Heat-induced formation of nitrogen oxides in water

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Abstract It was found by the fluorimetric method using 2,3-diaminonaphthalene that moderate heating of water (60–80°C, for up to 4 h) leads to the fixation of atmospheric nitrogen with the formation of nitrite. The kinetic parameters of this process were determined. The energy of activation of NO_2^- formation was estimated to be 139 kJ/mol. It was found that the amount of nitrite formed depends on the concentration of dissolved oxygen and nitrogen. It was shown by two independent methods (Griess reagent/ VCl_3 and 2,3-diaminonaphthalene/nitrate reductase) that heating of water (80°C, 1 h) results in the formation of nitrate; with the use of the fluorescent probe dihydrorhodamine 123, the generation of nitrogen dioxide (peroxynitrite) was revealed. Nitrite, nitrate, and nitrogen dioxide are formed in water upon heating in approximately equal amounts. A scheme of reactions proceeding with bidistilled water by the action of heat with the formation of nitrogen oxides is proposed.

Keywords Heat · Water · Nitrogen monoxide · Nitrite · Nitrate · Peroxynitrite

1 Introduction

Nitrogen is a key element of living and inorganic nature. Molecular nitrogen constitutes 78% of the earth's atmosphere and is fixed in the biogeochemical nitrogen cycle as reactive and biologically available forms. Nitrogen is incorporated into proteins, nucleotides, hormones, and vitamins and ranks fourth in abundance in living matter next to carbon, oxygen, and hydrogen.

Nitrogen oxides play an important role in normal physiological and pathological processes in living organisms. Nitrogen monoxide (*NO) is synthesized enzymatically by five-electron oxidation of L-arginine by NO synthases [1]. The spectrum of biological

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functions of $\bullet\text{NO}$ is very broad; it is involved in the regulation of vessel tone, intra- and intercellular signaling, neurotransmission, differentiation, immune responses, and other processes [2]. In inflammatory states, excessive $\bullet\text{NO}$ binds to a superoxide ($\bullet\text{O}_2^-$) to be converted to cytotoxic peroxynitrite (ONOO^-) and its derivative nitrogen dioxide ($\bullet\text{NO}_2$) [3]. Nitrite and nitrate were initially considered as biologically inert stable end-products of $\bullet\text{NO}$ oxidation in vivo. Recent studies have demonstrated that NO_2^- and NO_3^- can be reduced both enzymatically and chemically (under hypoxic conditions at acidic pH values), acting as a potential reservoir and an important alternative source of $\bullet\text{NO}$ in organisms [4, 5]. It was shown that nitrite and peroxynitrite are involved in intracellular signaling [3, 6]. The average concentration of nitrate in human blood plasma is normally $\sim 30\ \mu\text{M}$ and that of nitrite is by two orders lower [7].

The main exogenous sources of nitrogen oxides for humans are food (primarily of plant origin), drinking water, and air pollutants [8]. The amount of nitrate contained in 80 g of spinach leaves is greater than its amount formed in the human body in a day as a result of the functioning of all NO synthase isoforms [9]. It was shown that nitrogen oxides are formed non-enzymatically in biological tissues and water solutions of different compositions by the action of physical factors, such as ultrasound [10, 11], UV light [12], blue light (453 nm) [13], as well as upon electrochemical oxidation of nitrogen dissolved in water [14]. Electromagnetic fields are believed to increase the life time of radicals and hence the probability of their involvement into chemical reactions [15].

We have previously found that the exposure of air-equilibrated water solutions to heat [16–19] and light [17, 20] results in the formation of reactive oxygen species (ROS); some details of this complex process have been studied and a possible physical nature of this phenomenon has been considered [16, 18].

The goal of this work was to determine whether nitrogen oxides can be formed in water by the action of heat, the ambient electromagnetic radiation of a wide spectral range. It was found that, upon moderate heating, the fixation of atmospheric nitrogen in water with the formation of nitrogen oxides (nitrite, nitrate and nitrogen dioxide) occurs. Some kinetic parameters of this process were determined and the effect of some factors on the process was studied.

2 Materials and methods

2.1 Reagents and chemicals

The reagents 2,3-diaminonaphthalene (DAN) (ICN, Aurora, OH, or Sigma, St. Louis, MO, USA), nitrate reductase from *Aspergillus niger* (NR), β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH Na_4), dihydrorhodamine 123 (RhH_2), rhodamine 123 (Rh), L-glutathione reduced, sodium urate (Sigma, St. Louis, MO, USA) and vanadium chloride (Aldrich, St. Louis, MO, USA) were used without additional purification. Other chemicals were of reagent grade. We used 99.5% oxygen and 99% nitrogen (Institute for High Energy Physics, Russian Academy of Sciences, Protvino, Russia). A gas passageway included a water-trap to capture any adventitious particulate matter.

2.2 Water and saturation of water with gases

Bidistilled air-equilibrated water with a specific electric conductivity of 120–130 $\mu\text{S/m}$ and a pH of 5.6–5.8 was used in all experiments. Heating water at 80°C for 4 h changed its pH by

no more than 0.1 unit. For saturation with gas, water was intensively bubbled with oxygen or nitrogen for 30 min at a gas supply rate of no less than 2 l/min. The concentration of dissolved oxygen in water equilibrated with air was measured with a Clark electrode at 20°C and was on average 0.28 mM; on additional saturation of water with oxygen, it was 0.59 mM and on saturation with nitrogen, it was 0.14 mM. Also, the content of dissolved oxygen and nitrogen in water was analyzed by gas chromatography as described earlier [21]. Briefly, 2-ml samples of bidistilled water were transferred to 8.8-ml sealed vials pre-filled with argon and incubated at 30°C for 10 min in a shaker bath. The O₂ and N₂ concentrations in the gas phase were measured with an LKhM-80 gas chromatograph (Production Association 'Manometer', Moscow, Russia) equipped with a thermal conductivity detector and a 1 m × 3 mm column with molecular sieves (40°C, argon as a carrier gas).

2.3 Heating of water

Water was heated in three ways. (1) Samples of bidistilled water in 7- or 20-ml screw-capped sealed polypropylene vials for liquid scintillation counting (Beckman, Fullerton, CA, USA) were incubated in a U 10 ultrathermostat (Prufgerate-Werk Medingen, Dresden, Germany) for different periods of time at different temperatures as specified below. (2) Bidistilled water in a chemical glass (200 ml) was brought to boiling on an electric hot plate (3 kW), boiled for 10 min, and cooled to ambient temperature, following which the 5-ml samples were taken for analysis. It has been shown earlier that the concentration of dissolved oxygen in boiling water decreases to 50 μM [22]. (3) Bidistilled water in a glass flask (500 ml) was autoclaved (120°C, 0.11 MPa) for 1 h in a medical vapor sterilizer VK-75-01 (Plant of medical equipment, Tyumen, Russia), chilled to room temperature, and the 15-ml samples were tested.

2.4 Determination of nitrite by means of 2,3-diaminonaphthalene (DAN)

Nitrite concentration was determined by the highly sensitive fluorescent method using DAN [23]. DAN was dissolved in methanol at a concentration of 27 mM and stored at -20°C until used. Prior to the experiment, a DAN solution was diluted with a 0.62 M HCl solution to a concentration of 270 μM. Samples of bidistilled water (2 ml) equilibrated with atmospheric air in polypropylene vials were heated in a liquid thermostat for 0.5–4 h at a temperature from 60 to 80°C to an accuracy of ±0.1°C. The vials were then cooled in tap water, a DAN solution was added to a final concentration of 25 μM and the samples were incubated for 10 min at room temperature. The reaction was terminated by the addition of a NaOH solution to a final concentration of 130 mM (pH of a sample ~11). The fluorescence intensity of samples was measured on an SFM 25A (Kontron Instruments, Milan, Italy) or a Cary Eclipse (Varian, Mulgrave, VIC, Australia) spectrofluorometer at λ_{ex} = 365 nm and λ_{em} = 410 nm in a mirror quartz cuvette (Institute for Biological Instrumentation, Russian Academy of Sciences, Pushchino, Russia) at room temperature. The use of the mirror cuvette increased the sensitivity of the method ~3 times. Control samples were treated in a similar manner but were not heated. In calculations of the content of NO₂⁻ in heated water, the fluorescence intensity values for control samples were subtracted from those for test samples. Calibration curves were constructed using solutions of NaNO₂ of known concentrations (2–50 nM) in bidistilled water. The concentration of the stock solution was controlled assuming the coefficient of molar absorption of nitrite to be 2,400 M⁻¹ cm⁻¹ at 230 nm [24].

2.5 Determination of nitrite and nitrate using vanadium (III) and Griess reagent

Samples of bidistilled water (15 ml) in polypropylene vials were incubated in a liquid thermostat at $80 \pm 0.1^\circ\text{C}$ for 1 h and cooled. In another set of experiments, samples of bidistilled water were autoclaved. The volume of each sample in a vacuum rotor evaporator was brought to 100 μl , i.e., the solution was concentrated 150 times. The content of nitrite and nitrate in samples was determined using the Griess reagent by the method described previously [25]. To determine the total concentrations of nitrite and nitrate (NO_x) in specimens, experimental, control, and calibration samples (100 μl each) were introduced into the wells of a 96-well flat-bottom polystyrene plate (Corning Costar, Cambridge, MA, USA), then a freshly prepared saturated solution (100 μl) of VCl_3 (8 g/l in 1 M HCl) was added and immediately after this 100 μl of the Griess reagent (1 M HCl containing 10 g/l of sulfanilamide and 1 g/l of N-1-naphthylethylenediamine hydrochloride) was introduced. The plate was kept at 37°C for 1 h and then the absorbance at 546 nm was measured with a plate reader (Titertek Multiscan MCC/340, Flow Laboratories, Helsinki, Finland). The content of nitrite was determined in a similar way but without the addition of VCl_3 . For calibration, sodium nitrite and nitrate solutions of known concentration were used (concentration range 0.5–20 μM). The level of nitrate in samples was calculated as the difference between NO_x and nitrite concentration. The content of NO_2^- and NO_3^- in initial samples was calculated by taking into account that the volume of samples was reduced before the analysis 150 times.

2.6 Determination of nitrate using nitrate reductase

Samples of bidistilled water in polypropylene vials (5 ml) were incubated in a thermostat at $80 \pm 0.1^\circ\text{C}$ for 1 h and cooled by tap water to room temperature. In another series of experiments, samples of bidistilled water were boiled. The total NO_x concentrations were determined by the method described earlier [23, 26], by converting nitrate into nitrite with the use of NR. NADPH and then NR dissolved in bidistilled water were added to samples to a final concentration of 100 μM and 105 U/l, respectively. Samples were incubated at 37°C for 1 h and NO_x was determined using DAN as described above. In calculations, the values for control samples (water without heating + NADPH + NR) were subtracted from experimental data. For building a calibration curve, sodium nitrite solutions of the known concentrations with the added NADPH (100 μM) were used. During the experiment, the content of NO_2^- was concurrently determined using DAN. The concentration of nitrate in samples was calculated as the difference between NO_x and nitrite concentration.

2.7 Determination of nitrogen dioxide using dihydrorhodamine 123

The formation of nitrogen dioxide ($\bullet\text{NO}_2$) was determined from the oxidation of dihydrorhodamine 123 to fluorescing rhodamine 123 as described previously [27]. RhH_2 and Rh were dissolved in methanol at a concentration of 5 and 10 mM, respectively, and stored at -20°C . Samples of bidistilled water (5 ml) in polypropylene vials were incubated in a liquid thermostat at 60 and $80 \pm 0.1^\circ\text{C}$ for 0.5–2 h, quickly cooled by tap water and an RhH_2 solution in a phosphate buffer was added (final concentration in a sample: RhH_2 5 μM , phosphate buffer 10 mM, pH 7.4). The calibration curve was built using Rh solutions of known concentrations (5–20 nM) in the phosphate buffer (10 mM, pH 7.4). The fluorescence intensity of samples was measured on a Cary Eclipse

spectrofluorometer (Varian, Mulgrave, VIC, Australia) at $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 530 \text{ nm}$ in a mirror quartz cuvette at room temperature. In calculations of the content of $\bullet\text{NO}_2$ in heated water, the fluorescence intensity values for control samples were subtracted from those for experimental samples.

2.8 Ambient air conditions

It is known that nitrogen oxide air pollutants and trace contaminants from the containers used in the experiments may interfere with the measurement of nitric oxide in aqueous solutions, contributing to NO_x levels in specimens [28]. To ensure an accurate measurement of NO_x , we used only freshly prepared solutions, kept sample-containing vials tightly closed whenever possible and minimized sample exposure to exhaled air and ambient light. In our experiments, all the background interferences were incorporated into appropriate controls.

2.9 Data processing

In each experiment, samples were routinely analyzed in triplicate. The results are presented as the means and standard errors of means (SEM). Statistical comparisons were performed using an unpaired Student's t test or one-way analysis of variance (ANOVA) followed by the post hoc Fisher's least significant difference test, as appropriate. A value of $P < 0.05$ was considered significant.

3 Results

The fluorescent method of the determination of nitrite content [23] is based on the reaction with DAN whose product, 1-(H)-naphthotriazol, intensively fluoresces at alkaline pH values of the medium. The dependence of the fluorescence intensity of calibration samples on the content of nitrite was linear throughout the concentration range used (2–50 nM). The sensitivity of the method in our conditions was 1–2 nM NO_2^- .

Fig. 1 Kinetics of NO_2^- formation in bidistilled water during heating at different temperatures ($^{\circ}\text{C}$): 1–80, 2–70, 3–60. Nitrite concentrations were determined from fluorescence intensity measurements. Values are means $\pm$ SEM, $n = 2 - 6$. *Inset:* The Arrhenius plot for NO_2^- formation in bidistilled water upon heating. T is temperature (K) and k is the reaction constant for nitrite generation ($\text{M}^{-1} \text{s}^{-1}$)

Heating bidistilled air-equilibrated water at 60–80°C for 0.5–4 h leads to an increase in the content of NO_2^- (Fig. 1). Presumably, the generation of nitrite is a complex reaction of pseudo-second order:

The nitrite formation reaction occurs in two consecutive stages: slow (1) and fast (2), with the experimentally measured rate of this reaction being determined by its ‘bottleneck’, the rate-limiting first slow stage [30]. The reaction rate constants at different temperatures were calculated assuming that the generation of nitrite followed second-order kinetics. The initial linear portion of the kinetic curve was used when the concentration of the reaction product depends linearly on the rate constant k , the concentrations of reagents and time according to the formula:

$$C = kABt, \quad (3)$$

where C is the concentration of NO_2^- , A and B are respectively the stationary concentrations of nitrogen and oxygen in solution at a given temperature, k is the reaction constant, and t is the time. The concentrations of nitrogen and oxygen in water at different temperatures were taken equal to the reference values of solubility of these gases.

The activation energy of NO_2^- formation was determined from the slope of an Arrhenius plot (the logarithm of the rate constant for nitrite formation versus the reciprocal of the temperature):

$$-\log k = -\log k_0 + E_a/2.3 RT, \quad (4)$$

where E_a is the activation energy, R is the gas constant, and T is the absolute temperature (Fig. 1, inset). The activation energy of NO_2^- formation upon heating of water was estimated to be 139 kJ/mol.

The amount of nitrite formed in water during its heating depends on the content of dissolved oxygen and nitrogen (Fig. 2). The bubbling of water samples with oxygen or nitrogen before heating changes their concentrations in the solution. Listed in Table 1 are the results of the chromatographic analysis of these gases in samples before and after heating, with or without prior bubbling. The data show that (i) heating of control samples at 70°C for 1 h does not affect the content of O_2 and N_2 ; (ii) the saturation of water with oxygen

Fig. 2 Effect of additional saturation of water with oxygen and nitrogen on the amount of nitrite formed during heating (70°C, 1 h). Control samples were heated without additional saturation with gas; experimental samples were first saturated with gas and then heated. Values are means $\pm$ SEM, $n = 3 - 4$. *, Significantly different from the control (Student's t test, $P < 0.05$)

Table 1 Content of O₂ and N₂ in bidistilled water bubbled with oxygen or nitrogen and heated at 70°C for 1 h as measured by gas chromatography

Treatment	Content of O ₂ , μM	Content of N ₂ , μM
Water at 30°C (control)	202 $\pm$ 14 (5)	358 $\pm$ 19 (4)
Water heated at 70°C for 1 h	185 $\pm$ 7 (2)	396 $\pm$ 41 (2)
Water bubbled with oxygen for 30 min	577 $\pm$ 12 (4)*	337 $\pm$ 25 (3)
Water bubbled with oxygen for 30 min and heated at 70°C for 1 h	273 $\pm$ 22 (4) [#]	396 $\pm$ 21 (3)
Water bubbled with nitrogen for 30 min	130 $\pm$ 34 (2)*	458 $\pm$ 7 (2)*
Water bubbled with nitrogen for 30 min and heated at 70°C for 1 h	164 $\pm$ 10 (4)	496 $\pm$ 4 (3)

Data are the means $\pm$ SEM for the indicated number of replicates within one experiment and are representative of at least two independent experiments

* $P < 0.05$ versus group 1

[#] $P < 0.05$ versus group 3

increases the content of O₂ 2.9 times and decreases the content of N₂ 1.1 times; and (iii) the bubbling of water with nitrogen leads to a 1.6-fold decrease in the content of O₂ and increases the N₂ concentration 1.2 times. The study of the content of gases in samples saturated with oxygen or nitrogen and then heated shows that (i) the bubbling with oxygen causes a substantial (2.1-fold) decline in the content of O₂ after heating and a minor (1.2-fold) increase in the content of N₂; and (ii) saturation with nitrogen leads to insignificant changes in the content of both O₂ and N₂ after heating.

As shown in Fig. 2, the average amount of nitrite formed in heated control samples is equal to 3.1 nM, whereas the content of nitrite in samples bubbled with oxygen or nitrogen is 4.1 nM or 1.8 nM, respectively. Hence, the saturation of water with oxygen before heating results in a 1.3-fold increase in the nitrite content compared with the control, while the saturation with nitrogen decreases it to a value of 0.6 of the control. Experimental values of mean concentrations of O₂ and N₂ in samples before and after heating can be taken to estimate the expected yield of nitrite according to the formula:

$$C \propto AB, \quad (5)$$

where C is the content of NO₂⁻ and A and B are the mean concentrations of N₂ and O₂ in the solution during 1-h heating at 70°C, respectively. A comparison of these estimates obtained for control samples and solutions bubbled with oxygen and nitrogen indicates that the saturation with oxygen leads to a 1.7-fold increase in the nitrite content compared with the control and the saturation with nitrogen reduces it to a value of 0.8 of the control. Thus, measured values of the nitrite content and its calculated estimates differ by a factor of ~ 1.3 . Considering the approximate nature of averaged values of gas concentrations in heated samples, the calculated and measured values show good agreement.

The alkalization of bidistilled water to pH 11, as well as the addition of D₂O to 75% (v/v), which enhanced the heat-induced formation of hydroxyl radicals and singlet oxygen [16], did not increase the production of nitrite.

The content of nitrate in water heated at 80°C for 1 h was determined by two independent methods (Table 2). Because the sensitivity of the standard method of Griess is restricted to 1–5 μM of nitrite [31], water samples were concentrated 150 times before measurements. Whereas the nitrite concentrations determined fluorometrically and using the Griess reagent coincide within the limits of experimental error, the contents of nitrate determined by the

Table 2 Content of nitrite and nitrate in heated bidistilled water as measured by different methods

Conditions of heating	Method of measurement	[NO ₂ ⁻], nM	[NO ₃ ⁻], nM
Incubation in a thermostat (80°C, 1 h)	VCl ₃ /Griess reagent (after concentrating)	4.6 ± 0.6 (6)	17.7 ± 6.3 (4)
	NR/DAN	5.0 ± 0.6 (4)	6.0 ± 0.6 (5)
Boiling (100°C, 10 min)	NR/DAN	17.3 ± 0.9 (3)	25.8 ± 2.9 (3)
Autoclaving (120°C, 0.11 MPa, 1 h)	VCl ₃ /Griess reagent	10.8 ± 5.8 (2)	12.5 ± 4.2 (2)
	(after concentrating)		

Number of separate experiments is given in parentheses

different methods considerably differ. The application of DAN and nitrate reductase shows that NO₂⁻ and NO₃⁻ are formed in water upon heating in the ratio 1:1, whereas upon the application of VCl₃ and the Griess reagent, their ratio is ~1:3. This difference is probably due to the features of the second method. Indeed, the 150-fold concentrating of heated water performed prior to the analysis is accompanied by active physical processes (mixing, evaporation, and others), which may facilitate the course of chemical reactions leading to the formation of NO₃⁻ in water. The alkalization of bidistilled water to pH 10 before heating does not increase nitrate production, as determined by the VCl₃/Griess method.

The elevation of temperature and pressure enhances the generation of nitrite and nitrate in water: the content of NO₂⁻ and NO₃⁻ in autoclaved bidistilled water (120°C, 0.11 MPa, 1 h) increases approximately twofold compared with heating at 80°C (Table 2). Boiling of water (10 min), which is accompanied by the formation of vapor bubbles and is characterized by complicated thermo- and hydrodynamics, leads to a ~3.5-fold rise in nitrite concentration compared with heating at 80°C; in this case, NO₂⁻ and NO₃⁻ are detected in boiled water in the ratio 1:1.5 (Table 2).

The fact that dihydrorhodamine introduced into water after heating is oxidized to fluorescing rhodamine indicates that heating of water results in the formation of nitrogen dioxide. The time dependence of •NO₂ formation in water upon heating is non-linear (Fig. 3). The rate of •NO₂ generation in the initial portion of the kinetic curve was ~1.7 pM s⁻¹ at 60°C. The nitrogen dioxide scavengers urate (1 mM) and glutathione

Fig. 3 Kinetics of •NO₂ formation in bidistilled water during heating at different temperatures (°C): 1–60, 2–80. Rhodamine concentrations were determined from fluorescence intensity measurements. Values are means ± SEM, *n* = 3–4

(1 mM), added to water before heating (60°C, 1 h), decrease the formation of rhodamine by $86 \pm 8\%$ ($n = 6$) and $91 \pm 9\%$ ($n = 4$), respectively.

4 Discussion

We show in the present work that moderate heating of bidistilled water equilibrated with air results in the formation of nitrite, nitrate and nitrogen dioxide. The main reactions that are expected to proceed in the system are illustrated schematically in Fig. 4. In the construction of the scheme, we took the previously reported evidence into account indicating the formation of reactive oxygen species ($^1\text{O}_2$, $\text{O}_2^-/\text{HO}_2^\bullet$, H_2O_2) in water and water solutions under similar conditions [16, 19], as well as the weak acidity of bidistilled water (pH 5.6–5.8).

The half-life of $\bullet\text{NO}$ in pure water is inversely proportional to its concentration [29] and at a concentration of 0.05–1 μM ranges from 500 s to several hours [32]. Nitrite is the only stable final product of $\bullet\text{NO}$ autooxidation in an aqueous solution (Fig. 4, reaction 1) and is not oxidized further to nitrate [32]. In the reaction of nitrogen monoxide with a superoxide (Fig. 4, reaction 3; $k_3 \cong 1.6 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ [33]), peroxyntous acid ($\text{pK}_a(\text{ONOOH}) = 6.6$ [34]) is formed. This acid can also be formed in the reaction of nitrite with hydrogen peroxide (Fig. 4, reaction 2). Peroxyntous acid is unstable and at micromolar concentrations at $\text{pH} < \text{pK}_a$ is largely isomerized to form nitrate (Fig. 4, reaction 4) [35]. Part of ONOOH undergoes hemolytic cleavage under the same conditions (Fig. 4, reaction 5). The resulting nitrogen dioxide enters into a fast reaction with $\text{HO}_2^\bullet$ (Fig. 4, reaction 6; $k_6 = 1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [36]). The reaction product peroxyntic acid ($\text{pK}_a(\text{O}_2\text{NOOH}) = 5.9$ [36]) is stable in water (half-life up to 70 min [34]). The decomposition of peroxyntic acid results in the generation of $\text{HO}_2^\bullet$ and $\bullet\text{NO}_2$ (Fig. 4, reaction 7; $k_7 \cong 0.03 \text{ s}^{-1}$ [37]).

Dihydrorhodamine 123 was introduced in the late 1980s and was originally thought of as a sensitive and efficient *in vitro* and *in vivo* probe for peroxyntic acid production [27, 38]. Subsequent studies showed that the probe suffers from potential interference from both nonspecific oxidants ($\bullet\text{OH}$ and $\text{CO}_3^{\bullet-}$, in particular) and antioxidants, especially in cellular systems and biomimetic models [39, 40]. Despite its inherent limitations, the RhH₂ assay seems suitable for the detection and measurement of $\bullet\text{NO}_2$ in simple chemical cell-free systems.

In our experiments with dihydrorhodamine, nitrogen dioxide formed in reaction 7 (Fig. 4) was apparently registered since the probe was introduced into water after heating. The relatively high concentrations of rhodamine formed in heated water are probably partially explained by the fact that dihydrorhodamine itself introduces perturbation into

Fig. 4 Possible scheme of the main reactions proceeding in bidistilled water by the action of heat. All the explanations are given in the text

the system under study [41]: its intermediate radical product enters into a fast reaction with dissolved molecular oxygen ($k \cong 7 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) to form the superoxide

which facilitates the formation of peroxynitrous acid and eventually nitrogen dioxide. This is a peculiar way of signal amplification, which maintains the cascade of reactions (Fig. 4).

Thus, in a relatively simple chemical system, as is bidistilled water with dissolved atmospheric gases, thermal energy initiates a number of successive and parallel chemical reactions that lead to the formation of nitrogen oxides. It was shown earlier that the exposure of water to heat [16–19] and visible light [17, 20], including laser irradiation [20], results in the formation of ROS. The impact of light was demonstrated to have a resonance character because radiations beyond the absorption bands of molecular oxygen did not lead to the formation of ROS in water [20]. It was supposed [16, 18, 20] that the physicochemical mechanism of the transformation of the heat energy into highly energetic processes leading to the cleavage of chemical bonds and the formation of radical products is the adiabatic collapse of gaseous nanobubbles of atmospheric air contained in the solution [42], as it occurs by the action of ultrasound [43]. The triggering of the collapse of nanobubbles may result from a local electromagnetic perturbation induced by the transition of oxygen into a singlet state [20]. It is known that the temperature and pressure inside a collapsing bubble are sufficient to overcome the energy threshold ($E_a = 941.4 \text{ kJ/mol}$) of the break of triple bonds in the nitrogen molecule [44].

The experimental value of the activation energy of NO_2^- formation upon the heating of water, obtained in this study, agrees well with the estimate of the Gibbs free energy of NO_2 formation in the atmosphere (33.2 kcal/mol) given by Butcher and Charlson [45].

By extrapolating the rate constants of heat-induced NO_2^- generation at high temperatures to 37°C, the rate constant of NO_2^- formation by the action of heat in homoiothermal organisms was estimated to be $k_{37} = 2 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$ at a rate of $v_{37} = 2.9 \times 10^{-15} \text{ M s}^{-1}$, with the mean intratissue oxygen concentration in mammals being 30 μM [46] and the nitrogen concentration in human blood being about 1% ($\sim 490 \mu\text{M}$) [47]. Thus, the daily heat-induced production of nitrite in a human body weighing 60 kg (the water content in the body of an adult is $\sim 60\%$) can reach $4.1 \times 10^{-7} \text{ g}$, which accounts for $\sim 0.08\%$ of the content of nitrite ($\sim 0.5 \text{ mg}$) a human receives daily with plant food [8].

Assuming that nitrite, nitrate, and nitrogen dioxide are formed by the action of heat in water in approximately equal amounts, the above values of k_{37} and v_{37} must be increased several times to obtain a conservative estimate of heat-induced production of $\bullet\text{NO}$ in an aqueous medium.

With each liter of consumed boiled water, $\sim 0.8 \mu\text{g}$ of nitrite and $\sim 1.6 \mu\text{g}$ of nitrate enter the human body.

Heat is a constant physical factor of the internal medium in homoiotherms. It may be assumed that the heat-induced formation of nitrogen oxides is the minor pathway of nonenzymatic synthesis of nitrogen oxides in mammals. Presumably, local heat-induced changes in nitrogen oxide concentrations play important signaling and regulatory roles in various biological processes and underlie the curative effects of physiotherapy when it is accompanied by warming-up of tissues. It may appear that the amount of nitrogen oxides produced by the action of heat is too small to noticeably affect biological processes. However, it should be emphasized that the average concentration of a nitrogen oxide in a large water volume is registered. The local concentrations of heat-induced nitrogen oxides at the point of the collapse of a nanobubble can be substantial. Importantly, some

recent estimates place physiological •NO concentrations in vivo in the low nM range and below [48].

To summarize, we have shown for the first time that the exposure of water to heat results in the formation of nitrogen oxides: nitrite, nitrate, and nitrogen dioxide, and we have determined some quantitative parameters of this process. Further studies are needed to elucidate the nature, mechanism, and possible biological significance of these processes.

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