On the possibility of the nitric oxide formation upon biotransformation of hydroxamic acids

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Taking into account the data on oxidative transformation of hydroxamic acids to form nitric oxide and the similarity of their structure to the hydroxyarginine group being involved in the NO synthesis, we performed the study of biotransformation of salicylhydroxamic acid and *N*(4)-hydroxyasparagine in the liver tissues. According to the data from ESR spectroscopy, the compounds under study are inefficient NO donors in a biological system and their biological activities are mainly defined by the ability to chelate metal ions.

Key words: nitric oxide, hydroxamic acids, nitro compounds, biotransformation, ESR spectroscopy.

The problems concerning the formation of nitric oxide (NO) in an organism and mechanisms of its regulation are of current interest, since NO is essential in a number of important biochemical processes in cells and tissues by being involved in regulation of blood flow and immunity, neurotransmission and protection of an organism from the microbial damage.[1—5]

In this regard, the study of the properties of compounds capable of controllable effect on the NO level in an organism during their biotransformation is rapidly developed.

In the present work, we investigated compounds related to the class of hydroxamic acids (HAs). They are commonly used in medicine and biological studies.[6] The use of HAs in biological and medical assays is based on their ability to form stable chelate complexes with metal ions.[6—11] It is the chelating properties of HAs to which their influence on the active sites of metal-containing enzymes and inhibition of enzymes, such as prostaglandin-H-synthetase,[8] histone deacetylase,[9] urease,[10] and matrix metalloproteinase,[11] are assigned. In recent years, the data on the ability of HAs to form NO upon oxidative transformation in the model chemical systems were documented allowing one to regard HAs as the NO-donating compounds.[12,13] The interest in HAs as potential NO donors is also based on the similarity of their structures to that of *N*-hydroxyarginine, which is an intermediate in the enzymatic synthesis of NO in the cells involving NO synthase. However, the question on whether a compound is a donor of NO *in vivo* cannot be solved based only on the structural considerations and experiments in the model chemical systems.

The aim of the present work was to study the possibility of NO generation by the representatives of hydroxamic acids under the conditions of biological experiments.

Experimental

Salicylhydroxamic acid (**1**) (99%, Aldrich), hydroxylamine hydrochloride (**2**) (99%, Aldrich), and *N*(4)-hydroxyasparagine (**3**) were used as purchased. *N*-(2-nitroxyethyl)maleamidic acid $O_2NOCH_2CH_2NHCOCH{=}CHCOOH$ (**4**) was used as a control NO-producing compound. Compounds **3** and **4** were synthesized in the Institute of Problems of Chemical Physics of the Russian Academy of Sciences (see Ref. 14).

For the study of biotransformation of HAs, we used the isolated C_{57}/BL mice liver. The liver was isolated after decapitation of animals and disintegrated in a saline solution. The compounds under study were then added at the concentration of $1 \cdot 10^{-3}$ mol L^{-1} and the mixture was incubated at room temperature. Working solutions of hydroxylamine ($1 \cdot 10^{-3}$ mol L^{-1}) were prepared by dissolution of the corresponding amount of hydroxylamine hydrochloride in a saline solution and pH was adjusted to 6.8—7.0 using 2 *M* NaOH. At the same time, the control samples of liver tissues were incubated in a saline solution under the same conditions without addition of the preparations. The samples were taken at intervals and the samples for recording ESR spectra were prepared according to the standard procedure. The ESR spectra of the samples prepared were obtained on a ESP-300 spectrometer (Bruker-Analitische-Messtechnik, Germany). The conditions for spectral recording were

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as follows: the microwave radiation power was 20 mW, the magnetic field modulation amplitude was 5 G, and the temperature was 77 K. The NO formation was monitored by the appearance of the signals for the heme iron nitrosyl complexes (Heme-NO) in the ESR spectra. No additional traps were introduced.

In addition, the ability of HA to evolve NO was studied *in vivo* on the 20—22 g BDF_1 mice. Solutions of the compounds under study at the concentration of 40 mg kg^{-1} were administered intraperitoneally. The animals were decapitated at intervals (as for the experiments on the isolated liver), the liver was isolated, and the samples for recording ESR spectra were prepared analogously.

Results and Discussion

To detect NO that is produced in the liver tissue samples during their incubation with different HAs, we used endogenous traps, *viz.*, heme-containing proteins of the liver tissue preparations. It is known that the heme groups of proteins have a high affinity to NO by forming the stable nitrosyl complexes Hb-NO, which cause the appearance of the characteristic ESR signal with $g = 2.02$ and the triplet structure with a splitting constant of 17 G at $g = 2.01$. In our case, the endogenous NO traps were cytochrome P-450 localized in the hydroxylating system of the liver cell endoplasmic reticulum and hemoglobin (Hb) of the residual fragments of the liver circulatory system.

We studied the changes in the ESR spectra of the liver after incubation with hydroxamic acids **1**, **3** and the control nitro compound **4**. Figure 1 shows the ESR spectra of the C_{57}/BL mice liver samples incubated for 2 h at room temperature with compounds **4** and **3** and a saline solution (control). The ESR spectra of the mice liver samples display normally the signals for cytochrome P-450 with $g_1 = 2.42$ and $g_2 = 2.25$ and signals caused by the components of the mitochondrial respiratory chain: the singlet signal for flavosemiquinones in the free-radical region and the signal for the iron-sulfur centers of the NADH-dehydrogenase complex with $g_1 = 2.02$ and $g_2 = 1.94$, respectively. In the ESR spectra of the liver samples incubated in the presence of compound **4**, the high-intensity signal for the nitrosyl complexes of heme-containing proteins with nitric oxide was recorded even 2 h after initiation of incubation (the nitrosyl complex Heme-NO, $g = 2.01$) (Fig. 1, *a*), which evidences active biotransformation of this compound in the liver cells to evolve NO. At the same time, the ESR spectrum of the liver samples with addition of HA **3** after 2 h incubation (see Fig. 1, *b*) exhibits virtually no differences from the spectra of control samples (see Fig. 1, *c*). Only after incubation for 24 h, a slight increase in the intensity of the signal for Heme-NO compared to the control was observed (Fig. 2). In this case, the increase in the ESR signal for Heme-NO was assessed by subtraction of the ESR spectra of the control liver samples from the spectra of test samples incubated under the same conditions (see Fig. 2, spectrum *3*).

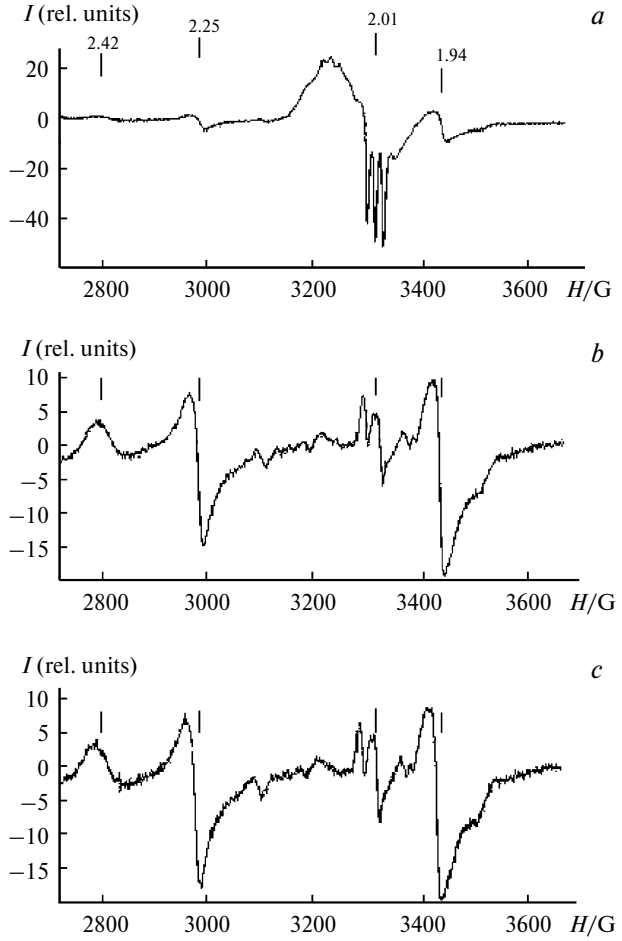

Fig. 1. The ESR spectra of the C_{57}/BL mice liver samples after incubation for 2 h at room temperature in the presence of compounds **4** (*a*) and **3** (*b*) and the spectrum of the control sample (incubation with a saline solution) (*c*).

In the analogous experiments with HA **1**, quite another pattern of the changes in the ESR spectra of Heme-NO were observed. In this case, after both 2 h and 24 h incubation, the intensities of the signals for Hb-NO in the ESR spectra of the test samples were less than those in the spectra of the control samples (Figs 3 and 4) and there was a significant increase in the intensity of the ESR signal for the low-molecular-weight iron complexes. Figure 5 shows the ESR spectra of the liver samples incubated with HA **1** in the low-field region where the ESR signals for MetHb with $g = 6.0$ and low-molecular-weight iron (Fe^{3+}) complexes with $g = 4.3$ are recorded. The signal for MetHb did virtually not change. The increase in the intensity of the signal for low-molecular-weight complexes observed for HA **1** suggests the appearance of an additional amount of iron in the system. This is probably caused by the chelating properties of HA **1**, which extracts the iron ions from the active sites of enzymes and hemoglobin. These data suggest also that HA **1** possesses more efficient chelating properties than HA **3**, for which the increase in this signal

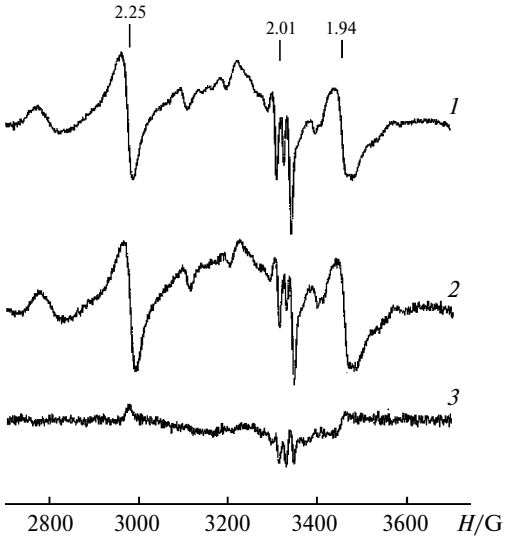

Fig. 2. The ESR spectra of the C_{57}/BL mice liver samples after incubation for 24 h with HA **3** (*1*), the control sample (*2*), and the difference ESR spectrum obtained by subtraction of spectrum *2* from spectrum *1* (*3*).

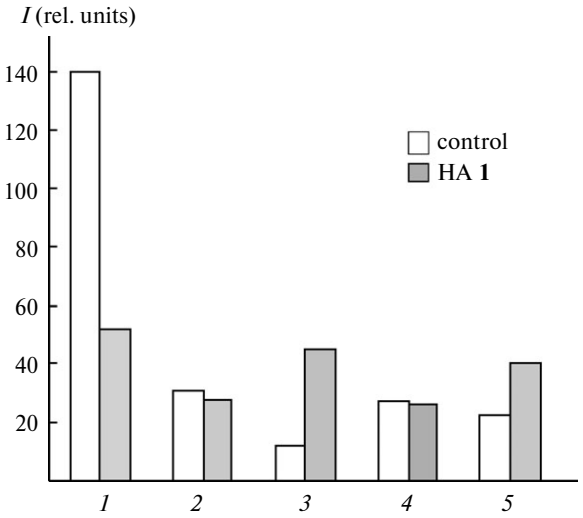

Fig. 4. The effect of hydroxamic acid **1** on the paramagnetic centers in the C_{57}/BL mice liver tissues after incubation for 24 h at room temperature: *1*, the nitrosyl complex Heme-NO (g = 2.01); *2*, Met Hb (g = 6.0); *3*, rhombic iron (g = 4.3); *4*, iron-sulfur centers (g = 1.94); *5*, cytochrome P-450 (g = 2.25). Here and in Fig. 6 *I* is the intensity of an ESR signal.

was negligible. These features of the ESR spectra reflect apparently a quite another character of the effect of HA **1** on cellular structures in contrast to HA **3** and compound **4**. For compounds **3** and **4**, metabolic transformation to form (to a greater or lesser extend) nitric oxide was predominantly observed, which was revealed by recording the signals for the nitrosyl complexes Heme-NO in these samples. Conversely, the action of HA **1** prevent the NO formation in the liver cells. As a result, the intensity of the signal for Heme-NO in the corresponding test samples is lower than that in the control samples. This effect can be explained by the fact that HAs being good complexones of metal ions can inhibit or weaken the action of metalloenzymes and, thereby, affect biochemical processes in a cell.[6,10,11,13]. The inducible NO synthase (iNOS) is one of such enzymes in the liver cells and thought to be responsible for the appearance of the nitrosyl complex Heme-NO in the control samples. To date, the NO gener-

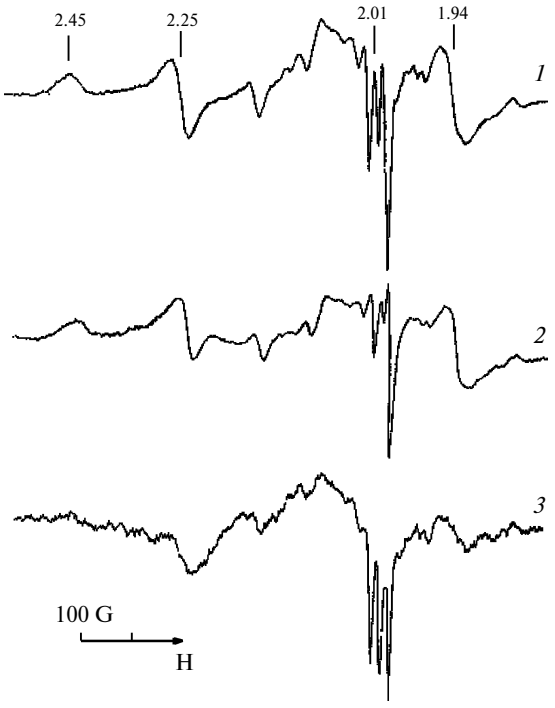

Fig. 3. The ESR spectra of the C_{57}/BL mice liver samples after incubation for 24 h without additives (*1*) and with HA **1** (*2*) and the difference ESR spectrum obtained by subtraction of spectrum *2* from spectrum *1* (*3*).

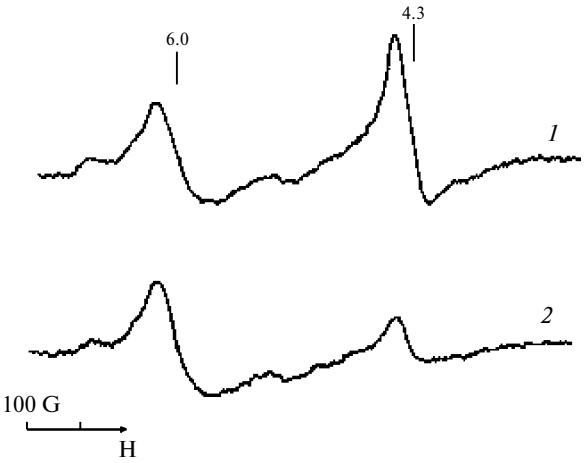

Fig. 5. The low field ESR spectra of the mice liver sample after incubation with hydroxamic acid **1** (*1*) and the control sample (*2*).

ation induced under different conditions has been observed in the liver cells of all types: hepatocytes, Kupffer cells, endothelial vascular cells, bile capillary cells, and labrocytes (Stellate and Ito cells). It is logically to assume that a decrease in the level of the nitrosyl complex Hb-NO in the samples with HA below its level in the control samples is due to the inhibition of the NO synthase function.

The obtained data indicated that biotransformation of HAs in the mice liver samples under study results in no NO formation, which is usually observed for NO donors as in the case of compound **4**. It is of note that in the case of HA **1**, there was even inhibition of the NO formation, as a less number of the nitrosyl complexes Heme-NO were recorded compared to those in the control liver samples. Documented data on the NO formation were obtained in the model system under the conditions of oxidative transformations of HAs, which are virtually not realized in a biological system.[12] Theoretically, one can assume another way of biotransformation of HAs, *viz.*, enzymatic hydrolysis to form hydroxylamine. Hydroxylamine will be oxidized somewhat easier and this reaction can lead to the NO formation. Indeed, in our experiments on incubation of the mice liver tissues with hydroxylamine (at the concentration of $1 \cdot 10^{-3}$ mol L^{-1}), the yield of NO was about twice greater than that in the control (Fig. 6). Probably, the formation of a small amount of NO in the experiments with HA **3** proceeds *via* this mechanism (see Fig. 2). For example, hydrogen peroxide can act as an oxidant, which is present in limited amounts in cells as a natural metabolic product.

In the experiments *in vivo*, where solutions of the compounds under study were administered intraperitoneally to mice, no appearance of the nitrosyl complexes Heme-NO upon biotransformation of HAs **1** and **3** was also detected. It is interesting to note that the combined administration of HAs and nitro compounds to the P-388 mice with lymphoid malignancy leads to the prolongation of inhibition of the cytochrome P-450 activity and an increase in the chemotherapeutic action of a known cytostatic agent, *viz.*, cyclophosphan.[15]

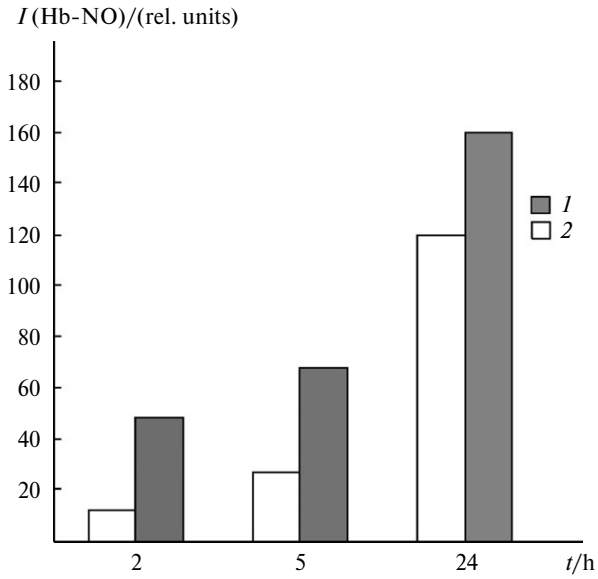

Fig. 6. The dynamics of the formation of the nitrosyl complex Heme-NO in the liver tissues upon incubation with hydroxylamine for 2, 5, and 24 h. *1*, incubation with hydroxylamine; *2*, the control sample.

Thus, in the present work we studied the possibility of the NO formation upon biotransformation of HA in the mice liver tissues (*ex vivo* and *in vivo*) by ESR spectroscopy. The obtained data evidence that the HAs under study are inefficient NO donors in the biological system, where reductive conditions are predominant, and the biological activities of HAs are mainly defined by their ability to chelate metal ions.

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