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Ultrasound-Induced Formation of S-Nitrosoglutathione and S-Nitrosocysteine in Aerobic Aqueous Solutions of Glutathione and Cysteine

I. I. Stepuro[1]*, R. I. Adamchuk[1], T. P. Piletskaya[1], V. I. Stepuro[2], and S. A. Maskevich[2]

[1]*Institute of Biochemistry, National Academy of Sciences of Belarus, Bulvar Tarashkevicha 50, Grodno, 230017 Belarus; fax: (0152) 33-2141; E-mail: stepuro@grsu.grodno.by*
[2]*Kupala State University of Grodno, ul. Ozheshko 22, Grodno, 230023 Belarus*

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Abstract—S-Nitrosocompounds are formed when aqueous solutions of cysteine or glutathione are exposed to ultrasound (880 kHz) in air. The yield of the S-nitrosocompounds was as high as 10% for glutathione and 4% for cysteine of the initial thiol concentrations (from 0.1 to 10 mM) in the aqueous solutions. In addition to the formation of S-nitrosocompounds, thiol oxidation to disulfide forms was observed. After the oxidation of over 70% of the sulfhydryl groups, formation of peroxide compounds as well as cysteic acid derivatives was recorded. The formation of the peroxide compounds and peroxide radicals in the ultrasound field reduced the yield of S-nitrosocompounds. S-Nitrosocompounds were not formed when exposing low-molecular-weight thiols to ultrasound in atmospheres of N_2 or CO. In neutral solutions, ultrasound-exposed cysteine or glutathione released NO due to spontaneous degradation of the S-nitrosocompounds. N_2O_3, produced due to the spontaneous degradation of the S-nitrosocompounds in air, nitrosylated sulfhydryl groups of glutathione manifested in the appearance of new absorption bands at 330 and 540 nm. The nitrogen compounds formed in an ultrasound field modified the sulfhydryl groups of apohemoglobin and serum albumin. The main target for ultrasound-generated oxygen free radicals were cystine residues oxidized to cysteic acid residues.

Key words: nitric oxide, nitrite, N_2O_3, S-nitrosocysteine, S-nitrosoglutathione, S-nitrosoalbumin, S-nitroso-apoHb, cysteic acid, ultrasound, pyrene fluorescence, cystine oxidation, fluorescence quenching

Many physicochemical factors, for example, exposure to light [1] or ultrasound [2], induce physiological responses (either positive or negative) on the body level. In this case, the most essential condition for physiological response formation is the triggering of biochemical reactions by biologically active products released from physiological depots under the influence of the physicochemical factors. For instance, the enhancement of blood flow caused by irradiation with red light may be related to the release of nitric oxide (NO) from nitrosohemoglobin (HbNO) or other photosensitive NO donors [1].

Nitric oxide is one of the most important biological regulators. It induces relaxation of blood vessels, modulates transmission of neural signals, and is responsible, along with oxygen free radicals, for the toxicity of phagocytes [3].

Nitric oxide is produced by NO-synthase from L-arginine with the participation of O_2 and NADPH. NO forms a complex with the iron ion of guanylate cyclase heme and activates the enzyme to increase synthesis of cyclic GMP, which mediates the effects of neuromediators and hormones [4].

NO is a very labile free radical compound; it effectively reacts with either O_2 in air or dissolved in water to form NO_2 [5]. The half-conversion time of NO to NO_2 is about 5-6 sec [6]. The generated nitrosyl radicals dismutate to N_2O_4, which forms NO_2^- and NO_3^- ions in water [7]. Under certain conditions NO can react with thiol groups to produce S-nitrosocompounds and S-nitrosylated proteins, for instance, serum albumin [8]. In arterial blood, NO is converted to NO_2^- accompanied by oxidation of oxyHb to metHb, whereas in venous blood the presence of NO leads to a production of small amounts of nitrosoHb due to the binding of NO to deoxyHb [9]. The oxidation of ferro forms of Hb by nitrites gives rise to NO and S-nitrosoglutathione in erythrocytes [10, 11].

S-Nitrosocompounds, especially S-nitrosoproteins, are more stable substances than free NO and are transferred via the blood flow over significant distances [12]. This probably ensures stability in supporting the tonus of blood vessels.

* To whom correspondence should be addressed.

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Therefore, the participation of NO in reactions with low-molecular-weight thiols, protein sulfhydryl groups, and the ferro forms of hemoglobin plays an important role in the mechanisms of transport, distribution, and stabilization of NO level in tissues.

Ultrasound is extensively used for diagnosis in clinical medicine, physiotherapy, and laboratory practice to obtain liposomes and to rupture cellular membranes. The effects of ultrasound on the body arise from a number of factors. These are primarily mechanical and thermal stimulation as well as chemical reactions [13]. The chemical effects are related to the production of cavitation bubbles in which water vapors are dissociated to form OH- and H-radicals [14-16].

The primary free radicals formed interact both with themselves and with dissolved gases and produce, along with hydrogen peroxides, a considerable amount of nitrogen oxides as well as nitrites and nitrates [13]. Enhancement of blood flow due to ultrasound-induced local relaxation of blood vessels has been found earlier [17, 18].

The changed blood flow remained for 30 min after ultrasonic treatment of a tissue section or an organ [17]. Ultrasound was shown to accelerate healing of chronic varicose ulcers on legs. The temperature increase was insignificant, which probably indicates that the mechanism of the effect was not thermal [19]. We suppose that in blood or single organs subjected to long-term treatment with a medical ultrasound apparatus S-nitrosocompounds could be formed. The possible induction of cavitation is connected with inhomogeneity of media that consists of cells, intracellular liquids with different oxygen saturation, etc. The concentration of glutathione, the main intracellular low-molecular-weight thiol, is high in cells (e.g., in erythrocytes it is 0.5-1.0 mM).

In this work, we studied the formation of S-nitrosoglutathione, S-nitrosocysteine, and S-nitrosoproteins under the influence of ultrasonic treatment of aqueous and aqueous–alcohol solutions of low-molecular-weight thiols and proteins in atmospheres of air or nitrogen. We also investigated the generation of NO and nitrogen oxides caused by spontaneous degradation of S-nitrosocompounds under anaerobic and aerobic conditions.

METHODS OF INVESTIGATION

Aqueous and aqueous–alcoholic solutions of glutathione (GSH) and cysteine (Cys) in an atmosphere of air were exposed to ultrasound with frequency 880 kHz and intensity ranging from 0.2 to 2 W/cm^2. The head of the ultrasonic device was cooled with water running through a hollow coaxial copper cylinder at 400 ml/min. The head of the ultrasonic device was built into the base of the cylinder.

Samples (5 ml, concentration 10^{-4}-10^{-2} M) in 25-ml glass flasks were placed on the surface of the sonication device and exposed to ultrasound for 30-300 min.

Solutions saturated with N_2 and CO were obtained by bubbling with molecular nitrogen or CO, respectively, for 10 min. Carbon dioxide was obtained by addition of sulfuric acid to an aqueous solution of formic acid in a Kipp gas generator.

The formation of S-nitrosoglutathione or S-nitrosocysteine after ultrasonic treatment of aqueous solutions of GSH or Cys, respectively, were recorded spectrophotometrically [20, 21].

Hydrogen peroxides as well as glutathione and cysteine peroxides were measured by the iodometric method using catalase [22].

The number of sulfhydryl groups in thiol solutions after exposure to ultrasound was recorded with Ellman's reagent using the molar absorption coefficient 13,600 $M^{-1}\cdot cm^{-1}$ [23]. Cystine formed in solutions was determined after preliminary treatment with $NaBH_4$. Then pH values of the solutions were sequentially lowered to pH 3.0 (to degrade excess $NaBH_4$), brought back to pH 7.0, Ellman's reagent was added, and absorption was recorded at 412 nm. The concentration of the cystine produced was determined by the increment in absorption in comparison with solutions not treated with $NaBH_4$. Cysteic acid and its derivatives were determined by a method described earlier [20].

Oxy-Hb was isolated from fresh donor blood by a method described previously [24]. OxyHb concentration in solutions was measured spectrophotometrically using the following absorption coefficients (in $M^{-1}\cdot cm^{-1}$): ε_{412} = 125,000; ε_{576} = 15,150; ε_{542} = 14,250 [25]. NO_2^- concentration was determined using the Griess reagent [26]. The concentration of nitrates was measured by absorption at 400 nm in the presence of phenol-2,4-disulfoacid [27].

NO_2 formation in aqueous solutions due to degradation of S-nitrosocompounds in air was monitored according to the amount of oxyHb oxidized to metHb considering that 1 mol of NO_2^- oxidizes 1 mol of ferrous cations in Hb [7].

N_2O_3 production was detected spectrophotometrically using the amount of nitrosylated GSH with absorption at 334 nm (ε = 780 $M^{-1}\cdot cm^{-1}$) as indicator.

The number of protons produced during the transition of nitrogen oxides NO_2 and N_2O_3 into aqueous solutions was determined spectrophotometrically using phenolphthalein as indicator and based on the Henderson–Hasselbach equation [28].

Formation of nitrotyrosine or nitrotyrosine residues within in human serum albumin (HSA) in water solutions of tyrosine or protein (pH 9.0) exposed to ultrasound was registered spectrophotometrically using molar absorption coefficient ε_{428} = 4200 $M^{-1}\cdot cm^{-1}$ [29].

NO release observed in degrading S-nitrosocompounds was monitored spectrophotometrically by nitrosoHb formation [10, 11] and by a method using the quenching of pyrene fluorescence. To record NO by the fluorescence quenching method, the desaturation device shown in Fig. 1 was used.

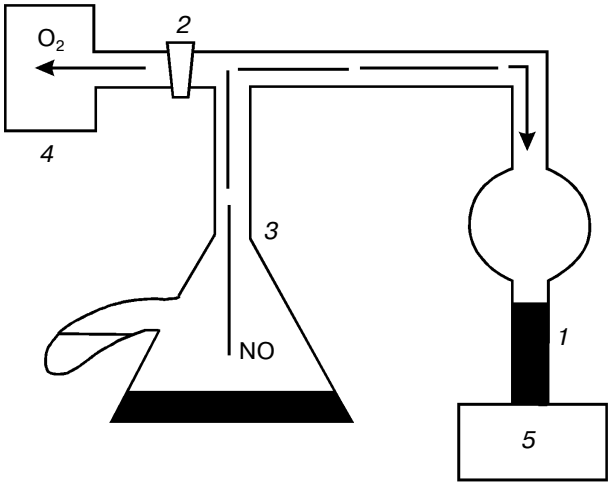

Fig. 1. Scheme of desaturating device for fluorometric (or spectrophotometric) determination of NO. It consists of a rectangular glass cuvette (l = 1 cm) (*1*), valve connecting the evacuated device either with vacuum pump or with atmosphere (*2*), Warburg vessel with sidearm (*3*), vacuum pump (*4*), magnetic mixer (*5*). After the removal of air, the desaturating device was placed into the spectrofluorometer (or spectrophotometer) sample holder.

The desaturation device is comprised of the following parts: right-angled glass cell (l = 1 cm) (*1*), valve which is used to connect the desaturation device with the vacuum pump or air (*2*), Warburg vessel with a sidearm (*3*), vacuum pump (*4*), and magnetic mixer (*5*). After removal of air, the desaturation device is placed into the cell compartment of the spectrofluorometer.

Pyrene solution (10^{-5}-10^{-6} M) was poured into the glass cell, whereas 2 ml of Tris or phosphate buffer (pH 7.0, 0.2 M) was placed into central compartment of the Warburg vessel. The solution of S-nitrosoglutathione or S-nitrosocysteine obtained in an ultrasound field was placed into the sidearm of the Warburg vessel. Air was pumped off with the vacuum pump to a pressure of 0.2 torr, and the reaction was started by mixing of the corresponding S-nitrosothiol with 0.2 M buffer, pH 7.0. The concentration of dissolved NO was measured from the quenching of pyrene fluorescence.

A similar device (Fig. 1) was also used to record NO spectrophotometrically using deoxyHb [10, 11].

A single-photon-counting fluorimeter with nanosecond time resolution [30] was used to measure fluorescence decay lifetimes. An solution of POPOP (1,4-bis[5-phenyl-2-oxazolyl]benzene) in ethanol was used as a standard with lifetime of 1.35 nsec. The nonlinear least-squares method with the Marquardt algorithm was employed for analysis of the fluorescence decay data [31].

Absorption spectra were recorded using a Specord M40 (Germany) spectrophotometer, and steady-state fluorescence spectra were measured using a Solar spectrofluorometer (Belarus).

RESULTS

1. Formation of S-Nitrosocompounds from Low-Molecular-Weight Thiols and Proteins Exposed to Ultrasound

After exposure of aqueous solutions of GSH or Cys under air to ultrasound, a new band with maximum at 340 nm and a weaker band at 540 nm appeared in the absorption spectrum (Fig. 2). The long-wavelength absorption band is responsible for the pink color of sonicated solutions of GSH or Cys. This absorption spectrum is a characteristic feature of S-nitrosothiols [20, 32].

The yield of S-nitrosocompounds induced by an ultrasonic field for GSH was higher than that for L-cysteine (Fig. 3). At thiol concentration of 10^{-2} M, the amount of S-nitrosothiol produced approached 10% and ~4% of the initial thiol concentration in solutions for GSH and Cys, respectively. S-Nitrosoglutathione yield (absolute value) was maximal at GSH concentration of ~8 mM (Table 1). Besides the formation of disulfides and S-nitrosocompounds in an ultrasonic field, the formation of peroxide compounds was detected. Figure 4 shows kinetics of GSH (10^{-3} M concentration) transformation in an ultrasonic field. Due to the very low concentrations for these conditions, the amount of produced S-nitrosoglutathione is not shown in Fig. 4. The GSH content in aqueous solution decreased after the ultrasound treatment. The kinetics of the decrease in sulfhydryl group number measured using Ellman's reagent is shown (Figs.

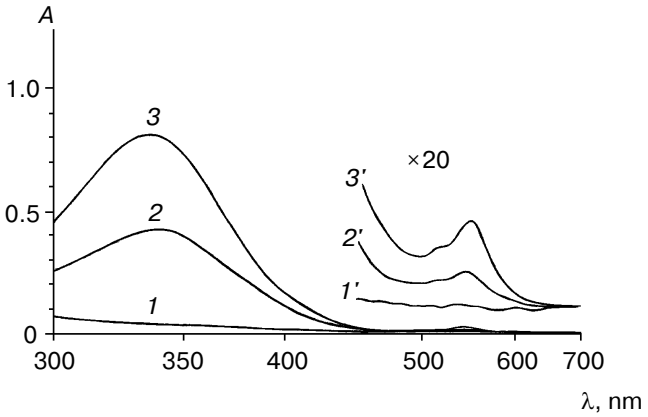

Fig. 2. Absorption spectra of GSH (*1*, *3*) and Cys (*2*) in aqueous solutions after exposure to ultrasound under nitrogen (*1*) or air (*2*, *3*). GSH and Cys concentrations were 10 mM. The time of the ultrasonic treatment (880 kHz, intensity 2 W/cm^2) of the aqueous thiol solutions was 30 min.

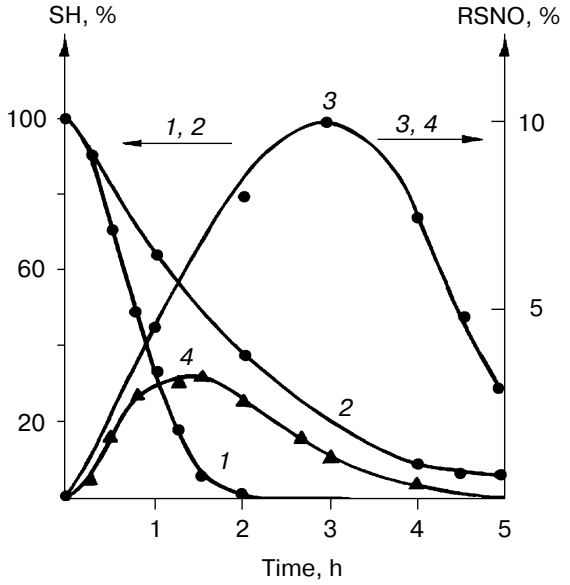

Fig. 3. Kinetics of the decrease in Cys (*1*) and GSH (*2*) sulfhydryl groups and formation of S-nitrosoglutathione (*3*) and S-nitrosocysteine (*4*) in aqueous solutions of GSH and Cys exposed to ultrasound under air. The concentration of GSH and Cys was 10 mM. Ultrasound intensity, 2 W/cm^2; frequency, 880 kHz. The initial sulfhydryl group concentration was taken as 100%. The yield of RSNO did not exceed 10% of initial thiol concentration.

3 and 4) for concentrations of 10 and 1 mM, respectively. After production of significant amounts of oxidized GSH (70% and more), peroxide compounds titrated iodometrically were found in the solution. Further sonication resulted in growth in peroxide compound concentration accompanied by a decrease in S-nitrosocompound production (Figs. 3 and 4).

The results indicate that formation of peroxide compounds in solution occurs after significant oxidation of the thiols to the corresponding disulfides, which is in good agreement with data obtained earlier [20]. Peroxide compounds or their intermediate forms probably interact with S-nitrosocompounds to eliminate them. Addition of hydrogen peroxide to the aqueous solution accelerates S-nitrosoglutathione degradation. Therefore, it can be suggested that peroxide compounds formed in an ultrasonic field (and consequent peroxide radicals) will decrease S-nitrosocompounds production.

In an ultrasonic field, amino acids and alcohols form peroxides that are easily detectable by the iodometric method [22]. Therefore, the presence of alcohols and amino acids led to a decrease of S-nitrosocompound yields in aqueous solutions of thiols exposed to ultrasound (Table 2).

The decrease in the production was inversely proportional to the concentration of peroxides produced by the ultrasound treatment of solutions of amino acids or alcohols. For example, the yield of amino acid peroxides was 22 and 36 μM for aqueous solutions of arginine and valine, respectively, exposed to ultrasound for 15 min.

In the N_2 atmosphere, S-nitrosocompounds were not formed. In water–alcohol solutions with 0.1 M ethanol (Table 2) as well as in the presence of valeraldehyde (1 mM), S-nitrosocompounds were practically not formed after exposure to ultrasound.

The effect of ultrasound on distilled water in the air atmosphere resulted in hydrogen peroxide formation, detectable by the iodometric method, and also nitrites and nitrates (Table 3).

The concentration of hydrogen peroxide after 30-min sonication of distilled water in the air atmosphere at ultrasound intensity 2.0 W/cm^2 was 3 mM. Our measurements showed that the amount of nitrite produced in water exposed to ultrasound for 30 min in air was 0.04 mM, and constituted only a few percent of the maximal quantity (0.6 mM) of the S-nitrosoglutathione formed (Table 1).

Lesser amounts of nitrites were produced in sonicated aqueous solutions of GSH. For example, in GSH solutions (10 mM) exposed to ultrasound for 90 min, 1.85 mM

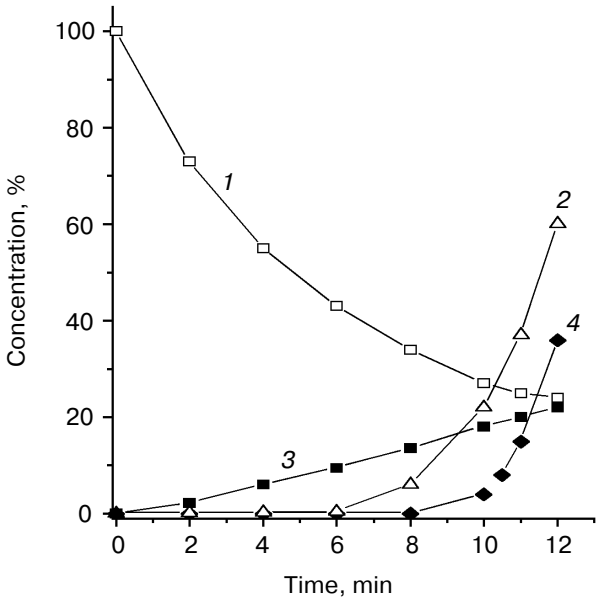

Fig. 4. Kinetics of decrease of GSH sulfhydryl groups (*1*), overall production of GSH peroxides and hydrogen peroxides (GSOOH + H_2O_2) (*2*), production of hydrogen peroxides in water (*3*), and GSH peroxides (*4*) after sonication of aqueous solutions of GSH (*1*, *2*, *4*) or water (*3*). The initial GSH concentration of 1 mM was taken as 100%. To determine the concentrations of GSH (*1*), GSOOH + H_2O_2 (*2*), H_2O_2 (*3*), and GSOOH (*4*) at different times, the percentage values for the concentration should be multiplied by 1 mM. The overall concentrations of hydrogen peroxides and GSH peroxides were measured iodometrically without a preliminary addition of catalase. The concentration of only GSH peroxides was determined after the preliminary incubation of catalase with sonicated GSH.

Table 1. S-Nitrosoglutathione formation in aqueous solutions with different GSH concentrations exposed to ultrasound (exposure time, 30 min)

Initial concentration of GSH, mM	Absorption at 340 nm	Concentration of S-nitroso-glutathione, mM	Fraction of GSH converted to S-nitrosoglu-tathione, %
2.0	0.165	0.20	10
4.0	0.345	0.40	10
6.0	0.485	0.60	10
8.0	0.520	0.65	8.1
10.0	0.510	0.64	6.4

Table 3. Formation of NO_2^- and H_2O_2 after ultrasound treatment of different intensities on distilled water in the air atmosphere (exposure time, 1 min)

I, W/cm^2	$[NO_2^-]$, μM	$[H_2O_2]$, μM
0	0	0
0.2	0.80	0
0.4	1.40	0.8
0.6	2.40	3.6
0.8	5.70	7.1
1.0	9.60	11.6
1.2	12.0	14.6
1.4	15.0	19.0
1.6	18.0	22.0
1.8	21.0	30.0
2.0	24.1	30.0

Table 2. Dependence of S-nitrosoglutathione (GSNO) and S-nitrosocysteine (CysNO) formation in aqueous solutions of GSH and Cys exposed to ultrasound on atmosphere composition and presence of organic compounds (concentrations of GSH and Cys are 10 mM; L-Arg and L-Val, 1 mM; exposure time, 30 min)

Composition of aqueous solution	Air atmos-phere	N_2 atmos-phere	CO atmos-phere	Yield of GSNO or CysNO, mM
GSH	—	—	+	0
GSH	—	+	—	0
GSH	+	—	—	6.1
GSH + ethanol (0.9 mM)	+			0.48
GSH + ethanol (1.8 mM)	+	—	—	0.44
GSH + ethanol (3.5 mM)	+	—	—	0.23
GSH + ethanol (35 mM)	+	—	—	0.02
GSH + ethanol (100 mM)	+	—	—	0
L-Cys	+	—	—	0.35
L-Cys + L-Arg	+	—	—	0.13
L-Cys + L-Val	+	—	—	0.08

Table 4. Production of S-nitrosoprotein after the sonication of apoHb aqueous solutions in air or nitrogen atmospheres for 60 min (the amount of NO released due to the spontaneous degradation of S-nitroso-apoHb was recorded spectrophotometrically by HbNO production; apoHb concentration is represented for tetrameric molecules)

Solution composition	Air atmosphere	Nitrogen atmosphere	HbSNO yield, μM
ApoHb (65 μM)	+	—	50
ApoHb (65 μM)	—	+	0

S-nitrosoglutathione and only 0.05 mM nitrite were formed.

S-Nitrosocompounds were not observed after mixing GSH or Cys with nitrite-containing sonicated water. These results indicate that S-nitrosocompounds are formed due to interaction of sulfhydryl groups not with nitrite, but with unstable intermediate products, e.g., the nitrogen oxides NO and NO^+ generated in an ultrasonic field.

After ultrasound treatment of aqueous solutions of HSA and apoHb, a weak absorption band at 340 nm appeared which was not observed in the initial protein solution. Sonicated proteins spontaneously released NO, which was registered spectrophotometrically by HbNO formation (Table 4).

Simultaneously with the modification of HSA sulfhydryl groups in an ultrasonic field, a fast oxidation of

Table 5. Oxidation of cystine residues to cysteic acid after sonication (30 min) of aqueous solutions of serum albumin (the amino acid content was determined after acid hydrolysis of the protein (110°C in 6 N HCl for 24 h) using an amino acid analyzer)

Amino acid	HSA	HSA + ultrasound
Cys	35	20.9
Gly	82	83
Met	6	4.3
Tyr	18	15
His	16	14
Cysteic acid	0	11

cystine residues to cysteic acid residues was observed. Besides cystine residues, the most significant decrease in content was observed for histidine and methionine residues as well as for aromatic amino acid residues (Table 5). The concentrations of other amino acid residues, for example of glycine, were not changed or changed insignificantly.

After ultrasound treatment of an aqueous solution of tyrosine or HSA, absorbance at 360-420 nm and weak fluorescence with maximum at 450 nm were observed.

Therefore, the results indicated that S-nitrosocompounds and products of oxidative transformation of thiols by oxygen free radicals are formed in ultrasound-treated aqueous solutions of low-molecular-weight thiols and of proteins containing sulfhydryl groups. Cysteic acid is mainly formed due to disulfide bond oxidation by hydroxyl radicals. This is suggested by the results of ultrasound treatment of low-molecular-weight thiols as well as by high yield of cysteic acid residues in the case of HSA (Table 5). It is known that S-nitrosocompounds are unstable in neutral media and degrade releasing NO, which in air forms a range of active compounds. Thus, we later registered the kinetics of NO release as proof of S-nitrosocompound formation in an ultrasonic field and as a tool to study the possible participation of S-nitrosocompound degradation products in secondary reactions. Measurements were carried out spectrophotometrically by monitoring HbNO formation and using the method of pyrene fluorescence quenching.

2. Spontaneous Release of NO from S-Nitrosocompounds under Anaerobic Conditions

2.1. Spectrophotometric monitoring. When S-nitrosoglutathione produced in an ultrasound field was located in a glass vacuum desaturator and air was removed, its spontaneous degradation accompanied by NO release could be seen. NO release was accelerated in neutral media and by addition of L-cysteine. In parallel, the decrease of absorption bands at 340 and 543 nm occurred. The amount of released NO was monitored by HbNO formation due to binding of NO with Hb. After long incubation times with deoxyHb under vacuum, practically all released NO was bound:

$$Hb + NO \rightarrow HbNO. \quad (1)$$

This is due to the extremely high value of the NO-binding rate. The equilibrium association constant of deoxyHb with NO is 1500 times higher than that with CO, and approximately 30,000 times higher than that with O_2. Therefore, after sufficiently long periods of time, NO is practically completely utilized from the desaturator. We found that the amount of spontaneously degraded S-nitrosoglutathione, as measured by a decrease of absorption (at 340 and 543 nm), satisfactorily coincided with the amount of HbNO produced.

In the same manner, measurements of S-nitrosoproteins produced after ultrasound treatment of water solutions of apoHb and HSA were carried out. The amount of NO released due to spontaneous degradation of S-nitrosoproteins was equal to the HbNO formed. Hb has six sulfhydryl groups [33], and HSA has one residue of cysteine, Cys-34 [34]. The yield of S-nitrosocompounds for

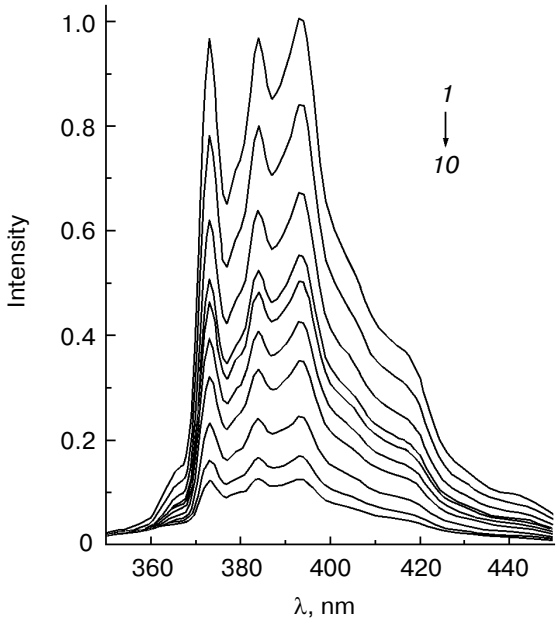

Fig. 5. Fluorescence of pyrene monomer in butanol under vacuum (*1*) and in air atmosphere at different pressures (*2-10*) (in torr): *2*) 22.5; *3*) 45; *4*) 72; *5*) 100; *6*) 140; *7*) 175; *8*) 240; *9*) 360; *10*) 760.

apoHb was 3% per mole of sulfhydryl group within the protein after a 30-min ultrasound treatment (Table 4). This is significantly lower than, for example, for GSH (Table 1).

The described spectrophotometric method for measurement of NO is very time-consuming (significant time is needed to reach complete binding of NO to Hb), and it can only measure amounts of NO that do not exceed the Hb concentration in the solution.

2.2. Measurements of NO concentration using pyrene fluorescence quenching. We developed a fast and sensitive method for monitoring of NO based on the quenching of fluorescence by NO molecules. Fluorescence intensity depends on quencher concentration [Q] according to the Stern–Volmer equation:

$$I_0/I = 1 + k_q\tau[Q],$$

where I_0 and I are fluorescence intensities in the absence and presence of quencher, respectively; k_q is bimolecular rate constant of quenching, and τ is the emission lifetime in the absence of quencher.

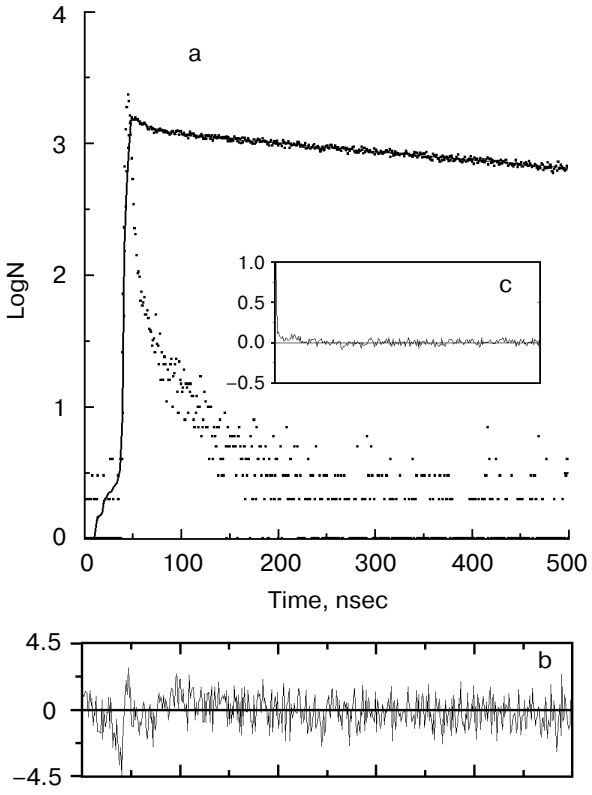

Fig. 6. Fluorescence decay of pyrene (10 μM) in butanol in the absence of oxygen. The decay lifetime is $\tau = 307 \pm 5$ nsec; $\lambda_{ex} = 337$ nm; $\lambda_{em} = 390$ nm. a) Experimental and calculated decay curves: dots correspond to experimental data; the solid curve is the result of approximation with a single exponential function; b) distribution of weighted residuals; c) autocorrelation function of weighted residuals.

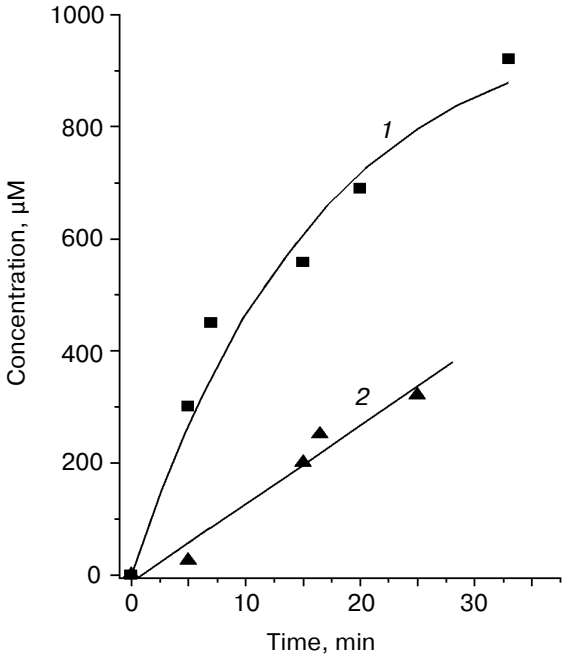

Fig. 7. Kinetics of NO formation during spontaneous degradation of S-nitrosocysteine (*1*) or S-nitrosoglutathione (*2*) measured by pyrene fluorescence quenching under anaerobic conditions. The concentration of pyrene was 10 μM, S-nitrosocysteine 2.5 mM, and S-nitrosoglutathione 6.0 mM. Besides the S-nitrosocompounds, thiols were also observed in solutions. The S-nitrosoglutathione/GSH ratio was 1 : 3, and the S-nitrosocysteine/Cys ratio was 1 : 2.

We used pyrene as the fluorescent molecule because of its high quantum yield and long emission lifetime. In dilute solutions, pyrene exists as monomers characterized by a fluorescence spectrum with vibronic structure. Pyrene fluorescence is strongly quenched by oxygen (Fig. 5). The fluorescence lifetime of the monomer in butanol in the absence of oxygen is ~300 nsec (Fig. 6). Since k_q both for oxygen and NO is ~10^{10} $M^{-1}\cdot sec^{-1}$ [35], one can easily calculate the concentration of quencher from fluorescence intensity measurements.

The solubility of quencher molecules in organic solvents and in water differs greatly, which results in different quenching of pyrene fluorescence at constant partial pressure of NO or O_2. Therefore, it is desirable to perform measurements in one organic solvent to exclude effects connected with different solubility of the gaseous ligands NO and O_2.

Air was removed from the desaturator volume with a vacuum pump, and pyrene fluorescence in the absence of quencher was measured. Then an aqueous solution of S-nitrosoglutathione, placed in the sidearm of a Warburg vessel, was mixed with phosphate buffer (pH 7.0) in the Warburg vessel. In weakly acidic media S-nitrosoglutathione is stable, but in neutral media in the presence of free GSH it easily degrades, releasing NO, which causes the quenching of pyrene fluorescence (Fig. 7).

These results indicate that in ultrasound-treated aqueous solutions of thiols, S-nitrosocompounds (NO donors) are formed. The rather fast degradation of the S-nitrosocompounds is probably due to the presence of low-molecular-weight thiols in solution [12, 21].

Under an atmosphere of air, nitric oxide produced during the degradation of S-nitrosocysteine and S-nitrosoglutathione undergoes more complicated transformations.

3. Formation of NO, NO_2, and N_2O_3 during Degradation of S-Nitrosoglutathione in Air. Kinetics of S-Nitrosoglutathione Degradation in Air Atmosphere

In air, S-nitrosoglutathione degradation accompanied by a decrease in absorption bands at 340 and 543 nm was also observed. The S-nitrosoglutathione degradation rate increased with increasing pH of the medium. Released NO interacts with air oxygen to form NO_2 in a three-component reaction (2) [7].

Nitrosyl radicals produced recombine to N_2O_4, which in water forms NO_2^- and NO_3^- (Eqs. (3) and (4)):

$$2NO + O_2 \rightarrow 2NO_2\,; \qquad (2)$$

$$2NO_2 \rightarrow N_2O_4\,; \qquad (3)$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+. \qquad (4)$$

At lower concentrations of oxygen in the desaturator atmosphere, the role of the interaction between NO_2 and NO molecules (reaction (5)) increases:

$$NO_2 + NO \rightarrow N_2O_3\,; \qquad (5)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+. \qquad (6)$$

As follows from reactions (2)-(6), the amount of NO_2 is equal to the increment of proton concentration in the solution. We determined changes in pH of the medium spectrophotometrically using phenolphthalein (pK_a = 9.6) and pyridoxine (pK_a = 3.7) dyes.

The aqueous solutions of phenolphthalein or pyridoxine were placed into a right-angled cuvette, whereas the solution of S-nitrosoglutathione was placed in a small flask. This flask served as a stopper that closed the desaturator hermetically. The volume of the air above solutions in the cuvette and flask was 20 cm^3. The phenolphthalein solution was bleached (Fig. 8), and in the pyridoxine spectrum the absorption band at 320 nm decreased and the band at 290 nm increased. These results indicate that the solutions became more acidic, and protonated forms of the compounds were produced. Using the Henderson equation and known concentrations of the protonated and deprotonated forms of the dye, one can calculate the increment of proton concentration in the solution [28]. It is known that NO_2^- oxidizes HbO_2 to Hb(III) [10, 11]. As it follows from comparison of data shown in Figs. 8 and 9, the amount of NO_2^- is approximately the same as the concentration of protons H^+.

The amount of additionally produced protons in the solution, as well as the Hb(III) concentration, were less than the amount of spontaneously degraded S-nitrosoglutathione. This is connected with reactions (2)-(6) between O_2 and NO in the aqueous medium in the vessel (3). After incubation for 24 h and complete S-nitrosoglutathione degradation, 0.9 mM nitrite was registered in the vessel (3).

If an aqueous solution of GSH was placed in the cuvette and an S-nitrosoglutathione solution was in the flask in the upper part of the desaturator, then after long-term incubation for 24 h or more in air, we observed the emergence of absorption bands at 334 and 543 nm. This indicates that GSH molecules were nitrosylated. The yield of nitrosylated GSH was low, not exceeding 1-2% of the total amount of released nitrogen oxides.

S-Nitrosoglutathione can also be produced due to the interaction of N_2O_3 with sulfhydryl groups of thiols and proteins [36].

Summarizing these results, we conclude that in air NO is mainly converted to NO_2, which is then transformed to NO_2^-.

On the base of these results, we suppose that also in ultrasonic fields in air the formation of S-nitrosocompounds probably is not connected with the interaction of sulfhydryl groups with N_2O_3. Indeed, the yield of S-nitrosocompounds in ultrasonic fields is rather high (Table 1). Therefore, we suppose that not N_2O_3, but nitric oxide (NO) and its redox form (NO^+) produced in ultrasound fields are mainly responsible for modification of sulfhydryl groups of low-molecular-weight thiols and also of sulfhydryl groups of Hb and HSA.

DISCUSSION

Formation of HO-, H-, and O_2^--radicals in water and aqueous solutions exposed to ultrasound has been proven by EPR spectroscopy [15]. Primary free radical products, produced in cavitational bubbles, interact with air oxygen and among themselves and form secondary products of reaction (hydrogen peroxides, nitrogen oxides, etc.). The yield of free radical products in an ultrasonic field depends significantly on the composition of the gas atmosphere and on the presence of volatile compounds [13, 14].

Among gas molecules inside the cavitational bubble, energy exchange is possible, which leads to dissociation and ionization of gas molecules [13].

In cavitational bubbles saturated with air, dissociation of N_2 and O_2 molecules takes place. Products of their degradation interact among themselves to form nitrogen oxide

and its redox forms, which diffuse to the interface between two media, and interact with sulfhydryl groups of low-molecular-weight thiols to form S-nitrosothiols that possess characteristic absorption at 340 and 540 nm (Fig. 2):

$$N + O \rightarrow NO; \quad (7)$$

$$N^+ + O \rightarrow NO^+. \quad (8)$$

In the absence of reactive sulfhydryl groups (Cys and GSH), nitric oxide in aqueous solution is mainly transformed to nitrite in reactions with oxygen (reactions (2)-(6)) or, interaction with superoxide anion, forms unstable peroxynitrite [29, 37]:

$$O_2^- + NO \rightarrow {}^-OONO. \quad (9)$$

Peroxynitrite is also formed as a product of the interaction between NO^+ and H_2O_2 [38]. Peroxynitrite is stable in alkaline medium, but if the *cis*-form is protonated (p*K* 6.8) [39], it isomerizes to nitrite with the lifetime ~1 sec at pH 7.0 [29].

Peroxynitrite oxidizes sulfhydryl groups of thiols [29, 36] and nitrates tyrosine [29]. The appearance of weak absorption at 360-420 nm in the spectrum of sonicated HSA suggests that ultrasound promotes modification of tyrosine residues.

The interaction of hydroxyl radicals with NO and NO_2 leads to the formation of nitrites and nitrates:

$$NO + OH \rightarrow HONO \rightarrow NO_2^- + H^+; \quad (10)$$

$$NO_2 + OH \rightarrow HONO_2 \rightarrow NO_3^- + H^+. \quad (11)$$

Therefore, in water exposed to ultrasound in air, hydrogen peroxides and nitric (Table 3) and nitrous acids are produced. The absorption maximum of nitrate ions is at 302 nm ($\varepsilon = 7\ M^{-1}\cdot cm^{-1}$) and that of nitrite ions at 340 nm. The yield of nitrate ions in the initial periods of exposure to ultrasound was low. The absorption spectra of NO_2^- anions and molecules of the corresponding acid HNO_2 are different (the absorption band of HNO_2 has fine structure).

Oxygen free radicals also diffuse to the interface between phases and interact with functional groups (primarily with sulfhydryl groups of Cys and GSH), causing their oxidation to disulfides, peroxides (Fig. 3), and, as shown earlier, to cysteic acid [20]. Formation of peroxides of GSH and cysteic acid does not occur until sulfhydryl group content is diminished and a significant concentrations of disulfides are produced [20]:

$$RSH + (OH, O_2^-) \rightarrow RS + (OH^-, H_2O_2); \quad (12)$$

$$RS + RS \rightarrow RS{-}SR; \quad (13)$$

$$RSH + RS \rightarrow RSHSR;\ RSHSR + RS \rightarrow RSSR + RSH. \quad (14)$$

We believe that a small production of RSO_2 in the presence of high concentrations of GSH is caused by efficient proceeding of reaction (14).

Simultaneously with sulfhydryl group oxidation by oxygen free radicals, S-nitrosocompounds are produced (Figs. 2 and 3), possibly due to interaction of thyil radicals with NO and nitrosonium ion NO^+ with RSH groups:

$$RS + NO \rightarrow RSNO; \quad (15)$$

$$RSH + NO^+ \rightarrow RSNO + H^+. \quad (16)$$

At sufficiently high concentrations of GSH, only reactions (15) and (16) proceed, and NO_2^- formation is negligible. The amount of NO_2^- produced under the action of ultrasound on distilled water in air (reactions (10) and (11), Table 3) was approximately 10 times lower than the maximal amount of S-nitrosoglutathione (6-6.5 mM) produced in GSH aqueous solutions exposed to ultrasound (Table 1). We think that this indicates the involvement of both NO and NO^+ in the formation of S-nitrosothiols.

Nitric oxide very effectively interacts with O_2^- to produce peroxynitrite (reaction (9)). The rate constant of this reaction is $6.7\cdot10^9\ M^{-1}\cdot sec^{-1}$ [37]. It results in a decrease in nitric oxide concentration. The same reactions probably occur between organic peroxide radicals and NO.

Aliphatic alcohols and amino acids, particularly in the beginning of sonication, lower the production of S-nitrosocompounds (Table 2). On ultrasound treatment of aqueous solutions of amino acids or alcohols, their peroxides are formed, these being easily detected by the iodometric method.

We suppose that the production of peroxide free radicals decreases the yield of S-nitrosocompounds due to the interaction of their forms with NO:

$$RSOO + NO \rightarrow RSO + NO_2; \quad (17)$$

$$RSO + RSH \rightarrow RSOH + RS; \quad (18)$$

$$RSOH + RSH \rightarrow RSSR + H_2O. \quad (19)$$

Glutathione peroxides are formed only after oxidation of 70% of GSH, when the role of reaction (14) is diminished due to the decrease of sulfhydryl group concentration.

In an atmosphere of N_2 or CO, we did not observe production of S-nitrosocompounds (Fig. 2, Table 2). Therefore, it can be assumed that O_2 is needed for formation of nitrogen oxides from primary radicals of N_2 dissociation.

Earlier it was shown that biologically active S-nitrosocompounds were able to spontaneously release NO, and its yield was increased after addition of thiols to the solution [21].

NO is a very active free-radical compound and effectively reacts with air oxygen or O_2 dissolved in water to form nitrite (reactions (2)-(6)).

However, when concentrations of GSH or Cys are significantly higher than S-nitrosoglutathione concentration in solution, then nitrite yield is decreased due to formation of conjugates between GSH and S-nitrosoglutathione, which then are transformed to GSSG, NH_3, and N_2O [21].

GSH or Cys solutions exposed to ultrasound contained besides initial thiol compounds and S-nitrosothiols, also disulfide forms of thiols, peroxides (Figs. 1-3), and cysteic acid, and products containing cysteic acid residues, etc. [20].

In aqueous solutions of serum albumin and apoHb exposed to ultrasound in air atmosphere, formation of protein S-nitrosocompounds capable of spontaneously releasing NO also occurred (Table 4). The yield of S-nitrosocompounds after sonication of apoHb was lower if we take into consideration the amount of sulfhydryl groups of protein and of low-molecular-weight thiols. For example, S-nitrosoglutathione production was approximately four times higher than HbSNO formation.

This could be connected with different accessibility of SH-groups in protein and/or with interaction of nitrogen oxides with other amino acid residues, e.g., tyrosine residues. In fact, as absorption spectroscopy data shows, ultrasound treatment modifies both free tyrosine and, probably, tyrosine residues in proteins. However, the number of nitrated tyrosine residues was low, not exceeding 2-5% of the number of protein S-nitrosothiols. Also, oxygen free radicals react with other targets in proteins, because, in contrast to concentrated thiol solutions, oxygen free radicals are not intercepted (reaction (12)) and not transformed to thyil radicals. The main targets for oxygen free radicals in HSA molecules are cystine residues, which are oxidized to cysteic acid residues during treatment with ultrasound (Table 5). HSA contains 17 cystine residues and one sulfhydryl group [34]. The same process of cysteic acid residue formation for low-molecular-weight thiols was also observed after production of a significant amount of disulfide compounds (70% and more) [20]. For HSA solutions, this process of cystine residue oxidation took place almost from the beginning of exposure to ultrasound.

It must be noted that both for solutions containing low-molecular-weight thiols and S-nitrosothiols and also for solutions of proteins and S-nitrosoproteins, the possibility of SH-group modification in secondary reactions with N_2O_3 exists in principle. The anhydride of nitrous acid can be formed in the reaction of NO with NO_2 after spontaneous degradation of S-nitrosothiols (reaction (6)).

Since compounds were exposed to ultrasonic fields for several hours, the produced S-nitrosocompounds partially degraded, releasing NO and forming new reaction products (reactions (2)-(6)).

Experimental methods based on pyrene fluorescence quenching by O_2 and NO (Figs. 5-7) and spectrophotometry using HbNO determination allow the amount of released NO to be determined.

Results of measurements indicate that S-nitrosoproducts obtained on exposure of aqueous solutions of thiols to ultrasound degrade spontaneously, releasing NO (Fig. 7). In air, nitric oxide formed during the degradation of S-nitrosocysteine or S-nitrosoglutathione undergoes more complicated transformation. Our measurements showed that NO was not found at oxygen partial pressure of 75 torr or more. At O_2 partial pressure of 140-150 torr, practically all of the NO was converted to NO_2. The amount of N_2O_3 did not exceed 1-2% of the total amount of NO_2. These data indicate that nitrous anhydride can be formed via S-nitrosocompound degradation in air atmosphere during long-term ultrasound exposure, but its contribution in thiol nitrosylation is small.

In water, NO_2 (N_2O_4) and N_2O_3 form NO_2^- and NO_3^-, respectively, and the same concentration of protons equal to the sum of NO_2^- and NO_3^- concentrations (Eqs. (2)-(6)). Coincidence of dye bleaching rate due to acidifying

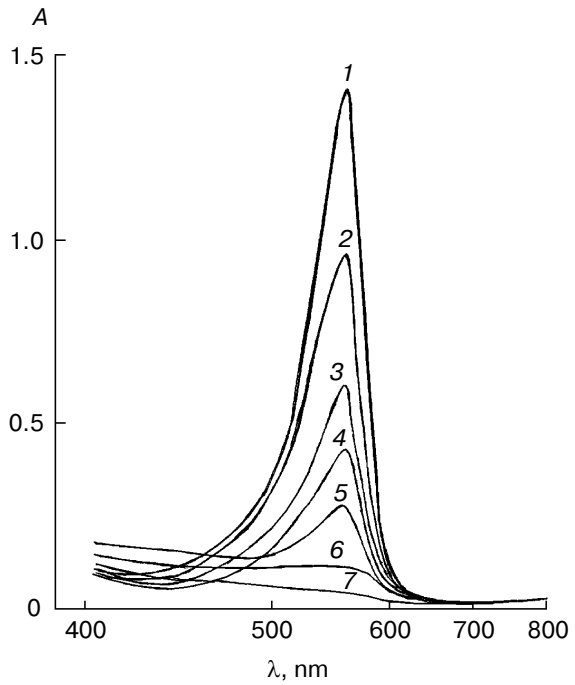

Fig. 8. Decrease in the deprotonated colored form of phenolphthalein in a desaturator (Fig. 1) because of acidification of the medium during spontaneous degradation of S-nitrosoglutathione: *1*) initial dye; *2*) after 20 min; *3*) 40 min; *4*) 60 min; *5*) 90 min; *6*) 120 min; *7*) 160 min. Phenolphthalein was placed in cuvette (*1*) and S-nitrosoglutathione in vessel (*3*) of the desaturator (Fig. 1). Phenolphthalein concentration, 0.1 mM; concentration of S-nitrosoglutathione obtained in the ultrasonic field was 10 mM. Air from the desaturator volume (20 ml) was not removed.

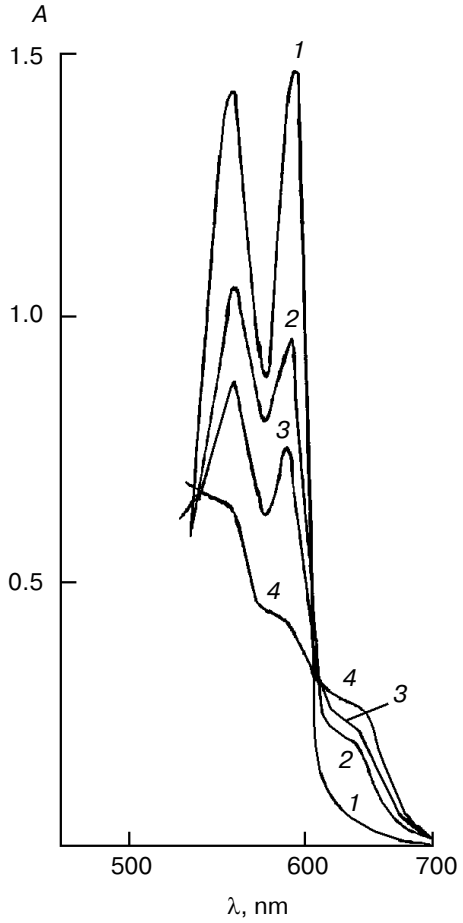

Fig. 9. Kinetics of HbO_2 oxidation by nitrite formed in cuvette (*1*) of a desaturator during spontaneous decay of S-nitrosoglutathione placed in vessel (*3*) (see Fig. 1): *1*) absorption spectrum of initial HbO_2; *2*) after 1-h incubation; *3*) after 2-h incubation; *4*) 3-h incubation. The concentration of S-nitrosoglutathione was 10 mM; the concentration of tetrameric HbO_2 was 25 μM.

of the medium when NO_2 penetrates into water (Fig. 8) and Hb(III) formation velocity as a result of HbO_2 oxidation by nitrite in air (Fig. 9), as well as HbNO formation or pyrene fluorescence quenching, indicate rather good coincidence of H^+ and NO_2^-, NO_3^- and NO concentrations. Therefore, we believe that after a sufficiently long period S-nitrosothiols completely degrade with the formation of nitrogen oxides. Probably under our experimental conditions the reaction producing conjugates between GSH and S-nitrosoglutathione, which then are transformed to NH_3 and N_2O and cause decreased NO_2^- yield [21], is insignificant and can be neglected.

Summarizing the results, we conclude that exposure of GSH and Cys to ultrasound leads to formation of S-nitrosocompounds with yields of 10 and 4%, respectively, with regard to the total amount (2-10 mM) of the thiol compounds in solution. In aqueous solutions of apoHb and HSA exposed to ultrasound, S-nitrosocompounds of proteins are also formed. Both low-molecular-weight S-nitrosothiols and protein S-nitrosocompounds spontaneously release NO. Beside S-nitrosocompounds, as main products in solution we found disulfides of thiols, peroxide compounds, and cysteic acid residues (Fig. 10).

As follows from the presented scheme, in the presence of sufficiently high GSH or Cys concentrations practically complete interception of oxygen free radicals and formation of thyil radicals are observed. S-Nitrosocompounds are formed both via the interaction of NO with thyil radicals and due to the interaction of NO^+ with sulfhydryl groups of thiols. Thyil radicals react with O_2 to form thioperoxide radicals, RSO_2, which effectively transform NO to NO_2 and, hence, decrease RSNO yield. In the presence of high (10 mM and more) concentrations of GSH or Cys in solution, cysteic acid or its derivatives are not formed because of effective transformation of RSOO to RS, and RSSR is the main product. However, at high RSSR concentrations and low concentrations of thiols, interaction of hydroxyl radicals with disulfides takes place to produce thyil radicals, which form RSO_2 in air. We noted earlier that, beside peroxynitrite, secondary reactive compounds (e.g., N_2O_3) resulting from transformation of NO during degradation of S-nitrosocompounds in air could contribute to the modification of protein sulfhydryl groups. But their contribution is small and should be taken into account only for long-term exposure to ultrasound fields. These reactions are not shown in the scheme. RSNO yield (Table 1) is approximately tenfold higher than the output of nitrite produced in water (in the absence of thiols) on exposure

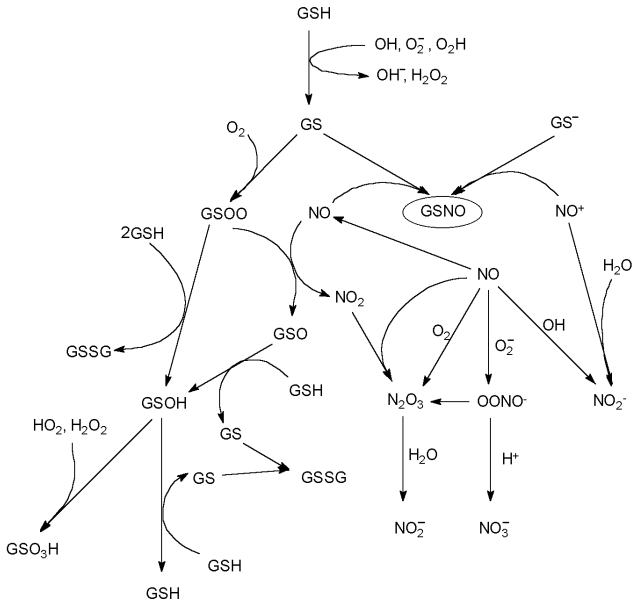

Fig. 10. Scheme of conversions of GSH in aqueous solutions exposed to ultrasound in an atmosphere of air.

to ultrasound (Table 3). On the base of these results, we suppose that mainly nitric oxide (NO) and its redox form (NO^+), produced in ultrasonic fields, are responsible for sulfhydryl group modification of low-molecular-weight thiols and proteins (Hb, HSA) and for S-nitrosocompound formation.

Ultrasound is widely used in medical practice, for example, in therapeutics, obstetrics, surgery, and for diagnosis of the vascular system [2]. During ultrasound irradiation, energy is conveyed to tissue via some contact medium, for example, a gel that is applied to the skin surface. There are dramatic maxima of intensity in the region of the radiator contact with tissue, and high radiation doses can be received. To avoid this, it is recommended to apply ultrasound with intensity of not more than 3 W/cm^2, to use interrupted exposures, and to move the sonicator head. In this work, during *in vitro* measurements, ultrasound in the range from 0.2 to 2.0 W/cm^2 was used. The yield of S-nitrosocompounds dramatically increased as the intensity of irradiation was increased above 0.3 W/cm^2 due to development of cavitation in the medium (Table 3).

However, S-nitrosoglutathione formation was observed even at the power of 0.2 W/cm^2 when ultrasound was imposed on GSH aqueous solutions in air.

We think that the favorable physiotherapeutic effect of ultrasound related to enhanced blood circulation may be due to, along with other factors, the formation of protein S-nitrosocompounds as well as low-molecular-weight S-nitrosothiols. The S-nitrosocompounds are able to spontaneously release NO, which is a vessel relaxation factor.

REFERENCES

1. Stamler, J. S., Jaraki, O., Osborne, J., Simon, D. I., Keanei, J., Vita, J., Singel, D., Valeri, C. R., and Loscalzo, C. R. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 7674-7678.
2. Bamber, J. (1989) *Physical Principles of Medical Ultrasonics* (Hill, K., ed.) Mir, Moscow.
3. Moncada, S. (1992) *Asta Physiol. Scand.*, **145**, 201-227.
4. Arnold, W. P., Mittal, C. K., Katsuki, S., and Murad, F. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 3203-3207.
5. Goldstein, S., and Czapski, G. (1995) *Free Rad. Med.*, **19**, 505-510.
6. Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991) *Pharmacol. Rev.*, **43**, 109-142.
7. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., and Byrns, R. E. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 8103-8107.
8. Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J., and Loscalzo, J. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 444-448.
9. Wennmalm, A., Benthin, G., and Petersson, A. S. (1992) *Br. J. Pharmacol.*, **106**, 507-508.
10. Stepuro, I., Chaikovskaya, N., Piletskaya, T., and Solodunov, A. (1994) *Pol. J. Pharmacol.*, **46**, 601-607.
11. Stepuro, I. I., Chaikovskaya, N. A., Solodunov, A. A., and Artsukevich, A. N. (1997) *Biochemistry (Moscow)*, **62**, 960-966.
12. Pietraforte, D., Mallozzi, C., Scorza, G., and Minetti, M. (1995) *Biochemistry*, **34**, 1177-1185.
13. Margulis, M. A. (1986) *Sonochemisty Reaction and Sonoluminescence* [in Russian], Khimiya, Moscow.
14. Kondo, T., and Riesz, P. (1995) *X Int. Congr. Radiation Research. Congress Lecture* (Hagen, U., Harder, D., Jung, U., and Streffer, C., eds.) Vol. 2, Wurzburg, Germany, pp. 1103-1106.
15. Riesz, P., and Kondo, T. (1992) *Free Rad. Biol. Med.*, **13**, 247-270.
16. Hengelein, A. (1987) *Ultrasonics*, **25**, 6-16.
17. Abramson, D., Burnett, C., Bell, Y., Tuck, S., Rejal, H., and Fleischer, C. (1960) *Am. J. Phys. Med.*, **39**, 51-62.
18. Haar, G. (1989) *Physical Principles of Medical Ultrasonics* (Hill, K., ed.) Mir, Moscow.
19. Dyson, M., Franks, C., and Suckling, J. (1976) *Ultrasonics*, **14**, 232-236.
20. Stepuro, I. I., Solodunov, A. A., and Sokolovskaya, S. N. (1995) *Biofizika*, **40**, 1158-1164.
21. Singh, S. P., Wishnok, J. S., Keshive, M., Deen, W. M., and Tannenbaum, S. R. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 14438-14443.
22. Gebicki, S., and Gebicki, J. (1993) *Biochem. J.*, **289**, 743-749.
23. Torchinski, Yu. M. (1977) *Sulphur in Proteins* [in Russian], Nauka, Moscow.
24. Benesch, R., Benesch, E., Renthal, R., and Maeda, N. (1972) *Biochemistry*, **11**, 3576-3582.
25. Kempen, G., and Zijlstrat, W. (1983) *Adv Clin. Chem.*, **23**, 199-257.
26. Green, D., Wagner, A., Glogowski, J., Skipper, P., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem.*, 1**26**, 131-135.
27. Terekhova, R. K., and Shishkina, I. N. (1962) *Izvestiya Vuzov. Khim. Khim. Tekhnol.*, **5**, 848.
28. Williams, V., and Williams, H. (1976) *Basic Physical Chemistry for the Life Sciences* [Russian translation], Mir, Moscow.
29. Pryor, W. A., Gueto, R., Jin, X., Koppenol, W. H., Schemllin, M. N., Squadrito, G. L., Uppu, P. L., and Uppu, R. M. (1995) *Free Rad. Med.*, **18**, 75-83.
30. Gachko, G. A., Zybelt, V. K., Kivach, L. N., Maskevich, S. A., and Maskevich, A. A. (1987) *Zh. Prikl. Spektr.*, **47**, 335-339.
31. O'Connor, D. V., and Phillips, D. (1984) *Time-Correlated Single Photon Counting*, Academic Press, London.
32. Vanin, A. F., and Malenkova, I. V. (1996) *Biochemistry (Moscow)*, **61**, 505-513 (Russ.).
33. Bunn, H. F., Forget, B. G., and Ranney, H. M. (1977) *Human Hemoglobins*, W. B. Sandress Company Philadelhia, London-Toronto.
34. Meloun, B., Moravek, L., and Kostka, V. (1975) *FEBS Lett.*, **58**, 134-137.
35. Calvert, J., and Pitts, J. (1968) *Photochemistry* [Russian translation], Mir, Moscow.
36. Wink, D. A., Cook, J. A., Kim, S. Y., Vodovotz, Y., Pacelli, R., Krishna, M. C., Russo, A., Mitchell, J. B., Jourd'heuil, D., Miles, A. M., and Grisham, M. B. (1997) *J. Biol. Chem.*, **272**, 11147-11151.
37. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1620-1624.
38. Halfpenny, E., and Robinson, P. L. (1952) *J. Chem. Soc. (A)*, 928-938.
39. Koppenol, W., Moreno, I., and Pryor, W. (1992) *Chem. Res. Toxicol.*, **5**, 834-842.