Inhibition of the nicotinamide adenine dinucleotide-oxidoreductase reaction by herbicides and fungicides of various structures

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The effect of herbicides (basagran, zenkor, kusagard, roundup, setoxidim, and lontrel and lontrel complexes with some doubly charged metal ions) and fungicides (tachigaren and tilt) on the activity of nicotinamide adenine dinucleotide (NADH)-oxidoreductase from the methylotroph *Methylococcus capsulatus* (strain M) was studied. All the herbicides and fungicides inhibit the enzyme, differing in the degree and type of inhibition. The inhibition constants K_i for these compounds and for lontrel complexes were determined. A correlation between the K_i values and the complexation constants of these pesticides with NADH was established. The studied compounds are toxic.

Key words: herbicides, fungicides, nicotinamide adenine dinucleotide, NADH-oxidoreductase, complexation, artificial electron acceptor.

Quite a few data concerning the influence of herbicides and fungicides on various components of the living cell,[1—3] in particular, on some enzymes,[4—11] have been reported. In our previous studies,[12—15] we demonstrated the formation of complexes of a number of pesticides with adenosine triphosphoric acid (ATP), nicotinamide adenine dinucleotide (NADH), and nucleic acids. Despite the enormous scale of production and use of chemical means for cultivated plant protection, there is still no unified view on the mechanism of their action. Probably, each pesticide acts through its own mechanism.

Nicotinamide adenine dinucleotide functions together with several vitally important enzymes. Therefore, it has been of interest to perform a kinetic study of some widely used pesticides on the activity of an enzyme acting together with NADH. As this enzyme, we chose NADH-oxidoreductase (NADH-OR, [EC 1.6.99.25]) from the methylotroph *Methylococcus capsulatus* (strain M),[16] which transfers electrons for mixed reduction of oxygen to water, methane transformation to give methanol in the active site of methane hydroxylase, and the reduction of dioxygen to water in the active site of cytochrome oxidase. The enzyme studied consists of four subunits, each including FAD and an iron sulfur cluster, 2Fe—2S;[17,18] NADH-OR functions according to the following scheme:

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

where A is acceptor.

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.

Here we present 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.

Experimental

Reagents. Commercial NADH (nicotinamide adenine dinucleotide) and NT (Sigma No. 2251, Reanal, Hungary) were used. The active substances of the herbicides and fungicides (their formulas are shown below) were isolated from commercial preparations by extraction.[12] The metal complexes of lontrel were synthesized by refluxing ethanolic solutions of lontrel with the corresponding divalent metal salts.[15]

Preparation of the bacterial biomass. 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}.[16] The flow rate was 0.24 m^3 h^{-1}.

* The authors are grateful to the VNIIsintezbelok for providing the possibility of growing the bacteria *M. capsulatus*.

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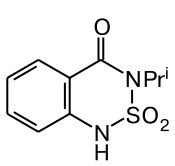
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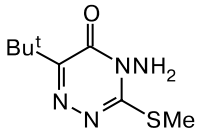
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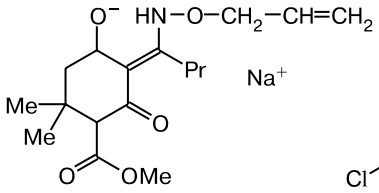
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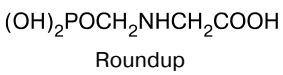
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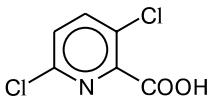
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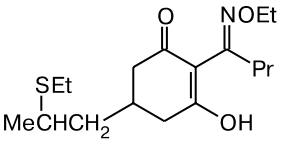
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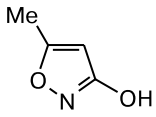
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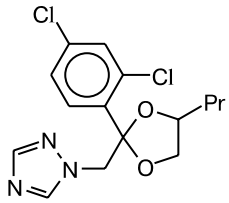
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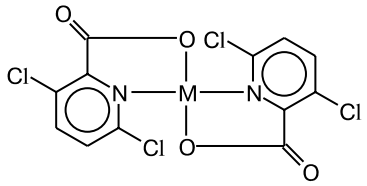
Lontrel metal complexes

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, and the supernatant was centrifuged for 1 h at 65000g, the precipitated membrane structures and the supernatant fraction ($SF_{65\text{-}1}$) were collected separately, frozen, and stored in liquid nitrogen until used.

Isolation and purification of NADH-OR. The fraction $SF_{65\text{-}1}$ (500 mL, 60 mg of protein mL^{-1}) was passed through a column (30×7 cm) with DEAE-cellulose 52 (Whatman, UK), 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 (4×70 cm) and a column with Sepharose 2B (4×80 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.

Determination of the 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[16] from the change in the absorbance at 550 nm using a Specord M-40 spectrophotometer. The reaction was carried out in 3-mL cells (10×10 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 concentration, and (2) at a constant NADH concentration and variable NT concentrations.

The K_i values were calculated from the equation[19,20]

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

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) equals

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

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

The Hill coefficients were determined by the Hill formula:[19,21]

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

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:

$$[E_h I_h] = K_h[E_h][I]^h;$$

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.

ESR spectra were recorded at 77 K on a 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. EPR spectra were recorded in 50% glycerol.

Results and Discussion

The effect of pesticides on the activity of NADH-OR is illustrated by Figs 1—5. The experimental kinetic curves for the rate of NADH-OR oxidation *vs.* the concentration of the NADH (S_1) substrate at an invariable NT concentration are presented in Fig. 1. The plots converted to the Lineweaver—Burk coordinates are shown in Figs 2—5. Figure 2 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. 2) 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).

It can be seen from Fig. 3 that the herbicide roundup does not compete with NADH for the enzyme binding site. It can be seen from Table 1 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}).

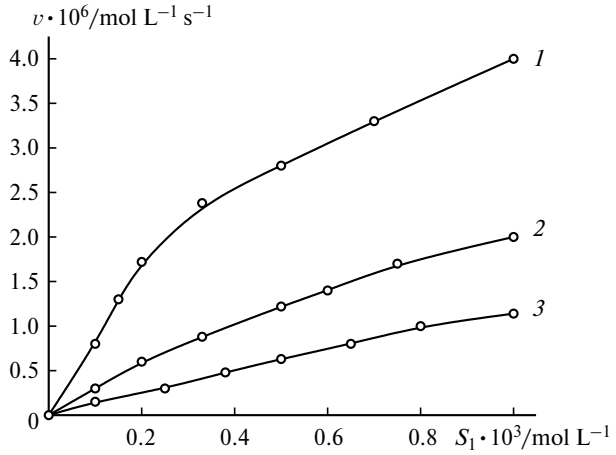

Fig. 1. Kinetic curves for the oxidation rate of NADH-OR *vs.* the concentration of NADH at a constant concetration of NT in the absence of an inhibitor (*1*) and in the presence of $0.33 \cdot 10^{-4}$ (*2*) and $1.00 \cdot 10^{-4}$ mol L^{-1} (*3*) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1};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

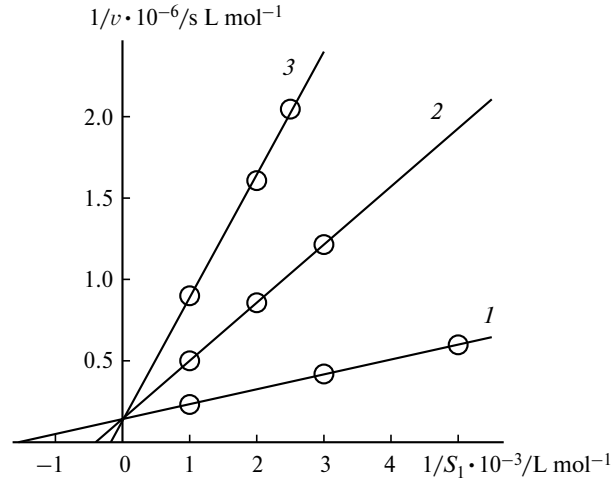

Fig. 2. Inhibition of the NADH-oxidoreductase by lontrel (in the Lineweaver—Burk coordinates) in the absence of an inhibitor (*1*) and in the presence of $0.33 \cdot 10^{-4}$ (*2*) and $1.00 \cdot 10^{-4}$ mol L^{-1} (*3*) of lontrel; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot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

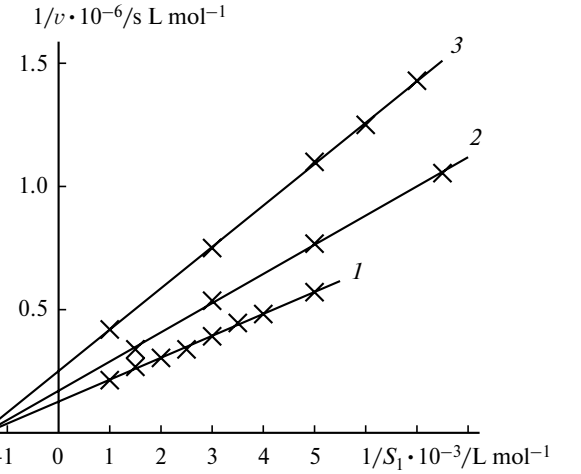

Fig. 3. Inhibition of the NADH-oxidoreductase with roundup (in the Lineweaver—Burk coordinates) in the absence of the inhibitor (*1*) and in the presence of $1.17 \cdot 10^{-3}$ (*2*) and $2.50 \cdot 10^{-3}$ mol L^{-1} (*3*) roundup; $C_{NT} = 2.467 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot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

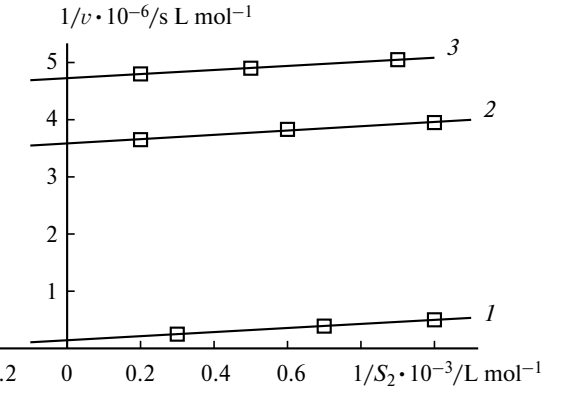

Fig. 4. Inhibition of the NADH-oxidoreductase with zenkor (in the Lineweaver—Burk coordinates) in the absence of an inhibitor (*1*) and in the presence of $3.33 \cdot 10^{-4}$ (*2*) and $5.00 \cdot 10^{-4}$ mol L^{-1} (*3*) of zenkor; $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot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

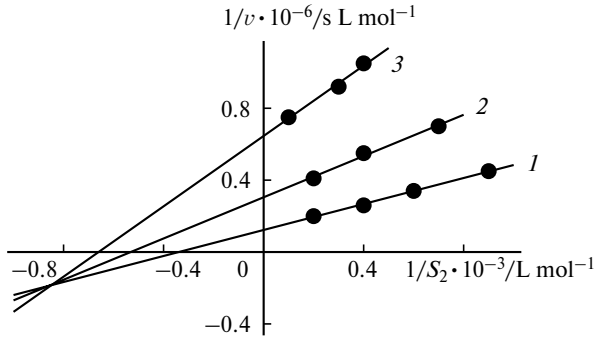

Fig. 5. Inhibition of the NADH-oxidoreductase with the cobalt complex of lontrel (in the Lineweaver—Burk reciprocal coordinates) in the absence of an inhibitor (*1*) and in the presence of $0.57 \cdot 10^{-3}$ (*2*) and $1.33 \cdot 10^{-3}$ mol L^{-1} (*3*) CoL_2 (L is lontrel); $C_{NADH} = 0.656 \cdot 10^{-3}$ mol L^{-1}; $C_{enzyme} = 1.0 \cdot 10^{-6}$ mol L^{-1}; $C_{NT} = 1.6 \cdot 10^{-5}$—$7.2 \cdot 10^{-3}$ mol L^{-1}.

The dependences of the reciprocal reaction rate on the reciprocal concentration of the NT electron acceptor (with NADH concentration remaining constant) are shown in Figs 4 and 5. The herbicides lontrel and zenkor (see Fig. 4, Table 1) also inhibit the rate of electron transfer from the NADH-OR active site 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. 5, Table 1).

The Michaelis constants (K_m) calculated without an inhibitor are $6.6 \cdot 10^{-4}$ and $2.47 \cdot 10^{-3}$ mol L^{-1} for NADH and NT, respectively.

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 1.

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 efficiently (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 1). Kusagard and setoxidim exhibit weak antireductase activities; they depress the enzyme activity when present in higher concentrations: $2.7 \cdot 10^{-2}$ and $1.7 \cdot 10^{-2}$ 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.[14]

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

Table 1. Effect of inhibitors on NADH-oxidoreductase*

Inhibitor	I_{50} /mol L^{-1}	h	For NADH				For NT			
			V_{max} /mol L^{-1} s^{-1}	S_1 mol L^{-1}	$K_i \cdot 10^4$	Type**	V_{max} /mol L^{-1} s^{-1}	S_2 mol L^{-1}	$K_i \cdot 10^4$	Type**
Lontrel (L)	$1.1 \cdot 10^{-3}$	1.726	—	$1.23 \cdot 10^{-3}$	1.00	A	$1.88 \cdot 10^{-6}$	$6.98 \cdot 10^{-4}$	7.42	B
Zenkor	$5.0 \cdot 10^{-4}$	1.952	—	$4.93 \cdot 10^{-3}$	0.25	A	$0.23 \cdot 10^{-6}$	$3.39 \cdot 10^{-4}$	8.94	B
Basagran	$6.0 \cdot 10^{-4}$	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
Roundup	$1.7 \cdot 10^{-3}$	1.328	$3.33 \cdot 10^{-6}$	$6.17 \cdot 10^{-4}$	22.00	C	$0.21 \cdot 10^{-6}$	$2.00 \cdot 10^{-4}$	42.90	B
Kusagard	$2.7 \cdot 10^{-2}$	1.575	—	$9.86 \cdot 10^{-3}$	14.00	A	—	$5.72 \cdot 10^{-3}$	158.90	A
Setoxidim	$1.7 \cdot 10^{-2}$	1.832	$2.00 \cdot 10^{-6}$	$7.59 \cdot 10^{-4}$	397.50	C	—	$11.00 \cdot 10^{-3}$	8.04	A
Tachigaren	$2.7 \cdot 10^{-3}$	1.920	—	$2.47 \cdot 10^{-3}$	21.00	A	—	$5.30 \cdot 10^{-3}$	4.55	A
Tilt	$2.2 \cdot 10^{-3}$	2.483	$1.25 \cdot 10^{-4}$	$5.98 \cdot 10^{-4}$	23.00	C	—	$13.00 \cdot 10^{-3}$	1.52	A
MgL_2	$2.0 \cdot 10^{-3}$	2.070	$1.66 \cdot 10^{-6}$	$8.97 \cdot 10^{-4}$	12.67	C	—	$23.83 \cdot 10^{-3}$	3.55	A
MnL_2	$3.0 \cdot 10^{-3}$	2.810	—	$4.93 \cdot 10^{-3}$	3.80	A	$1.72 \cdot 10^{-6}$	$1.81 \cdot 10^{-3}$	22.28	D
ZnL_2	$1.0 \cdot 10^{-3}$	2.070	$2.00 \cdot 10^{-6}$	$8.22 \cdot 10^{-4}$	10.19	C	$1.1 \cdot 10^{-6}$	$1.72 \cdot 10^{-3}$	2.46	D
CuL_2	$3.3 \cdot 10^{-4}$	1.900	—	$32.88 \cdot 10^{-3}$	0.06	A	$0.44 \cdot 10^{-6}$	$9.37 \cdot 10^{-4}$	4.01	C
CoL_2	$1.5 \cdot 10^{-3}$	1.909	$2.20 \cdot 10^{-6}$	$7.89 \cdot 10^{-4}$	13.73	C	$1.68 \cdot 10^{-6}$	$1.40 \cdot 10^{-3}$	13.05	D
NiL_2	$2.0 \cdot 10^{-3}$	2.070	$1.80 \cdot 10^{-6}$	$1.23 \cdot 10^{-3}$	12.36	C	$1.1 \cdot 10^{-6}$	$3.11 \cdot 10^{-3}$	11.67	C
FeL_2	$1.1 \cdot 10^{-3}$	1.750	—	$8.97 \cdot 10^{-3}$	1.13	A	$1.54 \cdot 10^{-6}$	$2.20 \cdot 10^{-3}$	11.67	C
MoL_2	$8.5 \cdot 10^{-4}$	1.840	—	$19.72 \cdot 10^{-3}$	0.13	A	—	$47.62 \cdot 10^{-3}$	0.41	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.

differences can be due to different structures of the pesticides used.

The metal complexes of lontrel are known[12,22] 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. The results of their influence on the enzymatic activity of NADH-OR are presented in Table 1. The inhibitory effect of the complexes ZnL_2, CoL_2, FeL_2, and MoL_2 (L is lontrel) 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.

Among the lontrel complexes with metal ions, only 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 lontrel complexes with Mn, Zn, and Co display a mixed type of inhibition. The change in the pattern of inhibition by lontrel complexes with doubly charged metal ions may be due to the fact that interaction of these complexes with the protein involves other protein ligands (thiol groups, the imidazole part of hystidine, and other amino acid residues of the peptide chain). In addition, the metal ions in these complexes can be reduced by the enzyme, as was shown by ESR for the lontrel complexes with copper ions (Fig. 6).

The results obtained indicate that the herbicides and fungicides 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. Additional interaction of these compounds beyond the enzyme active site also cannot be ruled out. The structure, the size, and the spatial configuration of the pesticide molecule are also significant. Apparently, the large size of the 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 compounds. 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 site.

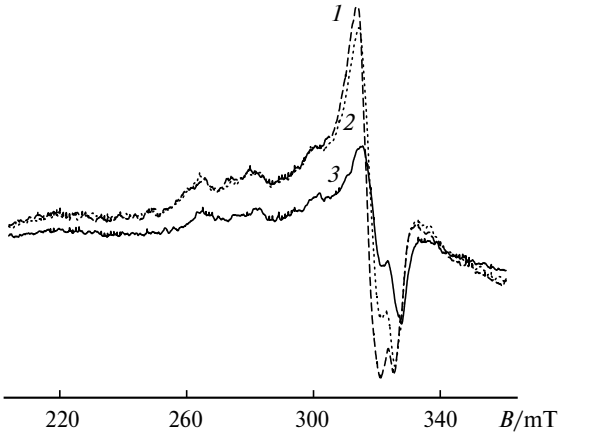

Fig. 6. EPR of the metal complex CuL_2 ($5.0 \cdot 10^{-4}$ mol L^{-1}) (*1*), CuL_2 ($5.0 \cdot 10^{-4}$ mol L^{-1}) in the presence of NADH ($6.0 \cdot 10^{-4}$ mol L^{-1}) (*2*), CuL_2 ($5.0 \cdot 10^{-4}$ mol L^{-1}) in the presence of NADH ($6.0 \cdot 10^{-4}$ mol L^{-1}) and enzyme (*3*); L is lontrel. The conditions of measurements: 77 K, microwave 10 mW, magnetic field modulation 0.4 mT.

Thus, despite the substantial differences in the chemical structures, all of the herbicides and fungicides 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 interruption of the electron transfer chain[23—25] by pesticides and the involvement of metals in this process.[26—28]

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.

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