Peroxynitrite, the reaction product of nitric oxide (NO) and superoxide (O2) is assumed to decompose upon protonation in a first order process via intramolecular rearrangement to NO2•−. The present study was carried out to elucidate the origin of NO2•− found in decomposed peroxynitrite solutions. As revealed by stopped-flow spectroscopy, the decay of peroxynitrite followed first-order kinetics and exhibited a pKₐ of 6.8 ± 0.1. The reaction of peroxynitrite with NO was considered as one possible source of NO2•−, but the calculated second order rate constant of 9.1 × 10⁴ M⁻¹ s⁻¹ is probably too small to explain NO2•− formation under physiological conditions.

Moreover, pure peroxynitrite decomposed to NO2•− without apparent release of NO. Determination of NO2•− and NO3•− in solutions of decomposed peroxynitrite showed that the relative amount of NO2•− increased with increasing pH, with NO2•− accounting for about 30% of decomposition products at pH 7.5 and NO3•− being the sole metabolite at pH 3.0. Formation of NO2•− was accompanied by release of stoichiometric amounts of O2 (0.495 mol/mol of NO2•−). The two reactions yielding NO3•− and NO2•− showed distinct temperature dependences from which a difference in E° of 26.2 ± 0.9 kJ mol⁻¹ was calculated. The present results demonstrate that peroxynitrite decomposes with significant rates to NO2•− plus O2 at physiological pH. Through formation of biologically active intermediates, this novel pathway of peroxynitrite decomposition may contribute to the physiology and/or cytotoxicity of NO and superoxide.

The reaction between nitric oxide (NO) and superoxide anion (O2•−) yields peroxynitrite with a second order rate constant near the diffusion-controlled limit (k = 4.3–6.7 × 10⁹ M⁻¹ s⁻¹) (1, 2). The reaction constitutes an important sink for O2•− because it is about twice as fast as the maximum velocity of SOD. Consequently, peroxynitrite has been implicated in many pathological conditions including stroke (3), heart disease (4), and atherosclerosis (5, 6). The potential cellular targets for peroxynitrite cytotoxicity include the antioxidants ascorbate, α-tocopherol, and uric acid (7–10), protein and non-protein sulfhydryls (11), DNA (12), and membrane phospholipids (13).

Decomposition of peroxynitrite is complex (14, 15). The anion is rather stable in alkaline solutions but decomposes rapidly (t½ = 1 s at pH 7.4, 37 °C) upon protonation to peroxynitrous acid (ONOOH) (pKₐ = 6.8) (16). Two pathways of ONOOH decomposition have been proposed. Some studies have argued that ONOOH is cleaved homolytically to generate hydroxyl and NO2 radicals. This hypothesis is based on the sensitivity to hydroxyl radical scavengers of certain peroxynitrite-induced reactions, including the formation of malondialdehyde from deoxyribose and the hydroxylation on the benzene ring of sodium benzoate, phenylalanine, and tyrosine (16, 17). Studies on decomposition of peroxynitrite by electron paramagnetic resonance spectroscopy with the spin traps 5,5-dimethyl-1-pyrroline-N-oxide and 4-pyridyl-1-oxide-N-tet-butylnitrone also provided evidence for the formation of free hydroxyl radicals (18, 19). Against this, Koppenol et al. (15) concluded from molecular dynamic calculations that homolytic cleavage of ONOOH is highly improbable. This was reinforced by the independence of the rate of ONOOH decomposition on solvent viscosity (20). Based on these results, it was suggested that decomposition of ONOOH to NO3•− involves formation of an activated intermediate (ONOOH+), which might account for the hydroxyl radical-like properties of peroxynitrite (15, 21).

There are several methods for the detection of peroxynitrite in biological systems. Since ONOOH decomposition yields an intermediate that nitrates phenolic compounds (22, 23), presence of nitrotirosine in proteins was proposed to be evidence of peroxynitrite production in tissues (24). However, using both a monoclonal antibody specifically recognizing peroxynitrite-modified proteins (24) as well as a published HPLC method (17), we failed to detect tyrosine nitration by authentic peroxynitrite. Spectrophotometric determination of dihydroxydihemamine 123 oxidation was described as another sensitive assay for the specific detection of peroxynitrite at submicromolar concentrations (25), but in our hands, interference of several redox-active compounds precluded application of this method in cell-free assay systems (26). Under certain experimental conditions, indirect evidence for peroxynitrite production can be obtained by comparing NO release in the absence and presence of SOD. The peroxynitrite donor compound SIN-1, for example, does not release detectable

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amounts of free NO unless SOD is present in amounts sufficient to outcompete the reaction with concomitantly produced O$_2$^•$ (26). Based on similar results obtained with purified neuronal NO synthase, we suggested that the enzyme generates NO and O$_2$ simultaneously and hence functions as peroxynitrite synthase if incubated in vitro (27). However, in contrast with the widely held view that peroxynitrite decomposes exclusively to NO$_2^–$ and O$_2$ in a 2:1 stoichiometry as a route of peroxynitrite decomposition at pH $\geq$ 7.5.

**EXPERIMENTAL PROCEDURES**

**Materials**—NO solutions were prepared by dissolving NO gas (Linde München, Germany, 99% pure) in deoxygenated water as described previously (29). All solutions were prepared freshly each day with Nano-pure water (Barnstead ultrafiltered type I, resistance 18 megohms cm$^{-2}$). Sulfanilamide, sodium nitrite, cadmium, and the Griess-Ilosvay reagent for postcolumn derivatization were from Merck, Darmstadt, Germany. All other chemicals were from Sigma, Vienna, Austria.

**Synthesis of Peroxynitrite**—Alkaline solutions of peroxynitrite (80–100 mM) were prepared from acidified NO$_2^–$ and H$_2$O$_2$ according to the Bayler-Villiger reaction (30) and quantified spectrophotometrically using an extinction coefficient of 1670 M$^{-1}$ cm$^{-1}$ (26, 30, 31). All solutions were diluted with H$_2$O to 10 mM immediately before the experiments. The tetramethylammonium salt of peroxynitrite ([Me$_4$N][ONOO]) was synthesized from [Me$_4$N][O2]; [Me$_4$N][ONOO] to NO3$–$ and O2 in a 2:1 stoichiometry as a paramagnetic (O2$^•$^-) impurities present. Purity of [Me$_4$N][ONOO] was also checked by $^{15}$N NMR spectroscopy, which indicated that no NO$^+$ was present. The salt was dissolved in 1 M NaOH to give a 24 mM stock solution, which was stored at $\sim$70°C and diluted with H$_2$O prior to experiments. With the exception of stopped-flow kinetics, all experiments described here were initially performed with conventional preparations of peroxynitrite and then repeated with [Me$_4$N][ONOO] to exclude that the results were due to unidentified contaminants.

**Kinetic Experiments**—Peroxynitrite decomposition was studied by stopped-flow absorbance spectroscopy at 302 nm (Bio-Sequential SX-172, Excelsior gas-jacketed ASDV spectrophotometer, Applied Photophysics, Leatherhead, U. K.). For simple decomposition experiments, reservoir 1 contained peroxynitrite in 0.01 M NaOH, and reservoir 2 contained the buffer solution (pH 3.0–6.0, 1 M acetic acid buffer; at pH 5.0–9.0, 1 M phosphate buffer; at pH 5.0–10.0, 1 M Tris-HCl; at pH $\geq$ 10, solutions of NaOH). The NaOH concentration in reservoir 1 was, in some cases, adapted to the requirements of the experiment: non-buffered experiments at pH 3.0, 10.0, and 11.0 were carried out with sufficiently low concentrations of NaOH.

The reaction of peroxynitrite with NO was studied by sequential stopped-flow, *i.e.* reservoirs 1 and 2 were premixed followed by mixing with contents of reservoir 3 with short delay time (10 ms). Reservoir 4 was used to push the mixed contents of reservoirs 1 and 2 forward into the main mixing chamber. Reservoir 1 contained buffer (pH 3.0–11.0; 4 × final concentration), reservoir 2 contained a solution of peroxynitrite in NaOH (4 × final concentration; typical final [NaOH] 5 mM), reservoir 3 contained a saturated solution of NO ($\sim$2 mM giving $\sim$1 mM final concentration), and reservoir 4 contained buffer (2 × final concentration). To vary NO concentrations, experiments were also done with 2-fold diluted peroxynitrite in reservoir 3 and NO in reservoir 4. The yield of residual, paramagnetic concentration of peroxynitrite but a 2-fold lower final concentration of NO ($\sim$0.5 mM). Samples of the NO solution were taken with a plastic syringe under helium gas and transferred directly into the stopped-flow reservoir. Experiments were carried out both with air-containing buffers and with buffers that had been thoroughly degassed. Degasging made no difference.

**Decomposition of Peroxynitrite and Determination of NO$_2^–$ and NO$_3^–$**—Unless indicated otherwise, peroxynitrite (1 mM or 0.5 mM) was decomposed by incubation in 0.1 M phosphate buffer for 1 h at pH 3.0–9.0. [Me$_4$N][ONOO] (0.25 mM or 0.1 mM) was decomposed in 0.5 M phosphate buffer under the same conditions. NO$_2^–$ was determined by the Griess assay. The samples (0.1 ml) were mixed with 10 M H$_2$O and 1 M solutions, an EDTA solution (0.5 M, pH 5.0) was mixed with 0.12 ml of freshly prepared Griess reagent (20 mg N-(1-naphthyl)-ethylenediamine and 0.2 g sulfanilamide dissolved in 20 ml of 5% (v/v) phosphoric acid) and measurement of the absorbance at 546 nm. For determination of NO$_2^–$ + NO$_3^–$, samples (0.2 ml) were adjusted to pH $\approx$7.5 and mixed with 20 M of an aqueous zinc suspension (100 mg/ml) and 20 M of an EDTA solution (0.5 M, pH 8.0). Samples were spun down for 5 min, and 0.12 ml of the supernatant was mixed with 0.12 ml of the Griess reagent, followed by determination of the absorbance at 546 nm. Calibration curves were established with NO$_2^–$ and NO$_3^–$ (10–50 mM each). The calculated amount of NO$_2^–$ present in stock solutions of conventionally prepared peroxynitrite agreed well with NO$_3^–$ measured after decomposition at pH 3.0. This amount was subtracted from the measured values.

The NO$_2^–$/NO$_3^–$ data were confirmed by HPLC analysis according to published protocols (33, 34). 50 M samples were injected onto a 250 × 4 mm C18 reversed phase column (LiChrospher 100 RP-18, 5 M particle size, Merck, Vienna, Austria) and eluted with 5% (w/v) NH$_4$Cl, pH 7.0, at a flow rate of 0.7 ml/min. NO$_2^–$ was detected by postcolumn derivatization with the stable Griess-Ilosvay reagent (Merck) (0.7 ml/min) with measurement of the absorbance at 546 nm.

For determination of NO$_2^–$ + NO$_3^–$, samples were reduced with a cadmium reactor (Cd, 0.3–0.8 mm, 20–50 mesh ASTM, Merck, washed with 0.1 M HCl, and packed in a Pharmacia HR 5/5 glass column) prior to postcolumn derivatization.

**Electrochemical Detection of NO and Oxygen**—NO and O$_2$ were measured with commercially available Clark-type electrodes (ISO-NO and ISO2, World Precision Instruments, Mauer, Germany) (27). NO and O$_2$ meters were connected to an Apple Macintosh computer via an analog to digital (A/D) converter (MacLab, World Precision Instruments). Release of O$_2$ from peroxynitrite was determined in 1.8-ml water-jacketed vials sealed with a rubber septum and maintained at 37°C. Experiments were performed in phosphate buffer (0.1 M or 0.5 M, pH 3.0–9.0), which had been gassed with argon to reduce the O$_2$ concentration to 20–40 µM. Aliquots of peroxynitrite stock solutions were injected through the septum to give concentrations of 0.5 mM (conventional peroxynitrite) or 0.25 mM ([Me$_4$N][ONOO]) and the output current was recorded at 0.33 Hz under constant stirring. Two-point calibration of the sensor was performed in air-saturated H$_2$O at 37°C (6.9 ppm; 0.216 mM O$_2$) and argon atmosphere (zero O$_2$).

To study the reaction of peroxynitrite with NO, 4 equiv peroxynitrite with NO$_3^–$ and aliquots of an ~2 mM NO solution were injected into 1.8-ml glass vials completely filled with 0.1 M phosphate buffer, pH 7.4, and sealed with a septum. At the indicated time points, 1.8–3.6 µl of peroxynitrite solution (0.5 mM) were applied to give concentrations of 0.25–1 µM. The output current was recorded at 1.66 Hz under constant stirring. The sensor was calibrated with NO$_3^–$ standards according to manufacturer recommendations.

**RESULTS**

Decomposition of peroxynitrite was monitored as decrease in absorbance at 302 nm at 20°C. As expected, decomposition at pH 3 was very fast and followed first order kinetics with a calculated rate constant ($k_{calc}$) of 0.86 ± 0.05 s$^{-1}$ but slowed down at increasing pH. The $k_{calc}$ values and corresponding Hill coefficients summarized in Table I demonstrate that peroxynitrite decay was first order under most conditions although Hill coefficients smaller than 1.0 were obtained at pH 8.0 (0.67 ± 0.02) and pH 11.0 (0.5 ± 0.1). Using the Hill equation for overall kinetic analysis of decomposition at pH 9–11, we calculated $k_{calc}$ of 6.8 ± 0.1, which agrees well with published data (35). The possible contribution of transition metals to peroxynitrite decomposition was studied with 0.6 M peroxynitrite in 0.5 M phosphate buffer (pH 7.4) in the presence of Cu(NO$_3$)$_2$, Fe(NH$_4$)$_2$(SO$_4$)$_2$, Fe(NH$_4$)$_2$(SO$_4$)$_2$, and the metal chelator DTPA. Rates of decomposition were affected neither by the metal salts (0.1 mM each) nor by DTPA (0.1 and 1 mM). At a concentration of 2.5 mM DTPA, the peroxynitrite decay rate was enhanced 10-fold.

Stopped-flow data showed that peroxynitrite decomposition
TABLE I

| pH | $k_{app}$ (s$^{-1}$) | Hill coefficient |
|----|----------------------|------------------|
| 3  | 0.86 ± 0.05          | 0.91 ± 0.07      |
| 4  | 0.82 ± 0.01          | 1.09 ± 0.07      |
| 5  | 0.71 ± 0.02          | 1.16 ± 0.05      |
| 6  | 0.61 ± 0.01          | 1.11 ± 0.03      |
| 7  | 0.39 ± 0.02          | 0.83 ± 0.03      |
| 8  | 0.08 ± 0.009         | 0.67 ± 0.02      |
| 9  | 0.0298 ± 0.009       | 1.10 ± 0.03      |
| 10 | 0.0003 ± 0.0001      | 1.16 ± 0.05      |
| 11 | 0.00009 ± 0.00001    | 0.5 ± 0.1        |

Decomposition of Peroxynitrite

Decomposition of peroxy nitrite (0.1–0.7 mM) was measured spectrophotometrically at 20 °C as decrease in absorbance at 302 nm at the indicated pH values. The $k_{app}$ were obtained by averaging the apparent first order rate constants that were calculated by dividing initial rates by the peroxy nitrite concentrations (mean ± S.D.; n = 8). Hill coefficients were calculated from the slope of plots of log $v_0$ versus log [peroxy nitrite].

was faster in the presence of −1 mM NO and that the increase in rate was dependent on the NO concentration. However, calculation of rate constants was difficult because the exact NO concentrations in these experiments were not known and the effect of NO was observed only as a relatively small increase of an already fast reaction. Therefore, we used an NO-sensitive electrode to measure the consumption of NO by known amounts of peroxy nitrite. Fig. 1 shows a representative trace obtained by addition of 4 μl of a saturated NO solution to 1.8 ml of 0.1 mM phosphate buffer, followed by two repetitive additions of peroxy nitrite yielding concentrations of 0.75 μM each. Peroxy nitrite induced a rapid consumption of NO with initial rates of 100 ± 9 nm s$^{-1}$ and a stoichiometry close to 1:1 (0.75 μM peroxy nitrite consumed 0.66 ± 0.06 μM NO). NO consumption (initial NO concentration 1–2 μM) was linear in the range of 0.25–1 μM peroxy nitrite with initial rates ranging from 20 to 167 nm s$^{-1}$ and a rate constant of 9.1 × 10$^{4}$ m$^{-1}$ s$^{-1}$.

We consistently observed that decomposition of peroxy nitrite or [Me$_4$N][ONOO$^-$] resulted in formation of about 70% NO$_2$ and 30% NO$_3^-$ at pH 7.4 and 37 °C. As the NO$_2$/NO$_3^-$ ratios were not affected by known metal chelators (Table II), our results do not support previous suggestions according to which formation of NO$_3^-$ is due to contamination of peroxy nitrite solutions with trace metals (36) but indicate that NO$_2$ release results from an as yet unrecognized pathway of peroxy nitrite decomposition. To address this issue, we measured NO$_2$ and NO$_3^-$ after peroxy nitrite decomposition at pH 3–9 and found that the relative amount of NO$_2$ increased with increasing pH (Fig. 2A). Assuming that these results were not due to a reaction of peroxy nitrite with contaminants in the stock solutions, our findings led us to speculate that 2 mol of peroxy nitrite decomposed to 2 mol of NO$_2$ and 1 mol of O$_2$. Indeed, using a Clark-type O$_2$ sensor, we found that the pH-dependent formation of NO$_2$ was accompanied by release of stoichiometric amounts of O$_2$. NO$_2$ and O$_2$ were determined after measurement of O$_2$ release in the same vials. Data are means ± S.E. of six experiments. B, correlation between O$_2$ and NO$_2$ production (slope = 0.495, correlation coefficient = 0.988).

FIG. 2. Decomposition of peroxy nitrite yields NO$_2$ and oxygen. A, peroxy nitrite (0.5 mM final initial concentration) was decomposed by incubation in 0.1 mM phosphate buffer (pH 7.0–9.0) at 37 °C for 1 h, followed by the determination of NO$_2$ and NO$_3^-$ as described under “Experimental Procedures.” NO$_2$ and NO$_3^-$ were determined after measurement of O$_2$ release in the same vials. Data are means ± S.E. of three experiments performed in duplicate.
Decomposition of Peroxynitrite

The present study was carried out to identify the pathways of formation of \( NO_2^- \) in the course of peroxynitrite decomposition. Stopped-flow kinetic experiments confirmed that peroxynitrite decomposed rapidly upon protonation with a \( pK_a \) of 6.8. The first-order rate constants calculated for peroxynitrite decomposition at different pH values agreed well with previously published data (11, 15, 37). Under physiological conditions (pH 7.4 and 37 °C), decomposition consistently yielded about 30% \( NO_2^- \) and \( NO_3^- \) were formed, whereas \( NO_2^- \) was the sole product at pH 1.6 and 24°C, respectively (39) and that peroxynitrite reacts with NO according to Equation 1 (40, 41).

\[
NO + ONOO^- \rightarrow NO_2^- + NO_3^-(\text{Eq. 1})
\]

Lewis et al. (28) observed that activated macrophages release more \( NO_2^- \) than expected and considered Equation 1 as a pos-
Decomposition of Peroxynitrite

ONOO\(^{-}\) + ONOOH → HNO\(_{2}\) + NO\(_{2}\) + O\(_{2}\)  \hspace{1cm} (Eq. 2)

This hypothesis was corroborated by determination of NO\(_{2}^{-}\) and O\(_{2}\) formed upon decomposition of two different peroxynitrite preparations. With both products, we obtained linear correlations between NO\(_{2}^{-}\) and O\(_{2}\) release with slopes close to the theoretical value of 0.50. At pH 3.0–11.0, peroxynitrite decomposition was first order at most. Moreover, even though the temperature dependence of the NO\(_{2}^{-}\)/O\(_{2}\) ratio clearly indicated that the two pathways have different activation energies (\(\Delta E_{\text{act}} = 26.2 \pm 0.9 \text{ kJ mol}^{-1}\)), the Arrhenius plot for overall peroxynitrite decomposition at pH 7.4 (30% NO\(_{2}^{-}\)) was strictly linear and yielded an \(E_{\text{act}}\) value that was virtually identical to that observed at pH 5.0 (10% NO\(_{2}^{-}\)). The \(E_{\text{act}}\) of overall peroxynitrite decomposition was found to be rather high (92 ± 2 and 90.0 ± 0.8 kJ mol\(^{-1}\) at pH 5.0 and 7.4, respectively). A similar value (77.5 kJ mol\(^{-1}\) at pH 5.0) was reported by Koppenol et al. (15).

From these observations, we conclude that the rate-limiting step in both reactions is the same, a conformational change of ONOOH to an activated intermediate that either rearranges to HNO\(_{2}\) (15, 35) or undergoes a reaction with peroxynitrite anion to yield NO\(_{2}^{-}\) and O\(_{2}\) (this study). Any potential model must account for the thermally characterized kinetics of peroxynitrite decomposition as well as the stoichiometries of the end products. Further, a bimolecular rate law for either of the product determining steps is excluded because the partitioning of the two pathways does not depend on the concentration of peroxynitrite. Fig. 5 shows a hypothetical pathway of peroxynitrite decomposition that appears to be most consistent with the data presented both here and in the literature. According to this scheme, activated ONOOH can either isomerize to NO\(_{3}^{-}\) or decompose to HO and NO\(_{2}\) radicals. At alkaline pH, the OH radical may react with peroxynitrite anion yielding O\(_{2}\), NO, and OH\(^{-}\), and NO could react with NO\(_{2}\) radicals to yield N\(_{2}\)O\(_{3}\) and finally nitrite.

The novel pathway of peroxynitrite decomposition described here could have important physiological consequences, as it possibly involves generation of intermediates with biological activities not attributed so far to peroxynitrite. In a recent paper, it was reported that peroxynitrite decomposition could lead to release of singlet O\(_{2}\) (44). If that observation were due to the novel reaction proposed here, peroxynitrite-dependent toxicity might be mediated by singlet O\(_{2}\) toxicity under certain pathophysiological conditions. Alternatively, decomposition to H NO\(_{2}\) and O\(_{2}\) may be responsible for the observed NO-like biological activity of peroxynitrite. At pH 7.4, peroxynitrite oxidizes hemoglobin to methemoglobin with an efficiency of about 20% (26), and it is tempting to speculate that this reaction represents scavenging by hemoglobin of the NO that is formed as intermediate during decomposition to NO\(_{2}^{-}\) and O\(_{2}\) (Fig. 5). Also, our working hypothesis involves intermediary formation of N\(_{2}\)O\(_{3}\), a potent nitrosating agent that could account for the observed peroxynitrite-induced nitrosoation of GSH, especially in light of our findings that the nitrosation reaction has a pronounced pH dependence and does not occur at significant rates below pH 7.5 (45). Accordingly, reactive intermediates formed in the course decomposition to NO\(_{2}^{-}\) and O\(_{2}\) could be responsible for stimulation of soluble guanylyl cyclase by peroxynitrite (45), resulting in cyclic GMP-mediated biological effects such as vascular smooth muscle relaxation and inhibition of platelet aggregation (46, 47).

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