ABSTRACT: The widespread opinion that N₂O₃ as a product of NO oxidation is the only nitros(yl)ating agent under aerobic conditions is based on experiments in homogeneous buffered water solutions. In vivo NO is oxidized in heterogeneous media and this opinion is not correct. The equilibrium in the system being dependent on temperature and ΔG(sol) for NO, NO₂, isomers of both N₂O₃, and N₂O₄. For polar solvents including water, ΔG(sol) for N₂O₃ is high enough, and a stationary concentration of N₂O₃ in the mixture with other oxides is sufficient to guarantee the hydrolysis of N₂O₃ to nitrite. In heterogeneous media, the mixture contains solvates NO₂(sol), N₂O₃(sol), and N₂O₄(sol) at stationary nonequilibrium concentrations. As far as ΔG(sol) is decreased in heterogeneous mixtures with low polar solvents and/or at increased temperatures, the equilibrium in such a system shifts to NO₂. Although NO₂ is a reactive free radical, it almost does not react with water. In contrast, the reaction with most functional protein groups efficiently proceeds by a radical type with the formation of nitrite and new radicals (X) further stabilized in various forms. Therefore, the ratio of the nitrosylated and nitrated products yields depends on actual concentrations of all NOx.

KEYWORDS: Nitrous Anhydride; Nitrogen Dioxide; Nitrosoylation; Nitric Oxide; Dinitrogen Tetroxide; Isomerism of Nitrogen Oxides; Micellar Catalysis; Micellar Oxidative Nitrosation

INTRODUCTION

Nitrosation constitutes one of the most important types of postsynthetic modification of proteins [1–8]. At the same time, nitrosation and subsequent deamination of nucleic acid bases is a well-known mechanism of mutagenesis [9]. Furthermore, carcinogenic nitrosamines are also the products of the nitrosation [10,11]. Naturally, understanding the molecular mechanisms of nitrosation is the most important task of biochemistry. The nitrous anhydride (N₂O₃) is the main intermediate agent in in vitro experiments involving nitrosation and nitrite formation under the action of NO in aerobic conditions [12–21]. It is widely held that a similar situation obtains in vivo. However, obstinate application of this hypothesis in the interpretation of nitrosation reactions yields basic difficulties, with nitration (in place of nitrosation) of tyrosine residues and some effects emerging with temperature changes among them. Alternative hypotheses have been suggested, one of which, for instance, presumes that the role of the principal nitrosant may be assigned to nitrosocomplexes of iron [22] or copper [23] and to peroxynitrite derivatives [24,25]. In fact, all of them show nitrosation activity, this circumstance, nevertheless, solving no problems which arise from the nomination of N₂O₃ for the role of the principal nitrosating intermediate.

Long before the discovery of the synthesis of NO from arginine, N₂O₃ was known to be an efficient electrophilic nitrosating agent [26–29]. In the biochemical literature, N₂O₃ has been widely cited in explanations of in vivo nitrosoamines formation in smokers’ lungs or in stomach acid (e.g., during nitrosation under the action of the nitrite present in food [10], or determination the conversion of 2,3-diaminonaphthalene to naphtho-triazole in Escherichia coli [30]).

REVIEW, RESULTS, AND DISCUSSION

Dinitrogen trioxide (nitrous anhydride) N₂O₃ is yielded during the oxidation of NO by oxygen [26,31]
TABLE 1. Equilibrium Constants [34,36–38]

<table>
<thead>
<tr>
<th></th>
<th>(H_2O)</th>
<th>(CH_3CN)</th>
<th>EtAc</th>
<th>(m)-Xylene</th>
<th>(Et_2O)</th>
<th>(CCl_4)</th>
<th>(C_7H_{14})</th>
<th>(C_{10}H_{22})</th>
<th>Gas Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_{N_2O_3}(10^4)^{a})</td>
<td>0.73</td>
<td>0.82</td>
<td></td>
<td></td>
<td></td>
<td>13</td>
<td>41 (estimation)</td>
<td>24200</td>
<td></td>
</tr>
<tr>
<td>(K_{N_2O_4}(10^4)^{a})</td>
<td>0.153</td>
<td>0.30</td>
<td></td>
<td></td>
<td></td>
<td>1.77</td>
<td>6.9</td>
<td>1510</td>
<td></td>
</tr>
<tr>
<td>(\frac{K_{N_2O_3}}{K_{N_2O_4}}(10^4)^{a})</td>
<td>3.48</td>
<td>2.23</td>
<td>5.24</td>
<td>8.56</td>
<td>67.8</td>
<td>98.1</td>
<td>249</td>
<td>414700</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)At 25°C.

in the following reactions:

\[2\text{NO} + \text{O}_2 = 2\text{NO}_2 \leftrightarrow \text{N}_2\text{O}_4\] (1)

\[\text{NO}_2 + \text{NO} = \text{N}_2\text{O}_3\] (2)

or from nitrite-ion in a highly acidic medium [27,32]

\[\text{NO}_2^- + \text{H}^+ = \text{HNO}_2\] (3)

\[\text{HNO}_2 + \text{H}^+ = \text{H}_2\text{NO}_2^+\] (4)

\[\text{H}_2\text{NO}_2^+ + \text{HNO}_2 = \text{N}_2\text{O}_3 + \text{H}_2\text{O} + \text{H}^+\] (5)

Light-blue solid \(\text{N}_2\text{O}_3\) is known to have two crystal forms [33] melting at \(\sim 100^\circ\) to form bright-blue liquid and subsequent heating results in NO evaporation and \(\text{N}_2\text{O}_4\) accumulation in the liquid phase because of decomposition according to the following reactions:

\[\text{N}_2\text{O}_3 = \text{NO} + \text{NO}_2\] (6)

\[2\text{NO}_2 = \text{N}_2\text{O}_4\] (7)

The dependence of the equilibrium constant \(K_{N_2O_3} = k_6/k_2\) (see graphic representation in Figure 1A) on temperature is described for pure compound (in gas phase) by the following equation [34]:

\[K_{N_2O_3} = \exp(47900/RT + 168/R)\] (8)

Therefore, at \(t = 37^\circ\), \(K_{N_2O_3} = 5.06\) and \([\text{N}_2\text{O}_3]\) is approximately \(2 \times 10^{-11}\) even at \([\text{NO}] = [\text{NO}_2] = 10^{-5}\). In passing, it is noteworthy, that concentrations of NO in exhaled air are on the order of 10 ppb \(\approx 5 \times 10^{-10}\) M [35]. Stability of \(\text{N}_2\text{O}_3\) increases considerably in solution [34,36], the equilibrium constant \(K_{N_2O_3}\) strongly depending on both the temperature and the solvent and is lowered by a factor \(0.5 \ldots 30 \times 10^3\) as compared with (8) (see Table 1 and Figure 1A).

At low temperatures \(\text{NO}_2\) mainly exists in the form of the symmetrical dimer \(\text{N}_2\text{O}_4\). The \(K_{N_2O_4} (= k_9/k_7\) for dissociation and dimerisation, respectively)

\[\text{N}_2\text{O}_4 = 2\text{NO}_2\] (9)

will also depend critically on the temperature and the solvent [37–39] (Table 1 and Figure 1B). Thus, \(\text{NO}, \text{NO}_2,\) and \(\text{N}_2\text{O}_4\) are inevitably in equilibrium with \(\text{N}_2\text{O}_3\), each showing a nitrosating activity of its own. However, the

---

**FIGURE 1.** Equilibrium constants as function of temperature for (A) \(K_{N_2O_3}\), (B) \(K_{N_2O_4}\), and (C) \(K_{N_2O_4} (K_{N_2O_3}^{-1})\). 1: alkane, 2: \(\text{CCl}_4\), and 3: \(\text{CH}_3\text{CN}\).
reactions’ mechanisms and stoichiometry are different from case to case:

\[ \text{N}_2\text{O}_3 + X^- (\text{or HX}) = \text{ONX} + \text{NO}_2^- (+\text{H}^+) \]  
\[ \text{(10)} \]

Fast, electrophilic; nitrite is the second product \((X = \text{SR, OR, NR}^1\text{R}^2)\)

\[ \text{N}_2\text{O}_4 + X^- (\text{or HX}) = \text{ONX} + \text{NO}_3^- (+\text{H}^+) \]  
\[ \text{(11)} \]

Fast, electrophilic; nitrate is the second product

\[ \cdot\text{NO}_2 + X^- (\text{or HX}) = \cdot\text{X} + \text{NO}_2^- (+\text{H}^+) \]  
\[ \text{(12)} \]

\[ \cdot\text{NO}_2 + \cdot\text{X} = \text{O}_2\text{NX} \]  
\[ \text{(12a)} \]

\[ \cdot\text{NO} + \cdot\text{X} = \text{ONX} \]  
\[ \text{(12b)} \]

\[ \cdot\text{X} + \cdot\text{X} = \text{X} - \text{X} \]  
\[ \text{(12c)} \]

Fast, radical; nitrite is the second product, nitrocompounds may be one of products

\[ 2\text{NO} + 2\text{HSR} = [\text{ONSR, NO}^- , \text{HONNOH}] \]  
\[ = \text{RSSR} + \text{N}_2\text{O} + \text{H}_2\text{O} \]  
\[ \text{(13)} \]

Slow, radical; NO plays as an oxidant, nitrous oxide is the second product.

But if a reagent is one-electron oxidant, the reaction may be very fast (see [26] for details and references):

\[ 2\text{NO} + \text{I}_2 = 2\text{NOI} \]  
\[ \text{(14)} \]

\[ \text{NO} + \text{Hb(Fe}^{+3}) = \text{NO}^+\text{Hb(Fe}^{+2}) \]  
\[ \text{(15)} \]

The existence of at least four isomers has been discussed for \(\text{N}_2\text{O}_3\) [33,40–42]. Two crystal forms of nonsymmetric I is known [33], whereas the other isomers have been identified by their spectra. The spectra suggest that I and II are in equilibrium in liquid Xe solution and that the energy difference between these two isomers is \(\Delta H = 1.8 \pm 0.2\) kcal/mol [40]. If \(\Delta H\) were independent of the environment, then II should be only \(~5\%) of the total \(\text{N}_2\text{O}_3\) in tissues at body temperature. \textit{trans-cis} \(\text{N}_2\text{O}_3\) (III) is the largest known isomer in energy \((\Delta H \approx 2\) kcal/mol compare II). It has nonequivalent \(\text{N}–\text{O}\) bonds \((1.4 \text{ and } 1.7 \text{ Å})\) and may be regarded as a NO molecule loosely attached to a \(\text{NO}_2\) molecule via a weak \(\text{N}–\text{O}\) bond. In symmetrical II species both \(\text{N}–\text{O}\) bonds are equivalent, with length’s intermediate between those in III [42]. \textit{cis-cis} \(\text{N}_2\text{O}_3\) (IV) are non-planar isomers predicted to be unstable [41], although solvation has not been taking into account by calculation. For example, a single proton would act as a stabilizer for a hexagon molecule or a ion with the \(\text{O}···\text{H}···\text{O}\) bridge.

Since the effects of temperature and solvent are individual for each isomer, the ratio I:II:III:IV is also determined by the solvent. Note that only II, III, and IV have nitrous anhydride \((\text{N}–\text{O}–\text{N})\) structures. When studying nitrosation under the action of \(\text{N}_2\text{O}_3\), most conventional procedures rely upon the mixture yielded in reactions (2) or (3)-(5). Goldstein and Czapski [17] have discussed the role of \(\text{N}_2\text{O}_3\) for the ferrocyanide (shown as S) oxidation, that competes with the nitrosation

\[ \text{N}_2\text{O}_3 + \text{S} = \text{NO} + \text{NO}_2^- + \text{S}^+ \]  
\[ \text{(16)} \]

and for the whole oxidation process

\[ 2\text{NO} + \text{O}_2 + 2\text{S} = 2\text{NO}_2^- + 2\text{S}^+ \]  
\[ \text{(17)} \]

It seems likely that it is I (if not \(\text{NO}_2\) or one of \(\text{N}_2\text{O}_4\)) which is effective as an oxidant in reactions of the (16) type, whereas the other isomers are more effective in nitrosation (see [26] for discussion of earlier results and mode of views on the isomerism of \(\text{N}_2\text{O}_3\) and the
reaction mechanisms). In subsequent work [18] it was shown that both NO\(_2\) and N\(_2\)O\(_3\) represent the intermediate nitrosating agents even in aqueous solution by high concentration of NO ([NO] \(\sim 10^{-4}\) M). Of six tested thiols, for three the principal pathway was radical (12, X\(^-\) = RS\(^-\)). Of course, both NO\(_2\) and N\(_2\)O\(_3\) were responsible for nitrosation process as their concentrations (see Figure 1A) and the rate constants (see Table 2) are both close to each other.

As is seen from Figure 1 and Table 1, N\(_2\)O\(_3\) without visible impurities of higher oxides ([N\(_2\)O\(_3\)]/[N\(_2\)O\(_4\)] + [N\(_2\)O\(_4\)] + [NO\(_2\)]) \(\geq 0.99\) could exist at \(t = 37^\circ\) in water and polar solvents only at [NO] \(> 10^{-2}\) M and in non-polar ones at [NO] \(> 0.5\) M. In other studies [12–21], experiments were conducted at \(10^{-4} > [NO] > 10^{-6}\) M with aqueous buffer solution as solvent in all cases; hence N\(_2\)O\(_3\) was not the sole NO oxidation product, yet was present in considerable amounts (but <1%, see below). These authors [13,20] concluded that N\(_2\)O\(_3\) was the only nitrosating intermediate with NO acting in aerobic conditions. In fact, if nitrite and ONX are the only reaction products, the contribution of other oxides will be negligibly small. Challis et al. [26] in 1977 found that both N\(_2\)O\(_3\) and N\(_2\)O\(_4\) gave the mixtures of nitro- and nitrosocompounds (see (12a) and (12b), X = N(CH\(_2\))\(_3\)), when piperidine was nitrosated in aqueous 0.1 M sodium hydroxide solution by “dilute (4.4\(\times 10^{-5}\) M) gaseous N\(_2\)O\(_3\)” (N\(_2\)O\(_4\) was diluted with air and oxygen-free nitrogen was used in the case of N\(_2\)O\(_3\)—it is clear, both N\(_2\)O\(_x\) were highly dissociated in the gas mixture, [NO\(_2\)] > [N\(_2\)O\(_3\)].) When [N\(_2\)O\(_3\)] was higher than \(10^{-3}\) M, nitropiperidine was “not detectable”, but the yields of the nitrosocompound were significant.

Kharitonov et al. [15] and Goldstein and Czapski [18] postulated at the same time that the formation of nitrosothiols in vivo could not proceed under the action of N\(_2\)O\(_3\) because the NO oxidation rate in reaction (1) was too small at physiological concentrations of the reagents even at the highest (in the authors’ opinion) NO concentration in vivo (values of 3 min [15] and 7 min [18] were calculated for the lifetime if [NO] = \(10^{-6}\) M).

This reasoning was no longer valid after the discovery of the micellar catalysis contribution to NO oxidation [47–50]. Since both NO and oxygen are relatively hydrophobic, both reagents become concentrated in the hydrophobic phases of lipid membrane or lipoprotein, and in hydrophobic cores of protein or RNA macromolecules. At low volume fractions of hydrophobic phases, such concentration efficiently accelerates the third-order reactions, and the reaction rate of NO oxidation in the hydrophobic phase grows \(k_{1b}Q_{NO}Q_{O_3}/k_{1w}\), times as compared with the surrounding aqueous phase (\(k_{1b}, k_{1w}\) are the rate constants in hydrophobic phase and in water, and \(Q_{NO}\) and \(Q_{O_3}\) are

**TABLE 2. Rate Constants**

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>Value</th>
<th>Details</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_1)</td>
<td>(2.9 \pm 0.1 \times 10^6) M(^{-2}) s(^{-1})</td>
<td>22(^{\circ}), pH 7.4</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td>(2.1 \times 10^8) (2.4 (\times 10^7) M(^{-2}) s(^{-1})</td>
<td>23(^{\circ}) (37(^{\circ})), pH 4.9, 7.4</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>(1.6 \pm 0.1 \times 10^6) M(^{-1}) s(^{-1})</td>
<td>20(^{\circ}), pH 7.75</td>
<td>[15]</td>
</tr>
<tr>
<td>(k_2)</td>
<td>1.1 (\times 10^6) M(^{-1}) s(^{-1})</td>
<td>Pulso-radiolysis in water, 20(^{\circ}), pH 5</td>
<td>[36]</td>
</tr>
<tr>
<td>(k_{1w})</td>
<td>8.1 (\times 10^4) s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>530 s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000 + (10^9) [OH(^-)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{10})</td>
<td>6.7 (\times 10^7) M(^{-1}) s(^{-1})</td>
<td>pH 7.4, PenSH</td>
<td>[18]</td>
</tr>
<tr>
<td>(X = HPO_4^{2-/2+})</td>
<td>9.4 (\times 10^7) M(^{-1}) s(^{-1})</td>
<td>pH 7.4, GSH</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>8.0 (\pm 1.8 \times 10^6) M(^{-1}) s(^{-1})</td>
<td>pH 8.9</td>
<td>[44]</td>
</tr>
<tr>
<td>(k_{1w})</td>
<td>1.86 (\pm 0.3 \times 10^6) M(^{-1}) s(^{-1})</td>
<td>pH 8.9 [NO] = 25 mM</td>
<td>[44]</td>
</tr>
<tr>
<td>(X = HNR,R_2) (morpholine)</td>
<td>7.5 (\times 10^7) M(^{-1}) s(^{-1})</td>
<td>Stopped-flow, 22(^{\circ}), pH 7.4</td>
<td>[18]</td>
</tr>
<tr>
<td>(X = HSR) (N-acetyl-penicillamine)</td>
<td>1.8 (\times 10^6) M(^{-1}) s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(X = \text{captopril})</td>
<td>3.5 (\times 10^6) M(^{-1}) s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(k_{1w})</td>
<td>1000 s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(</td>
<td>X = H_2NC\text{H}_6\text{H}^+</td>
<td>)</td>
<td>7.2 (\times 10^{-3}) s(^{-1})</td>
</tr>
<tr>
<td></td>
<td>4.8 (\times 10^{-3}) s(^{-1})</td>
<td>CH(_3)CN, 0(^{\circ})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.2 (\times 10^{-3}) s(^{-1})</td>
<td>EtAc: CH(_3)CN = 1:1, 0(^{\circ})</td>
<td></td>
</tr>
<tr>
<td>(</td>
<td>X = \text{ascorbat-ion})</td>
<td>8.6 (\times 10^6) M(^{-1}) s(^{-1})</td>
<td></td>
</tr>
<tr>
<td>(</td>
<td>X = p\text{-HOC}_6\text{H}_4\text{O}^-</td>
<td>)</td>
<td>6.7 (\times 10^7) M(^{-1}) s(^{-1})</td>
</tr>
<tr>
<td></td>
<td>1.6 (\times 10^7) M(^{-1}) s(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(</td>
<td>X = \text{Cys})</td>
<td>2.4 (\times 10^6) M(^{-1}) s(^{-1})</td>
<td>pH 9.22</td>
</tr>
<tr>
<td>(</td>
<td>X = D\text{TTI})</td>
<td></td>
<td>pH 7.9…9.5</td>
</tr>
</tbody>
</table>

\(^4\)In organic solvents the reaction was found to be of first order to N\(_2\)O\(_3\) and of zero-order to aniline. The observed variation in \(k\) values was explained in terms of the solvation of reactants. In all cases the rate-limiting step was solvation N\(_2\)O\(_3\) to NO\(^+\)NO\(_3\) (donor)\(_3\), species.
the partition coefficients). Because of the reagent flow to the hydrophobic phase, their concentrations fall commensurately in the aqueous phase. Correspondingly, the reaction rate in the aqueous phase appears to be smaller than that is observed in pure water with no hydrophobic phase and with the same content of the reagents in the system; in the overall, however, the NO oxidation reaction accelerates considerably. The combined acceleration of the reaction in a heterogeneous two-phase system is governed by the equation

\[ H = \frac{k_{\text{app}}}{k_{1w}} = \frac{Q_{\text{NO}}^2Q_{O_2}x + 1 - x}{(Q_{\text{NO}}x + 1 - x)^2(Q_{O_2}x + 1 - x)} \]  

(18)

where \( k_{1h}, k_{1w} \) and \( k_{\text{app}} \) are the reaction rate constants in the hydrophobic phase, in water, and an apparent rate constant in heterogeneous medium; \( Q_{\text{NO}} \) and \( Q_{O_2} \) are the partition coefficients; and \( x \) is a part of hydrophobic phase in a total volume.

It follows from (18) that the dependence of the NO oxidation reaction rate on the hydrophobic phase fraction is maximal at \( x_{\text{max}} \approx 1\% \) (depending upon the values of remaining parameters of (18), see [47,50] for details). It was shown that for multiphase systems, with all in vivo real systems included, the dependence of the oxidation acceleration on \( Q \) and \( x \) has some unusual features [50], but on the whole the dependence course retains the same: at small \( x \), the acceleration surface slopes upward steeply, then after having reached the maximum, it smoothly slows down. If \( Q_{\text{NO}} \approx Q_{O_2} \gg 1 \) with growing \( Q \) the maximally obtainable acceleration grows approximately as \( 4Q^2/27 \). The \( Q_{\text{NO}} = 9 \) values for the two-phase octanol: water system are known from the experiment [51]; analysis of the thermodynamic constants of NO dissolution in pure liquids yields the value of 70 for the higher limit of \( Q_{\text{NO}} \) [47,52]. The \( Q \) values for real multiphase systems in vivo have not been hitherto known, but the accelerating factor, equivalent to the \( k_{1h}Q_{\text{NO}}^2Q_{O_2}/k_{1w} \) for two-phase systems, was experimentally found for several model systems to be equal to the order of 300 [49] (see comment in [50]).

Thus, the typical half-life of NO oxidation reactions in heterogeneous medium at the physiological concentration of the reagents might appear to be under 1 s, with virtually all the NO being oxidized in a small volume of hydrophobic phases and the oxidation products formed in these phases.

At low NO concentrations (with up to \( 10^{-6} \) M traced in homogenous aqueous solution [12,52–55]), NO oxidation continues to be governed by the kinetic equations of the third-order reaction, but at [NO] \(< 10^{-6} \) \( N_2O_3 \) is by no means the main oxidation product of NO present in the equilibrium mixture; thus the conclusion that it should be the main nitrosating intermediate, most notably in heterogeneous medium, becomes groundless.

Indeed, if \( N_2O_3 \) will be the sole NO-dependent nitrosant in aerobic solutions by near-neutral pH (the pathways (11) and (12) not play), the stationary concentrations of \( N_2O_3, N_2O_5 \), and \( N_2O_6 \) can be obtained as trace intermediates by setting \( d[NO_x]/dt \approx 0 \), (compare [12]):

\[ [NO_2]_{\text{st}} = \left( 1 + \frac{k_6}{k_{1w}} + \sum k_{11h}^x[X] \right) \left( \frac{2k_1[NO][O_2]}{k_2} \right) \]  

(19)

\[ [N_2O_3]_{\text{st}} = \frac{2k_1[NO]^2[O_2]}{k_{1w} + \sum k_{11h}^x[X]} \]  

(20)

\[ [N_2O_4]_{\text{st}} = \frac{k_7}{k_9}[NO_2]^2 \]  

(21)

(symbol \([NO_x]_{\text{st}}\) used for stationary concentrations of the reagents).

Then

\[ [NO_2]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{2k_1[NO][O_2](1 + k_6/M)M}{k_2k_1[NO]^2[O_2]} = \frac{(1 + k_6/M)M}{k_3[NO]} \]  

(22)

\[ [N_2O_4]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{4k_7k_1[NO]^2[O_2]^2(1 + k_6/M)^2M}{2k_9k_2k_1[NO]^2[O_2]} = \frac{2k_1k_2[O_2](1 + k_6/M)^2M}{k_2k_9} \]  

(23)

where \( M = k_{1w} + \sum k_{11h}^x[X] \)

If \( k_6 \gg M \),

\[ [NO_2]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{k_6}{k_2[NO]} = \frac{K_{N_2O_3}}{[NO]} \]  

(24)

\[ [N_2O_4]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{2k_1k_2k_7[O_2]}{Mk_2k_9} = \frac{2k_1k_2k_7[O_2]}{Mk_2k_9} \]  

(25)

If \( k_6 \ll M \),

\[ [NO_2]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{M}{k_2[NO]} \]  

(26)

\[ [N_2O_4]_{\text{st}}/[N_2O_3]_{\text{st}} = \frac{2k_1[O_2]M}{k_2^2K_{N_2O_4}} \]  

(27)

Ratio (24) agrees with the equation for the dissociation constant \( K_{N_2O_3} \). Ratio (25) does not depend on
[NO], is linear with [O2], and falls off sharply when concentrations of the targets for nitrosation [X] grow. The contribution of the term in brackets is crucial to account for nitrosation in heterogeneous systems and the impact of the temperature (see below).

By assumption, the impact of N2O4 (pathway (11)) is negligibly small and [N2O4]st/[N2O3]st ≈ 0 because activities of N2O4 and N2O3 in electrophilic reactions are closely in order of magnitude (see Table 2, [26]).

In kinetics experiments, phosphate buffers are typically used. Because phosphate is effective as a catalyst for the hydrolysis of N2O3 [13,18,26,56], the values for M will be higher than those that would be obtained in pure water (see Table 2). While near-neutral pH in the equilibrium play HPO4− and HPO4−2, the impacts of each are not known. Caulfield et al. [44] used phosphate buffers in interval 7.4 > pH > 8.9 and found rates of nitrosation for morpholine. It is the authors’ opinion that this is an effect of OH−. But the impact of OH− in hydrolysis of N2O3 must be smaller. Indeed, if k10 for phosphate would be pH-independent, then for 0.01 M phosphate, the values of k_{10w} + k_{10p} (symbol P used for phosphate) will be

\[
\begin{align*}
\text{for } pH 7 & : 2000(w) = 10^8 \times 10^{-7} (OH−) + 400 \times 0.01 \times 2000(pH) = 10010 \\
\text{and for } pH = 9 & : 2000(w) = 10^8 \times 10^{-5} (OH−) + 400 \times 0.01 \times 2000(pH) = 11000.
\end{align*}
\]

Hence, impact of OH− in 0.01 M phosphate buffer solution must be <10% even without any other targets, when pH changes from 7 to 9. But Caulfield et al. found >80% fall for the rate of morpholine nitrosation, when pH changes from 7.4 to 8.9. Consequently, it was an effect of the change for the ratio [HPO4−]/[HPO4−2] (and may be [PO4−3] by the higher pH) because two-charged HPO4−2 must be more effective as catalyst.

Let us find the values of (24)–(27) for the near-neutral aqueous solution at 20–25°C without catalysts of hydrolysis of N2O3 and any targets for nitrosation (phosphate including), and for phosphate buffer solution with and without targets, using Table 2. Results are shown in Tables 3 and 4.

As is clear from Tables 3 and 4, all experimental results of the earlier works [12–21,53–55] are consistent with [N2O4]st/[N2O3]st < 0.01. Only in pure water without phosphate is this not true. [NO2]st/[N2O3]st >> 0, but without NO2-specific targets (as in [18]) NO2 was not an effective player. Accordingly, N2O3 was named the principal nitrosating intermediate, NO2 could be advantageous only in diluted phosphate buffers, such that when [NO] was small N2O4 did not play. The question is what are the implications of this result for the nitrosation in vivo?

For heterogeneous systems (including the all living systems) as a first approximation (Eq. (18)) rather k1 in (25), should be used k_{app} or the value should be multiplied by H. Because the factor H varied over more than two orders of magnitude according to Q and x, when the part of hydrophobic phase is small, acceleration may be significant and [N2O4]st/[N2O3]st may increase 100 times more (compare values in Table 3). Concentrations of phosphate, bicarbonate and other natural catalysts of the N2O3 hydrolysis are not very high in vivo and are dependent upon subcellular localization (see [44] for discussion). The contribution of the term in brackets for heterogeneous systems are the highest. When >99% of NO is oxidized in the hydrophobic phase NO2 and N2O3 are generated in the nonpolar environment (compare the values for water and hexane in Table 1). This term is critically dependent upon temperature (see Figure 1C). Ultimately, the values for M in hydrophobic phases decrease because there is essentially no water in these phases, rendering the impact of k_w negligible. Concentrations of targets are often very low as well.

Hence, in many cases [N2O4]st/[N2O3]st > 0 and our proposal that N2O3 is the sole nitrosating intermediate in vivo in the general case is incorrect. Similar reasoning shows that NO2 may be more active player in nonpolar environment as compared with homogeneous aqueous solutions because [NO2]st/[N2O3]st >> 0. For heterogeneous media this depends upon the values of Q_{NO} and the ratio of dissociation constants K_{N2O3} for both phases. In aqueous phase [NO] decreases by flow of NO to hydrophobic phase (Q_{NO} > 1), and so the impact of NO2 will be increased. If the increasing of [NO] in hydrophobic phase depending on Q_{NO} cannot compensate the increasing of [NO2] depending on change of K_{N2O3}, the impact of NO2 in the nitrosation will be increased in hydrophobic phase as well.

Researchers have attempted to ascribe all chemical processes proceeding under the action of NO oxidation products to the effect of one compound, and these attempts proved ineffective. Having analyzed all the NO oxidation products hitherto known, Wink et al. [54,55], actually stated that none of them (including N2O3) is universal. The authors inferred that some unknown intermediate existed and denoted it by NO4−. Note, cis-trans N2O3 (III) was unknown at that time.

Later Goldstein and Czapski [18] pointed to a number of errors in [55], thereby proving invalid the
deduction about the existence of NO₂. Nitrosation was associated with N₂O₃ and/or NO₂; N₂O₄ was also taken into consideration, but was rejected for reasons of stoichiometry. The conclusion of Lewis et al. was more categorical: all data were consistent with N₂O₃ being the principal nitrosating agent at physiological pH [13]. It is noteworthy that none of the teams determined nitrate in the reaction products, despite some references to earlier works where nitrate was found. Nonetheless, study [13] shows experimental data on the decrease and accumulation of nitrite (determined by absorption at 280 nm) and nitrosomorpholin [NOMor] (see Figures 5 and 6 of the quoted article). At an [NO]₃ initial concentration of 28 mM, by 20 min its concentration falls to make 1 mM, and by 30 min to be below 0.1 mM; throughout, the nitrite concentration grows and the sum of [NO₂⁻] + [NOMor] does not exceed 22 mM by the 30 min. The remaining 28 – 22 = 6 mM of the oxidized nitrogen is to be accounted for.

The presence of diverse catalysts largely interferes with the determination of the real contribution of N₂O₃ in nitrosation in vivo. Chloride, bicarbonate, acetate, and other carboxyl group-containing compounds and many sulfur-containing compounds are efficient for nitrosation [29,57–59]. For example, diazotization of l-methionine or of S-methyl-l-cysteine occurs approximately 100 times faster than that of alanine, suggesting that initial S-nitrosation occurs, followed by an internal S- to N-rearrangement of the nitroso group [57]. At the same time, many of them, as well as phosphate, accelerate the hydrolysis of N₂O₃ (nitrosation of water), which decreases stationary concentrations of N₂O₃ and the yield of nitrosation products.

In electrophilic nitrosation under the effect of N₂O₄ either isomers ONONO₂ (VI, VII) or the “excited” symmetric O₂NNO₂ (VIII) that may be conceived as NO⁺NO₃⁻ are active intermediates. Isomeric nitroso nitrates VI and VII may be the secondary products of nitrosation under the effect of other NOX, their fraction therefore being likely to differ strongly from their fraction in model experiments with pure V. In all cases of nitrosation under the effect of N₂O₄ nitrate must formed simultaneously.

Beside V–VIII for N₂O₄, six structures of ONONOISOI ners can be suggested (cis–trans isomerism similar both for N₂O₃ and peroxynitrite). If some of them are nitrosating agents, peroxynitrite must be formed at the same time. When discussing the nature of the nitrosating intermediate in oxidative nitrosation, Goldstein and Czapski [18] considered this latter possibility and showed that is cannot be realised. The conclusion was drawn here from that other N₂O₄ (including VI–VIII) take no part in nitrosation.

NO₂ acts as the initiator of radical reactions, with the nitrosation of thiols and the nitration of amines or tyrosine residues in proteins among them [26,60,61]. NO itself might be an effective nitrosating agent only for compounds of the disulphide type able to form the thyl radical, with S(I) as oxidizing agent in this case. Under the action of NO on thiols in anaerobic conditions, N₂O forms, i.e. nitrogen is reduced [62–64]. With amines or alcohols the reactions proceed very slowly [26].

If nitrosation proceeds rapidly and reactivity is largely different from isomer to isomer, the equilibrium in the mixture of NO₂ and I–VIII may be disturbed. In investigations of competent nitrosation reactions, such as nitrosation of a mixture of two amines, thiole or amine in phosphate buffer solution, when nitrosation competes with the hydrolysis of N₂O₃ or N₂O₄, each of I–VIII makes a peculiar contribution. By this reason, the calculation of nitrosation rate constants in experiments with competing substrates is possible only if the equilibrium between I–VIII in the course of the experiment be proved to exist. Otherwise, a situation can evolve when each of the substrates will be nitrosated by its own agent and the competition will be ineffective.

Nothing is known about any kinetic studies of nitrosation at low NO concentrations, and the application of results obtained at large concentrations of NO must be done very carefully because [NO] is involved in the denominators of (24) and (26). On the whole, however, at NO concentrations possible in vivo, in heterogeneous medium each reagent makes a notable contribution to the total of nitrosation observed. It is noteworthy that since NO₂ initially formed during NO oxidation in reaction (1) only partially binds to form higher oxides in reactions (2) and (7) the reagent for the synthesis of nitrotyrosine and for the launch of radical reactions in vivo is always present, but its concentration are determined by the concentrations of NO and oxygen, by the parameters accelerating NO micellar oxidation, by the nitrosation and nitration targets shifting the equilibrium in the system of nitrogen oxides. Note that temperature
increase leads to N₂O₃ and N₂O₄ decomposition and NO₂ accumulation. Therefore, nitrotyrosine formation must progress with heating. This mechanism is likely to be the key process for the heat shock phenomenon in NO-producing organisms, their symbionts, and parasites (Nedospasov, Beda, and Suntsova, manuscript in preparation).

Under close examination of the NO-dependent nitrosation in heterogeneous aerobic systems, multiplying the k₁ by H (Eq. (18)) is inadequate. Indeed, by this expedient, we can allow for impact of the acceleration of NO oxidation, with no distribution of the higher oxides over the phases. We deduced Eq. (18) from the assumption that diffusion is faster where the chemical reactions and concentrations of the reagents in each phase are coordinate-independent. Because the rate constant for oxidation is small, this is true at least for the small objects (i.e., for membrane, hydrophobic cores of the protein or RNA, or for lipoprotein, but not for cholesterol plaque). But for nitrosation, the situation reverses: the rate constants for nitrosation are much greater (which is why oxidation is rate-determining for nitrosation), and some reagents, including several interconverted oxides, are distributed between phases in nonequilibrium, coordinate-dependent concentrations. The values of thermodynamics and kinetics constants will depend crucially upon the composition of each phase (for example, there are sets of concentrations for which, in the hydrophobic phase at equilibrium, [NO₂⁻] or [NO] + [NO₂] > [N₂O₃] or [N₂O₄] + [N₂O₅], whereas the opposite obtains for the aqueous phase) (see Figure 1).

Known attempts to find a nitrate by NO oxidation in heterogeneous medium have proven inadequate. Liu et al. [49] found that the final content of reaction products (overnight incubation) [NO₂⁻]/([NO₂⁻] + [NO₃⁻]) was 0.968 ± 0.031 and 0.926 ± 0.022 for 0.1 M phosphate +0.1 M KCl buffer pH 7.4 along and for buffer +4% Triton X-100 (the second phase), using 50 mM DEA-NONOate (NaEt₂NN(NO)_2, NO-donor, half-life ≈ 2.5 min) as starting concentration (see comment reporting NO-donors in models of oxidation of NO in [48]). Because phosphate and chloride in high concentrations were used, the rate of hydrolysis of N₂O₃ was roughly 50 times faster, then in pure water (see Table 2), but the catalytic effect of phosphate and chloride on the N₂O₄ hydrolysis is not known. As a first approximation, in heterogeneous medium the value of (N₂O₃/NaEt₂NN(NO)_2) increased only 2.3-fold. Because there were some principal errors in [49], the value for k_{app} of 4% Triton X-100 solution is unknown (see comment in [50]). Espey et al. found a distinction between nitrosating mechanisms within human cells and aqueous solution by using NONOate at low concentration (([NaEt₂NN(O)NO]₀ = 0.5 mM) and 4,5-diaminofluorescein as a target for nitrosation [65]. Authors discussed an impact of NO₂ in the nitrosation and oxidation chemistry in biological systems. Nitrate or other products of reactions of NO₂ and/or N₂O₄ were not detected.

Chen et al. [66] found both nitrite and nitrate by using macrophages attached to microcarrier beads with and without superoxide dismutase (where SOD was used as a peroxynitrite scavenger). Without SOD, [NO₂⁻]/([NO₂⁻] + [NO₃⁻]) was 0.47 versus 0.74 with SOD. In their reaction-diffusion model, the authors [66] did not use N₂O₄, and nitrate was seen as the product of peroxynitrite only, in experiments with SOD including (because a “film region” was beyond the reach of SOD). Recall that one of the key discoveries, therefore, the recognition of NO as a non-random metabolite, was the synthesis of nitrite, nitrate and nitrosoamines by activated macrophages [67,68]. Nitrite forms from the hydrolysis of both N₂O₃ and N₂O₄, whereas nitrate forms only from the hydrolysis of the latter (or from peroxynitrite).

Nitric acid is produced industrially by solving of the “nitrous gases” (mixture of oxides of nitrogen with N₂ and O₂) in water. Challis et al. [26] show that by lowering the concentration of N₂O₃, both nitrosation and nitrification take place independent of NO in the gas phase because it is not an effective scavenger for NO₂ to N₂O₃. Consequently, in heterogeneous gas-liquid media NO is oxidized to nitrite and nitrate. In heterogeneous liquid–liquid living systems, all must be the same: NO acts as a scavenger for NO₂, and its effectiveness is dependent upon environment and temperature.

Given the assumption that the rates of nitrosation in heterogeneous medium depend on the rate of NO oxidation as before, let us consider two limiting cases: (2) Nitrosation takes place in both phases, so that the flow of the higher oxides through the phase interface is zero (F_{NO₂^-} = 0), and each reacts in the phase where they were generated (Figure 2a). In this case, we have considered two one-phase systems with the stationary concentrations of oxygen and all oxides of nitrogen including NO (because [NO₁] are [NO]-dependent). Values of the ratios between their concentrations in both phases depend on the partition coefficients Q, consequently, if all F_{NO₂^-} = 0, concentrations of targets must be stationary as well. Using Eqs. (24) and (25) on the assumption that k_w = 0 in hydrophobic phase (because there is no water in this phase) we can find that the values of (24) and (25) can be much greater then 1 in the hydrophobic phase, if \( \Sigma k_{10} \times [X] \) is small. \( \Sigma k_{10} \times [X] + \Sigma k_{11} \times [X] + \Sigma k_{12} \times [X] \) must be used for the calculation in this case; all \( k_{10}, k_{11} \) and \( k_{12} \) values are dependent upon environment; for electrophilic reactions the dependence can be very sharp—even faster in water—and the values of \( k_{10}, k_{11} \) for aqueous solutions can not be used for nonpolar phases. In nonaqueous solvents,
nitrosation and other reactions may be zero-order to targets (compare values for aniline and N₂O₄ in Table 2). The observed variations in k values for the nitrosation and diazotization of aniline in the three solvents has been explained in terms of the solvation of reactants. It was expected that the reaction rate constant is greater in CH₃CN than in EtAc, but because CH₃CN is a stronger solvating agent than EtAc, the (donor)ₙNO⁺ species is more stable in the former while the liberated NO⁺ ion is free in the latter, as indicated by UV spectra; hence, there is a better chance of interaction with aniline resulting in a higher k value than that in CH₃CN. In case of a mixture of EtAc/CH₃CN, both the decrease of dissociation of N₂O₄ due to lowering of dielectric constant compared with pure CH₃CN, and the effect of greater solvation of NO⁺ by CH₃CN [45].

Consequently, in the aqueous phase there were no principal changes for homogeneous solutions, but in the hydrophobic phase (where the almost all NO will be oxidized), both NO₂ and N₂O₄ will be active players; if [NO] is small, NO₂ will be the main nitrosating and oxidizing intermediate.

(2) nitrosation takes place in the aqueous phase only because there are no targets for nitrosation (and nitration and oxidation) in the hydrophobic phase and all higher oxides of nitrogen must diffuse for reaction to the aqueous phase. Consequently, the hydrophobic phase is a principal source of the higher oxides for the aqueous phase and a jump of concentrations on the phase interface depends upon partition coefficients (Figure 2b).

In the aqueous phase, concentrations of the higher oxides depend on the distance from the hydrophobic phase, because nitrosation is rapid and a diffusion cannot equalize the concentration gradients. The result will depend upon the size and geometry of phases (e.g., for a planar or spherical membrane, or for a ball, as a model of lipoprotein or cholesterol plaque, the results will be very different). Only for the smallest objects and very slow nitrosation could the gradients equalized, but if [NO] is not very low, this is impossible, because the rate of nitrosation is dependent on NO oxidation.

Only if we ignore possible equilibrium disturbances in the N₂O₃–N₂O₄–NO₂ system, and solvation effects can we obtain the contribution of each component into the nitrosation of a model compound using common equations for competitive reactions.

Thus, in homogeneous aqueous buffer solutions under model physiological aerobic conditions (t ≈ 37°C, pH ≈ 7, [NO] ≈ 10⁻⁵ M) N₂O₃ is the main nitrosating intermediate. In heterogeneous systems, including all living systems, the competition of NO and NO₂ for NO₂ to yield more complex nitrogenous species is significant. Both N₂O₃ and N₂O₄ at physiological NO concentrations may be the effective intermediates in the electrophilic nitrosation reaction, with nitrite or nitrate being the second reaction product. The concentration of NO in the aqueous phase will be lower than in a homogeneous water system with the same total NO content, and the relation between [N₂O₃] and [N₂O₄] will be smaller. In the hydrophobic phase, the stability of N₂O₃ and N₂O₄ are smaller, than in the aqueous phase, when an equilibrium between NO₂, N₂O₃ and N₂O₄ is shifted to NO₂ and N₂O₄. In order to understand nitrosation regulation in high animals and in man, one must bear in mind that the reduction of nitrite back to NO may be fast in tissues, while the loss of nitrate from the nitrogen oxide cycle is almost irreversible (e.g., with urine in mammals [48,69–71]). Thus, during N₂O₃-induced nitrosation, the nitrogen oxides pool replenished under the action of NO synthases is renewed, while in nitrosation effected by N₂O₄, half of the oxygenated nitrogen is lost in each turn of the cycle [48]. NO concentration reduction decreases the ratio of N₂O₃ and N₂O₄, and raises the contribution of N₂O₄ in nitrosation. In higher animals, the nitrate formed cannot effectively be recovered to NO and, therefore, the contribution increase of N₂O₄ in nitrosation leads to a fall of NO resynthesis from nitrite and ONX. Taking into account
that the rate of NO oxidation is proportional to [NO]², dependence of the nitrosation rate upon NOS activity may be sharper as a quadratic in vivo in the substitution process of the nitrosating intermediate (autoinhibition of NOS under the action of NO is likely to lessen this effect.) Both decreases in NO concentration and decreases in temperature lead to a growth of the NO₂ ratio and, therefore, radical reactions (including tyrosine nitration), are accelerated with heating. Decrease in temperature and increases in NO concentration give the process a contrary course.

On the whole, many more experiments in heterogeneous media are required to gain an understanding of metabolism of NO and of higher nitrogen oxides.

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