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STERICALLY-HINDERED HYDROXYLAMINES AS BIOACTIVE SPIN LABELS

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The use of sterically hindered hydroxylamines for the regulation of free radical reactions in biological systems and for the pharmacological correction of pathological conditions is described. They are shown to possess a number of advantages over nitroxyl radicals. They are more soluble in water, are less toxic and are easily oxidized to nitroxyl radicals in aqueous solutions. Hindered hydroxylamines can be also used for the study of pharmacokinetics of bioactive spin labels by the EPR technique. Pharmacokinetic parameters for spin-labeled analogues of tetronal are evaluated. Sulfur-containing hindered hydroxylamiens are effective bioantioxidants, inhibiting efficiently the LPO of the microsomal fraction of liver and ADP- and thrombin induced plasma platelet aggregation. They increase also the survival of animals under ischemic shock. A cyclic mechanism for its antioxidative action is suggested. Recent data on the influence of the nitroxyl $\rightleftharpoons$ hydroxylamine moiety on bio-activity are summarized.

KEY WORDS: Nitroxyl free radicals, platelet aggregation, shock treatment.

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INTRODUCTION

Nitroxyl radicals (nitroxides) have been studied as a novel class of potential medicines for a long time.[1-3] Such kinds of biological activity of nitroxyl radicals as antitumor,[1,2,4-6] radiosensitizing,[7] neuro-psychotropic,[8] and antiaggregative were investigated. It is also well-known that nitroxyl radicals are reduced by chemical[10] and biological[11] agents (Scheme 1), and are disproportionized under the treatment by acids[12] (Scheme 2):

$$>\dot{N}\text{-}O \xrightarrow{[H]} >N\text{-}OH \qquad (1)$$

$$2 >\dot{N}\text{-}O + 2HX \longrightarrow >\overset{+}{N}H\text{-}OH\ X^- + >\overset{+}{N}{=}O\ X^- \qquad (2)$$

However, nitroxyl radicals not only can be reduced *in vivo* by sulfhydryl-containing compounds (glutathion, peptides and proteins) or electron transport membranes,[11] but can also be formed in cells and tissues as a result of the oxidation of corresponding sterically-hindered hydroxylamines by oxygen, superoxide radical,[13] different dehydrogenases,[14] and etc (Scheme 3):

$$>N\text{-}OH \xrightarrow{[O]} >\dot{N}\text{-}O \qquad (3)$$

Aqueous solutions of sterically-hindered hydroxylamines are characterized by an

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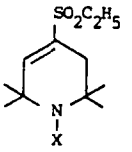
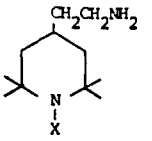
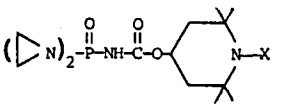
ESR spectrum of corresponding nitroxyl radicals, the amplitude of ESR lines depending upon the nitroxyl structure and the composition of the medium.[2,15] Thus, it doesn't matter what is injected into the system, at least for pharmacological experiments *in vivo*: a nitroxyl radical or corresponding hindered hydroxylamine. The animal experiments testify for neuro-psychotropic and some other kinds of activity of nitroxyl radicals and corresponding hydroxylamines being very much alike the fact resulting from our experiments.[2,8,16,17] Indeed one should consider the influence of $>N\text{-}O \rightleftharpoons >N\text{-}OH$ moiety on biological activity. Our aim is to suggest the sterically hindered hydroxylamines as potential bioactive spin labels for usage of the regulation of free radical reactions and for the pharmacological correction of pathological conditions (biomembrane lipid peroxidation).

THE ADVANTAGES OF USING STERICALLY HINDERED HYDROXYLAMINES AS POTENTIAL BIOACTIVE PARAMAGNETICS

Potential medicines based on nitroxyl radicals – bioactive spin labels – can be injected in living beings in reduced form as corresponding hindered hydroxylamines but not as nitroxyl radicals. The basic grounds for this conclusion are as follows:

1. Hydroxylamines used as salts are more soluble in aqueous solutions.
2. Hydroxylamines possess obviously less toxicity. As shown from Table 1, the

TABLE 1
The acute toxicity of nitroxyl radicals and corresponding hydroxylamines.

No	Compound	Ld_{50}, mg/kg	References
			
1	X = O	690	8
2	OH ·HCl	740	8
			
3	X = O	90	8
4	OH ·HCl	105	8
			
5	X = O	150	4
6	OH ·HCl	-*	6

*Data on the action of substances **5** and **6** onto "the increase in life span" (%ILS) values (mice murine lymphoid leukemia L1210) are available only. %ILS for **5** and **6** are 40 and 26, respectively.

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values of acute toxicity (for mice) for nitroxyl radicals are a little less then the corresponding values for hindered hydroxylamines in the case of the substances characterized by neuro-psychotropic (compounds **1** to **4**) and antitumor (**5** to **6**) activity.

3. Hydroxylamines react with superoxide radical $O_2^{\cdot-}$ to give rise to corresponding nitroxyl radicals.
4. Hindered hydroxylamines may be oxidized in aqueous solutions with the formation of nitroxyl radicals.
5. Bioactive hindered hydroxylamines are usually more easily synthesized, as protective group.

THE SYNTHESIS OF BIOACTIVE HINDERED HYDROXYLAMINES

Hindered hydroxylamines are usually synthesized from nitroxyl radicals by bubbling hydrogen chloride through its ethanol solutions. The simplicity of the synthesis of bioactive hindered hydroxylamines can be illustrated by the synthesis of spin-labeled organophosphorus cholinolytic as an example. This bioactive spin label was synthesized[18] by the addition of P–H hydrophosphoryl bond of O-alkylphenylphosphonite to the carbonyl group of triacetonamine **7** followed by the oxidation of the adduct into nitroxyl radical **8** (Figure 1). The authors were not able to fulfil the addition of O-alkylphosphonite directly to triacetonamine nitroxyl. However, they succeeded in this reaction with 1-hydroxytriacetonamine hydrochloride **9** (Figure 2). It enabled them to conduct the synthesis of this bioactive nitroxyl hydroxylamine as a one-stage process.[19]

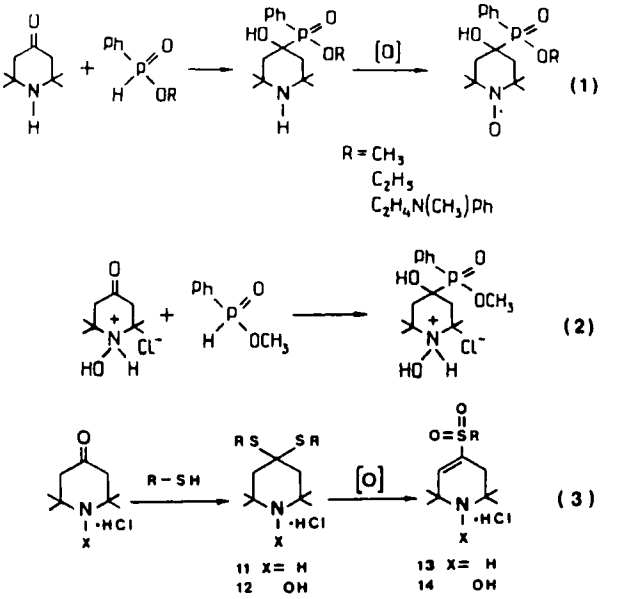

FIGURE 1 Schemes for the synthesis of bioactive nitroxyl radicals, sterically hindered amines and hydroxylamines. In line (3): R = ethyl to *n*-hexyl.

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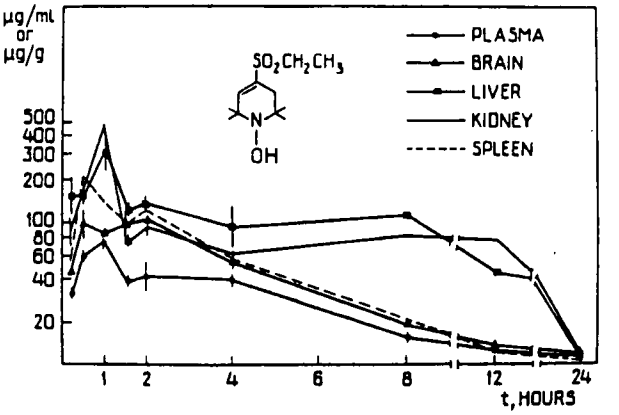

FIGURE 2 Concentration of hindered hydroxylamine **2** in blood plasma, in the brain, liver, kidneys, and spleen of rats, in mcg/ml of blood plasma or in g of tissue. Abscissa is time, hrs. Aqueous solutions of agent **2** were i.p. injected into rats weighing 180–200 g at doses of 90 mg/kg. The rats were decapitated 15, 30 min and 1, 2, 4, 12, and 24 hrs after injection. The number of rats for each point on the distribution curve ranged from 4 to 6. Blood plasma was separated by centrifuging. Tissues were homogenized in 3 volumes of water. Equal volumes of an oxidizer 0.02 N of NaOH and 0.06% of H_2O_2 (1:1) were added to homogenates and plasma. ESR spectra were registrated 60 min later.

The scheme for synthesis of sulfur-containing sterically-hindered amines and hydroxylamines of peperidine series is presented on Figure 3. The condensation of triacetonamine **8** or 1-hydroxy-triacetonamine **9** with alkanthiols affords thioketals of triacetonamine **11** or of 1-hydroxy-triacetonamine **12**, accordingly. Thioketals **11** can be oxidized to corresponding monosulfoxides by hydrogen peroxide or pertungstate.

HYDROXYLAMINES FOR STUDYING PHARMACOKINETICS BY ESR TECHNIQUE

Hindered hydroxylamine **14** (R = C_2H_5) was used for studying pharmacokinetics of nitroxyl radicals with the proved neuro-psychotropic activity.[16] To measure the concentrations of hydroxylamine **14** (R = C_2H_5) in rat blood and in the homogenates of brain, liver, kidneys and spleen the authors made use of its ability to be oxidized with hydrogen peroxide in alkaline medium with the formation of nitroxyl radical. The brain concentration of the radical attained its maximum as early as 0.5 hr after injection, remained at the constant level (100 mcg/g) within 2 hr, and after this started declining. For the excretory organs, there is a second maximum of the nitroxyl concentration detected, which is seemingly conditioned by the elimination of metabolites. By 24 hr after injection very low concentrations of the agent are found, but they are absolutely undetectable in the liver and kidneys. The data on the dynamics of the concentration of agent **14** in the blood plasma could be fairly well described within the frame of the two-compartmental model (with the account taken of absorption).[17,21] Hydroxylamine **14** (R = C_2H_5) is rapidly absorbed from the peritoneum (a half-time of absorbtion is about 20 min) and distributed in the organs and tissues ($t_{1/2,a}$ = 1.5 hr). Its elimination from the orgism also proceeds rather slowly ($t_{1/2}$ = 8.8 hr). The values of the blood plasma clearance are found to be close to those

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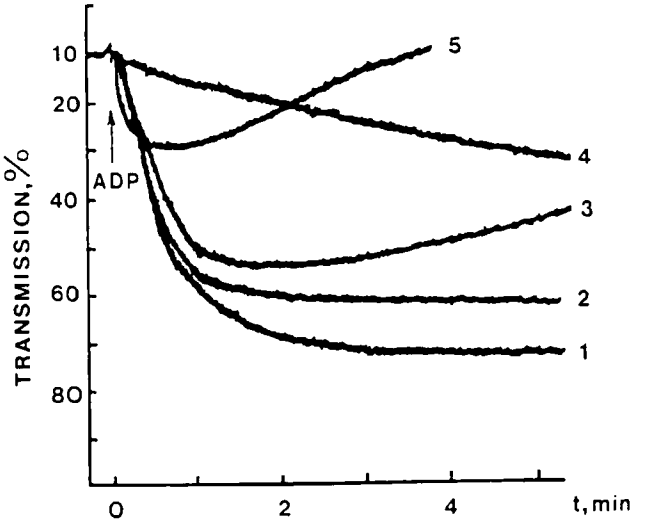

FIGURE 3 Aggregation curves (inducer ADP 0.01 mM) of rabbit platelet-rich plasma in the presence of 2.4 mM TMP (curve 2 - triacetonamine hydrochloride **7** and curve 4 - 4, 4-bis-(thio-n-propyl)-2,2,6,6-tetramethylpiperidine hydrochloride **11**) or 1-hydroxyl-TMP (curve 3 - 1-hydroxyl-triacetonamine hydrochloride **9** and curve 5 - 1-hydroxy-4,4,bis(thio-n-propyl)-2,2,6,6-tetramethylpiperidine hydrochloride **12**); curve 1 - control. In order to study aggregation the rabbit blood was taken with 3.8% sodium citrate. The anticoagulant : blood volume ration was 1:9.[24] Platelet-rich plasma was obtained by blood centrifugation at 120–160 g for 12 min. The final ADP concentration necessary to induce irreversible aggregation amounted to $10^{-2} \div 10^{-1}$ mM. ADP solution was injected at a dose of 10 mcl, and the solutions of the substances under investigation were injected at a dose of 5–50 mcl of PRP. The aggregation of platelets was investigated with Chroholog Corp. aggregometer following the Botn's method.[23]

of the glomerular filtration in rats, which proves that the nitroxyl is eliminated from the organism predominantly *via* the renal way. The nitroxyl hydroxylamine is effectively absorbed by the tissues. This is evidenced by the high value of the distribution volume V_{ss} = 2.2 l/kg, which is twice as much as the weight of the experimental animals.

HINDERED HYDROXYLAMINES AS BIOANTIOXIDANTS

It was found during pharmacological investigations of nitroxyl radicals and corresponding hydroxylamines were effective inhibitors of lipid peroxidation (LPO) in the preparations of liver endoplasmic reticulum at the same time being a novel group of bioantioxidants.[22] The intensity of lipid peroxidation was judged according to the accumulation of products that react with 2-thiobarbituric acid (TBA-active products, malonic dialdehyde, MDA). We investigated the influence of the substances **1**, **2** and **11**, **12** (R = n-propyl) on the enzymatic NADPH-dependent and spontaneous (storage for 2–4 days at 4°C) LPO of the microsomal fraction of the liver, which is a convenient model for assessing the effectiveness of the inhibiting action of various chemicals on the LPO of biomembranes. It was found that the concentrations of substances at which 100% inhibition was observed in NADPH-dependent LPO of the microsomal fraction are equal to: 10^{-2} M for **1**, 10^{-3} M for **2**, 10^{-2} M for **11**, and 10^{-5}

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M for **12**. Thus, hindered hydroxylamines **2** and **12**, existing in redox equilibrium with stable nitroxyl radicals in aqueous solutions inhibit NADPH-dependent LPO substantially more strongly than the corresponding tetramethylpiperidines **1** and **11**. Moreover, hydroxylamine **12**, containing both hydroxylamino and *n*-propyl sulfide groups, exhibited an inhibiting effect even greater than that of butylated hydroxytoluene (BHT) ionol (100% inhibition at 10^{-4} M).[22]

Analogous results were obtained in a study of the influence of these substances on the rate of spontaneous LPO and the retention of membrane-bound protein (cytochrome P-450) under conditions of storage of microsomes. Hindered hydroxylamine **12** in contrast to amine **11** lowers substantially the rate of accumulation of MDA-like material. Hydroxylamine **12** also substantially lowered the rate of destruction of cytochrome P-450. Hindered hydroxylamine **2** also lowered the level of MDA-like material during the storage of the microsomes, in contrast to hindered amine **1**. After four days of storage the content of MDA-like material in the control microsomes was 1.8 nmoles of MDA per mg protein, while in the presence of 10^{-3} M of hindered hydroxylamine **2** it was 0.6 nmole MDA per mg protein.

NITROXYL HYDROXYLAMINES ARE EFFICIENT ANTIAGGREGANT AGENTS[9,17]

Since bioantioxidants are effective inhibitors of platelet aggregation we screened a number of nitroxyl radicals and corresponding sterically hindered amines and hydroxylamines[9] using ADP-induced platelet aggregation as a model system.[23] A few of the substances are found to possess high inhibiting activity.[9] Sulfur-containing hindered hydroxylamines are the most efficient inhibitors of platelet aggregation among them. From Figure 3, which presents curves of the ADP-induced platelet aggregation in the presence of compounds **2**, **7**, **9** and **13** (R = ethyl), it follows that the inhibiting ability of hindered hydroxylamines **2** and **9** is greater in comparison with that of TMP **7** and **13** (R = ethyl). It is interesting to note that hindered hydroxylamines not only lower the aggregation degree, but also induce desaggregation of the aggregates.

TABLE 2

Influence of sterically hindered amines and hydroxylamines on the formation of MDA-like materials in platelet aggregation induced by thrombin. PRP was produced as described in Figure 3 legend. The platelet concentration, determined by the method of phase contrast microscopy in a Goryaev chamber, was $(5 \div 7) \cdot 10^5$ per mcl. Thrombin-induced formation of MDA-like material was studied as described previously.[25] The data citead in the work represent average values of three to four measurements. Compounds were synthesized as in[2,17,20]

Composition	Content of MDA-like material		Suppression of the formation of MDA-like material in thrombin-induced aggregation, %
	nmoles MDA per 10^9 cells	% of control	
Without thrombin (control)	0.52	100	–
+ thrombin	1.88	362	–
+ **7** + thrombin	1.20	231	50
+ **9** + thrombin	0.94	181	69
+ **11** (R = n-C_3H_7) + thrombin	0.88	169	74
+ **12** + thrombin	0.66	127	90

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The substances synthesized influence similarily the platelet membrane metabolism induced by thrombin. Substances **9** and **2** suppress the formation of MDA-like materials to a greater degree than compounds **7** and **13** (R = ethyl) in thrombin-induced platelet aggregation, which can be seen from Table 2. Although hindered hydroxylamines **9** and **2** lower the level of MDA by 69% and 90%, respectively, TMP **7** and **13** (R = ethyl) corresponding to them lower it only by 47% and 74%.

Besides, substances of this series lower the surface platelet charge, and increase a number of surface sulfhydryl groups of platelet membrane and its "fluidity", i.e. they increase the structural flexibility of membrane phospholipids.[9] It seems that the action of these substances on platelet membrane is the inhibition of cyclooxygenase and thromboxane B production is similar to that of aspirine.

PROTECTION BY HINDERED HYDROXYLAMINE AGAINST ISCHEMIC SHOCK

In the cases of local ischemia of various organs LPO proves to be responsible not only for the damage caused to the ischemiased organ itself, but also for the systematic lesions of the organism which develop because of the generalization of peroxidation processes in the whole organism. The latter phenomenon is observed when a massive organ is exposed to reperfusion; this frequently leads to the development of severe, sometimes lethal pathology termed "ischemic shock".[26] One of the promising directions in the screening of the antishock preparations is testing and studying the mechanisms which underlie the action of the LPO inhibitors bioantioxidants.[27]

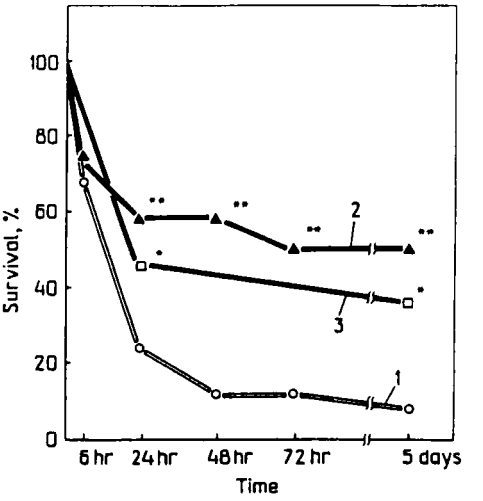

FIGURE 4 The influence of hindered hydroxylamine **12** and ionol on the survival of rats affected by ischemic shock. 1 - without substances (control); 2 - substance **12** (35 mg/kg of body weight 1 hr prior to tourniquet removal), 3 - BHT,ionol (30 mg/kg 4 hr prior to tourniquet removal). *; ** - statistically significant differences from the control: $P < 0.05$; $P < 0.01$, correspondingly. Experiments were carried out on 60 Wistar rats weighing 200–260 g. Ischemic shock was induced by a 6 hour tourniquetting of both hind limbs with subsequent reperfusion. Substance **12** was injected into the animals at a dose of 35 mg/kg 1 hr prior to removal of tourniquets.[26–28]

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The influence of hindered hydroxylamine **12** on the survival of rats has been studied, as well as its protective effect on the myocardium in the case of ischemic tourniquet shock. We used a simple model for the ischemic shock: a long-term torniquetting of both hind limbs of the experimental animals with the subsequent removal of the tourniquet (reperfusion).[28] It has been established that the use of hindered hydroxylamine **12** (Figure 4, curve 2) within the frame of the indicated schedule increases the survival of the animals affected by ischemic shock by 45% as compared to the control (curve 1). Substance **12** appeared to be even more effective than BHT ionol, which had to be injected much earlier (4 hr to the removal of the tourniquets) (curve 3), since the BHT injection given 1 hr before the tourniquet removal failed to give any effect.[26] This can be attributed to the slow absorbtion of BHT. Thus, the experiments conducted have shown that hindered hydroxylamine **12** significantly increases the percentage of the survived animals, when injected intraperitoneally at a dosage of 35 mg/kg 1 hr before the tourniquet removal. This substance proved to have a protective effect on the myocardium in the case of ischemic shock.

HYDROXYLAMINES AS A TOOL FOR THE REGULATION OF FREE RADICAL REACTIONS: CONCLUSIONS

The results obtained testify to the fact that sulfur-containing hindered hydroxylamines of piperidine series prove to be efficient bioantioxidants, which was shown on the membrane models of lipid peroxidation *in vitro*. At concentrations of $10^{-6} \div 10^{-5}$ M

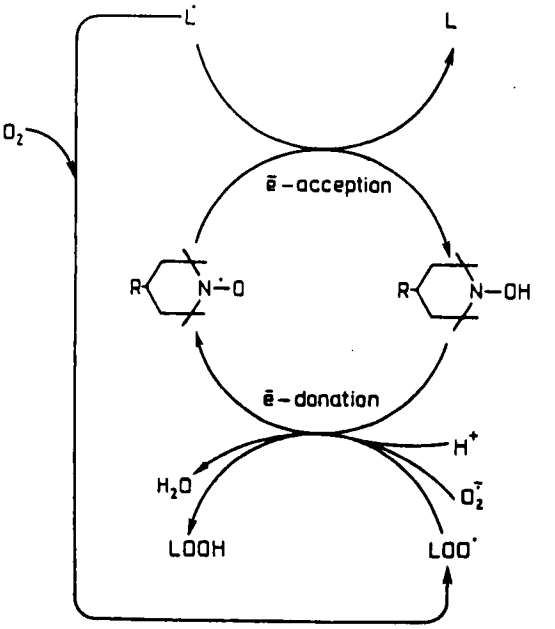

FIGURE 5 Possible cyclic mechanism for pharmacological action of sterically hindered hydroxylamines.

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these substances lower significantly the rate of peroxidation, whilst the corresponding hindered amines fail to exhibit this effect. As was shown, hindered hydroxylamines are oxidized to the corresponding nitroxyl radicals in water solutions. Therefore, the antioxidant effect observed in this case can be conditioned by the substances of both types. The existence of such efficiently functioning equilibrium between the nitroxyl (the electron-acceptor antioxidant) and its 1-hydroxy-derivative (the electron-donor antioxidant) was found in the course of the inhibition of free radical oxidation of polyolefines.[29] Figure 5 gives a schematic view of a hypothetical mechansim of the LPO inhibition induced by such systems in biomembranes.

The presence of nonoxidized atom of sulfur gives an important impact to the anti-oxidative effect of sulfur-containing hindered hydroxylamines. There is also a possibility of a synergetic effect of these two functional groups: NHOH and –S–, similar to that found in the study of the influence of the compounds of this series on red blood cells.[30] The hydrochloride of 1-hydroxy-4,4-bis(thrio-*n*-propyl)-2,2,6,6-tetramethylpiperidine **12** exhibited the highest antixodative activity. An increase in the length of the alkyl radical at the atom of sulfur up to a hexyl was found to diminish the antioxidative activity. These hindered hydroxylamines proved to be a good tool for the pharmacological correction of ischemic and reperfusive injuries, as well as a number of other pathologies, which appear to be caused predominantly by the LPO in biomembranes.

Table 3 summarises the main results on bioactivity of nitroxyl radicals and hindered hydroxylamines (animal, cell and biomacromolecule experiments). It represents the influence of nitroxyl heterocycle and N–O ⇋ N–OH moiety as well as the directions of activity variations after the modification by nitroxyl radicals. In our opinion, the nature of nitroxyl heterocycle influences the bioactivity in all cases, except radiosensitizing and haemolytic activities. According to our cell and animal experiments nitroxyl ⇋ hydroxylamine moiety affects all kinds of bioactivity described except substrate activity of spin-labeled effectors. The presence of nitroxyl moiety doesn't influence the inhibiting activity of anticholinesterase agents and substrate activity of nucleotides and phospholipids. For all kinds of bioactivity except acute toxicity and substrate activity the enhancement resulting from the modification by nitroxyl radical

TABLE 3
The influence of nitroxyl radicals and hindered hydroxylamines on bioactivity. Animal and cell experiments

Type of bioactivity or biocompounds	Influence of nitroxyl heterocycle	Influence of N–O ⇋ N–OH moiety	Directions of Activity variations	References
acute toxicity	+	+	−	1, 4, 5, 8, 16, 17
anticancer	+	+	+	1, 4, 5, 2, 17
radiosensitizing	−	+	+	7
neuro-psychotropic	+	+	−+	8, 16, 17
antiischemic	+	+	+	27, 28
antishock	+	+	+	27, 28
antiaggregating	+	+	+	9, 22
haemolytic	−	+	−+	30
anticholinesterase*	+	−	−+	18, 2, 17
nucleotides*	+	−	−+	2, 17
phospholipids*	+	−	−	2, 17, 31

The strengthening of bioactivity +, the easing of bioactivity − • Biomacromolecule experiments

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was found. In some cases the modification was characterized by the easing of bioactivity for neuro-psychotropic and haemolytic activity as well as for substrate activity.

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