontrel complexes with doubly charged metal ions may be due to the fact that the interaction of these complexes with the protein involves other protein ligands (thiol groups, the imidazole part of histidine, and other amino acid residues of the peptide chain). In addition, the metal ions in these complexes can be reduced by the enzyme, as it was shown by ESR for the lontrel complexes with copper ions (Fig. 14).

Our results indicate that the herbicides, fungicides, and lontrel metal complexes can react with NADH-OR in the cavity of the protein matrix in which either NADH binding or electron transfer to a natural or artificial electron acceptor takes place. An additional interaction of these compounds beyond the enzyme active center cannot also be dismissed. The structure, size, and spatial configuration of the pesticide molecule are also significant. Apparently, the large size of kusagard and sedoxidim molecules prevents them from entering the cavity of the protein globule, which may account for the weak inhibition of NADH-OR by these com-

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pounds. Apparently, the combination of these factors is responsible for the different mechanisms of NADH-OR inhibition by the considered compounds.

The Hill factor (*h*) is nearly equal or close to 2 for all of the compounds, which indicates that two inhibitor molecules can be attached simultaneously to the enzyme both inside and probably outside the active center (Table 3). This parameter substantially distinguishes pesticides and metal complexes from metal salts. The Hill factor for the considered metal salts is close to unit (Table 5), indicating the possibility of the addition of only one metal cation to the enzyme.

Evidently, the metal cation tends to negatively charged groups of amino acids, namely, carboxyl, sulfide, and amide groups. In addition, Mn, Mg, and Zn for the least stable complexes with carbonyl, and Ni and Fe form the strongest complexes with this group (Holtzclaw & Collman, 1957; Isatt et al., 1954), which agrees with our data. Thus, the interaction of this kind near the active center of the enzyme can result in conformational changes in the region of electron transfer, which is manifested as noncompetitive and mixed types of inhibition. It is known (Tsuprun et al., 1987; Fitzpatrick et al., 2005) that the 2Fe-2S cluster is in the composition of the active center of NADH-OR. The change in the character of inhibition on going from divalent metal salts to their lontrel complexes can additionally be due to the interaction of the complexes not only with the protein matrix but also with the metal of the 2Fe-2S cluster.

Figure 15 represents the scheme of direction of the inhibitor attack. The intramolecular electron transfer in the active center of NADH-OR proceeds from flavine adenine dinucleotide (FAD) to the iron-sulfur cluster 2Fe-2S and further to an artificial electron acceptor (Bayer et al., 1996; Du et al., 2000b; Ganson & Jensen, 1988). For competitive inhibition, the cation occupies the site of NT and this breaks the chain of electron transfer. The fact that the metal salts have much lower I_{50} compared to the complexes indicates easiness of this interaction. The determining factor in the behavior of the metal salts is the structure of the electronic shells of the cation.

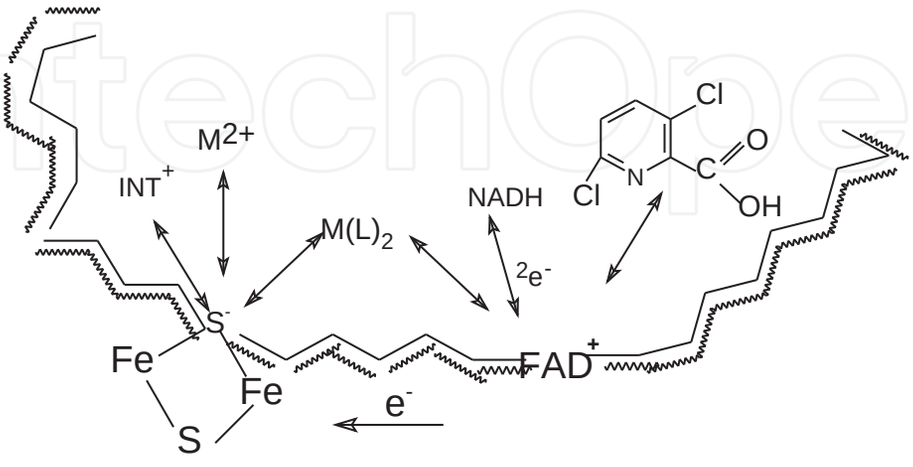
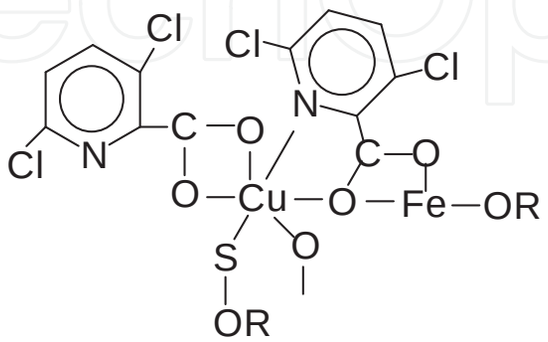


Figure 15. Scheme of direction of the attack from different inhibitors.

Present in the composition of the complex, the metal cannot act as a free cation, since it is significantly affected by the ligand environment. The considered ligand (lontrel) is capable of occupying the site of NADH, donor of two electrons, in the active center of the enzyme. Probably, the interaction with iron of the cluster occurs through the carboxyl of the ligand and due to a high electron density of chloropyridine. As a result, the ligand or complex inhibits the NADH-binding region of the electron-transfer chain.



We have previously reported (Aliev et al., 1988) that the lontrel complexes can exist in solution in both the dimeric and polymeric forms. The structures of the complexes allow them to play the role of both the electron-donor and electron-acceptor. It can be assumed that the complexes form polymer chains in which the ligands acts as a "bridge" and the metal of the complex pulls electrons of the 2Fe-2S cluster of the active center of the enzyme. The existence of two binding sites in the active center likely explains the fact that K_i of the complex (in the case of Mo, with respect to the electron acceptor; in the case of Cu, Mo, and Fe, with respect to the electron donor) is one to two orders of magnitude lower than that of the salt.

Obviously, the evaluated compounds, herbicides, fungicides, and metal complexes of herbicide lontrel, can retard oxidation processes in plants and living organisms. However, the effect of these compounds on various components of the ecosystem is manifested in a diverse character of reactions of the organisms, in the multiphase character of these reactions, and in the possible transition of one effect to another. To analyze the influence of the studied compounds on the living organisms, we chose hydrobionts – marine "luminescent" bacteria *Benechea harveyi* (strain B 1 7 – 667F), (Zhmur & Orlova, 2007; Kuz`mich et al., 2002). It is validly considered (Tsvetkov & Konichev, 2006) that hydrobionts are the most appropriate objects for the study of biochemical adaptations to the toxic action and, as a consequence, they are the most popular laboratory test-objects and object-indicators for calibrations of contaminations under natural conditions. On the other hand, when the effect of chemical toxicants on biological systems is studied, the time of the toxicological analysis itself compared to the rate of formation of metabolites in the living organism is significant. Bacteria *Beneckea harveyi* allow researchers to obtain a fast response and to compare their effect on the change in the enzymatic (luciferase) activity. It is important that the inactivation of only one enzyme is controlled *in vitro* on a model system.

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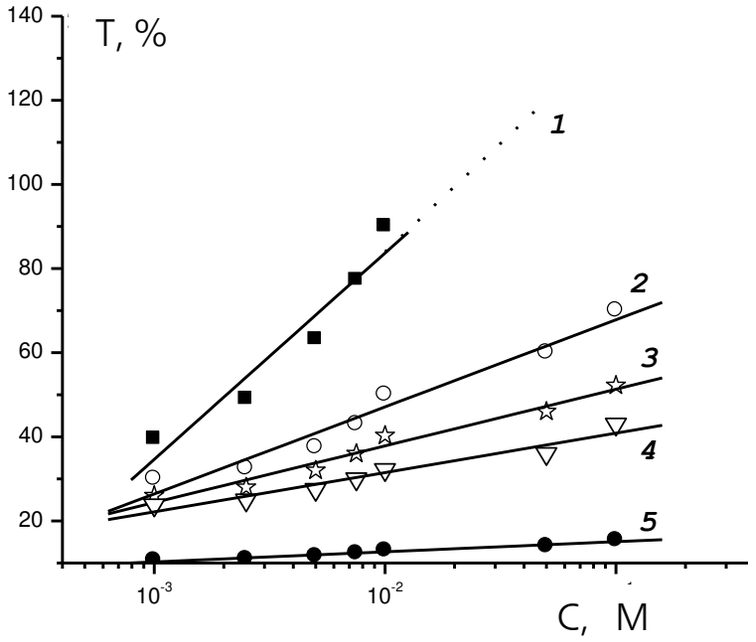

Figure 16. Change in the toxicity of the pesticides towards "luminescent" bacteria *Beneckea harveyi vs.* concentration of the pesticide in the concentration range from 10^{-1} to 10^{-3} M. (1) zenkor; (2) lontrel; (3) roundup; (4) basagran; (5) tachigaren.

The results of measurements of toxicity of herbicides and fungicide tachigaren with respect to luminescent bacteria *Beneckea harveyi* are presented in Fig. 16. The plots show that the toxicity of solutions increases proportionally to the concentration with an increase in the pesticide concentration. However, the rates of toxicity increase differ for different substances: the rate of zenkor is considerably higher than those of other compounds, whereas for tachigaren the toxicity coefficient (T, %) increases with the lowest rate. Zenkor has the highest toxicity of all the compounds evaluated. Lontrel, roundup, and basagran differ from each other to a lower extent. The lowest toxicity was determined to be tachigaren. In fact, even at the highest of the studied concentrations, 10^{-1} M, tachigaren remains to be a nontoxic compound with T < 19%. In the concentration range from $3 \cdot 10^{-3}$ to 10^{-1} M basagran exhibits a weak toxicity: $24 < T \leq 40\%$. In the concentration range from 10^{-3} to $3 \cdot 10^{-3}$ M, all compounds (except zenkor) are lowly toxic with $T \leq 50\%$. Beginning from the concentrations 10^{-3} M (zenkor), 10^{-2} M (lontrel), and 10^{-1} M (roundup), they become highly toxic compounds.

The results of measurements of pesticide toxicity are presented in Table 6. Parameter EC_{50} corresponds to the toxicant concentration resulting in the 50% decrease in the luminescence of bacteria. The values of EC_{50} increase in the order zenkor < lontrel < roundup < basagran < tachigaren. An analysis of the data in Table 6 shows that the pesticides are arranged in the decrease in toxicity in the same sequence, which is retained at all concentrations studied.

It was discussed previously that the metal complexes of herbicide lontrel are characterized by a considerable antireductase activity. However, there are no literature data on the quantitative estimation of their toxicity towards hydrobionts. The results of the study of the influence on *Beneckea harveyi* are given in Fig. 17. It is seen that all the metal complexes are toxic even at a

concentration of 10^{-7} M. The toxicity increases linearly with an increase in the concentration for all complexes. Curves 1–4 corresponding to the complexes of different metals are parallel, indicating the same rate of toxicity increasing on the concentration of ML_2. At all concentrations the toxicity coefficients of the complexes decrease in the series $CuL_2 > CoL_2 > MnL_2 > MgL_2$. As follows from Table 6, EC_{50} measured by the "probit analysis" method (Loshadkin et al., 2002) change in the same order: $CuL_2 > CoL_2 > NiL_2 > MoL_2 > MnL_2 > ZnL_2 > L > MgL_2$. It should especially be mentioned that the toxicity of almost all lontrel complexes with respect to *Beneckea harveyi* turned out higher than that of the starting herbicide. The value of EC_{50} for CuL_2 is more than two orders of magnitude lower and that for ZnL_2 is four orders of magnitude lower than that for lontrel. The exclusion is the MgL_2 complex, whose toxicity is insignificantly lower than that of the starting lontrel.

№	Pesticide	EC_{50}		ML_2	EC_{50}	
		M	g/l		M	g/l
1.	Zenkor	$(4.4\pm0.1)\cdot10^{-3}$	0.94	CuL_2	$(1.3\pm0.1)\cdot10^{-5}$	0.0058
2.	Lontrel	$(8.0\pm0.3)\cdot10^{-3}$	1.54	CoL_2	$(3.0\pm0.2)\cdot10^{-4}$	0.13
3.	Roundup	$(2.0\pm0.1)\cdot10^{-2}$	3.38	NiL_2	$(5.0\pm0.2)\cdot10^{-4}$	0.22
4.	Basagran	$(2.9\pm0.1)\cdot10^{-2}$	7.01	MoL_2	$(7.0\pm0.3)\cdot10^{-4}$	0.33
5.	Tachigaren	$(1.0\pm0.2)\cdot10^{-1}$	9.91	MnL_2	$(1.6\pm0.1)\cdot10^{-3}$	0.69
6.				ZnL_2	$(2.0\pm0.1)\cdot10^{-3}$	0.89
7.				MgL_2	$(1.0\pm0.1)\cdot10^{-2}$	4.06

Table 6. Toxicity values (EC_{50}) for the pesticides and lontrel metal complexes (ML_2) measured by the *Beneckea harveyi* biotest.

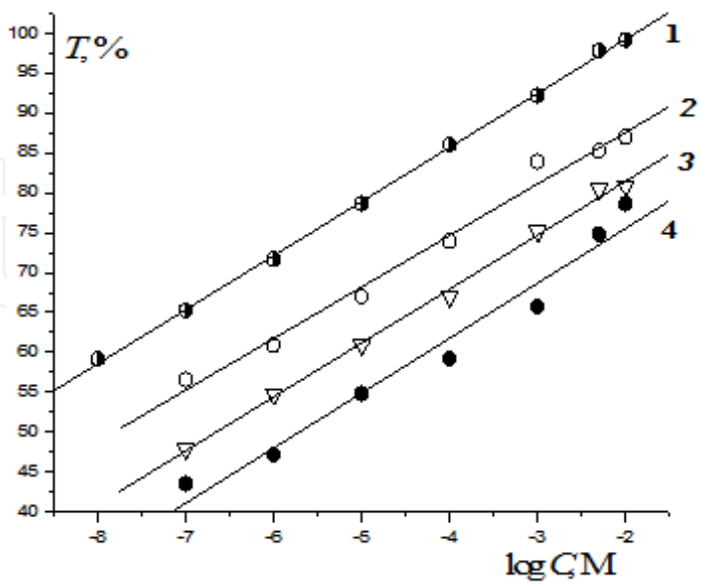

Figure 17. Change in the toxicity index determined on "luminescent" bacteria *Beneckea harveyi vs.* logarithm of the concentration of the metal complexes: (1) CuL_2; (2) CoL_2; (3) MnL_2; (4) MgL_2.

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A comparison of the data in Table 6 shows that metal complexe toxicity is manifested at concentrations two orders of magnitude lower than that of any pesticide considered. Similarly, the EC_{50} parameters of the pesticides and metal complexes indicate thatthe values of toxicity of the latter are two orders of magnitude higher than that of all pesticides and, particularly, herbicide lontrel. In the range of the concentrations studied, both the pesticides and the complexes of seven metals are arranged in the same sequence by toxicity decreasing.

The study performed of the toxicity of herbicides, fungicides, and metal complexes of lontrel towards bacteria *Beneckea harveyi* showed high toxicity. This fact indicates the antiluciferase activity of these compounds. The determined values of EC_{50} correlate with both the complexation constants (K) of these compounds with NADH and inhibition constants (K_i) of enzyme NADH-OR by these compounds.

4. Conclusion

Thus, despite the substantial differences in the chemical structures, all of the herbicides, fungicides and lontrel metal complexes studied inhibit NADH-OR at both the electron-donor and the electron-acceptor sites. These compounds inhibit the NADH-binding region and, perhaps, the intramolecular electron transfer from FAD to the 2Fe-2S iron-sulfur cluster and further to an artificial electron acceptor. This conclusion is consistent with the published data on the interruption of the electron transfer chain (Tissut et al., 1984; Macherel et al., 1982; Higgins et al., 1981) by pesticides and the involvement of metals in this process (Bayer et al., 1996; Du et al., 2000b; Ganson & Jensen 1988). The character of inhibition changes on going from the metal salts to their complexes: all the metal salts compete with an electron acceptor for the binding site, and the complexes compete with an electron donor. It is very important that in some cases, of Mo, Cu, and Fe, the strength of the bond with the enzyme increases on going from the metal ion to the ligand and to the complex ($M^{+2} < L < ML_2$), which should result in an increase in the toxic properties of the complexes compared to the metals and pesticides.

The enzyme NADH-OR is abundant in nature and is found in both unicellular and multicellular organisms; therefore, broad-scale practical use of herbicides and fungicides may entail their accumulation in living organisms and severe environmental consequences.

Evidently, one of the mechanisms of formation of toxicity of herbicides seems to be inhibition of redox processes in organisms of different trophic levels. In birds and mammals (including people), the inhibition of the oxidative enzymes decreases the protective functions of the organism and results in various maladies. Moreover, almost all well understood diseases of modern man are caused by a poor ecological state of the environment (Gichev, 2003; Klyushnikov, 2005; Mogush, 1984; Isaev, 1997; Lisichkin & Chernov, 2003).

Several thousands of pesticides are produced in the world. About 180 pesticides are widely used (Mel'nikov, 1987). Maximum allowable concentrations are substantiated only for 30 of them (Fomin, 2000). The assertion about an exclusive importance of their use for the enhancement of agricultural productivity is not substantiated (Yablokov, 1990; Fisher et al., 2002;

Yudanova, 1989; Khan, 1980; Moses, 1988; Paasivitra, 1988). The use of pesticides is an example for gaining a short-term profit of chemical companies owing to the long-term detriment for all society (Skurlatov et al., 1994; Yablokov, 1990; Suley & Uilcoks, 1983; Kurdyukov, 1982).

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