Reactions of Peroxynitrite with Phenolic and Carbonyl Compounds: Flavonoids are not Scavengers of Peroxynitrite[1])

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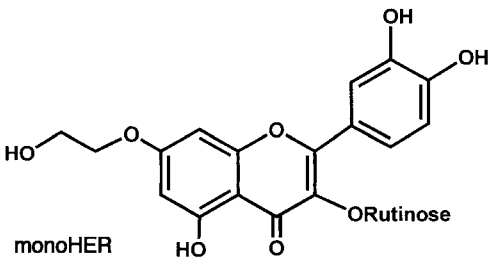
Dedicated to Professor *Albert Eschenmoser* on the occasion of his 75th birthday

Peroxynitrite ($ONOO^-$, oxoperoxonitrate(1−)), an isomer of nitrate that oxidizes and nitrates biomolecules, is likely to be formed *in vivo* from the reaction of superoxide with nitrogen monoxide. To determine whether flavonoids scavenge peroxynitrite as postulated in the literature, we studied the reactions of peroxynitrite with phenol, hydroquinone, catechol, and the flavonoid monoHER. These reactions are first-order with respect to peroxynitrous acid and zero-order with respect to the organic compounds, and proceed as fast as the isomerization of peroxynitrous acid to nitrate. *In vivo*, a large fraction of all peroxynitrite is likely to react with carbon dioxide to form an unstable adduct, the 1-carboxylato-2-nitrosodioxidane anion ($ONOOCO_2^-$). The presence of phenolic compounds did not influence the rate of disappearance of this adduct, which was *ca.* 4×10^2 s^{-1}. On the basis of these kinetic studies, it can be concluded that flavonoids are not scavengers of peroxynitrite. The products from the reaction of peroxynitrite with hydroquinone (benzene-1,4-diol) and catechol (benzene-1,2-diol) are *para*-benzoquinone and *ortho*-benzoquinone, respectively; no nitrated products were found. In a subsequent reaction, *ortho*-quinone reacted with nitrite, a common contaminant of peroxynitrite preparations to form 1,2-dihydroxy-4-nitrobenzene. We also investigated whether carbonyl compounds could redirect the reactivity of peroxynitrite toward nitration, as carbon dioxide does. The reaction with acetone is first-order with respect to peroxynitrite and first-order with respect to the carbonyl compound. The rate constant is 1.8 $M^{-1}s^{-1}$ at neutral pH and 20°; peroxynitrite does not react with the carbonyl compounds dimethyl acetamide, L-alanylalanine, or methyl acetate. It is not likely that the carbonyl compounds or the mono-, di-, or polyphenolic compounds can scavenge peroxynitrite *in vivo*.

1. Introduction. – The chemistry of the inorganic toxin peroxynitrite (oxoperoxonitrate(1−), $ONOO^-$) is presently under intense scrutiny. Peroxynitrite is formed near activated macrophages from the diffusion-limited reaction of nitrogen monoxide ($NO^{\bullet}$) and superoxide ($O_2^{\bullet -}$) with a rate constant of $0.5-1.9 \times 10^{10}$ $M^{-1}s^{-1}$ [1–3]. Although peroxynitrite may play an important protective role in the immune system, it may also damage tissues. For this reason, there is considerable interest in finding a scavenger of peroxynitrite. It has been proposed that the beneficial aspects of bioflavonoids, or vitamin P as named by *A. Szent-Györgyi*, could be ascribed to scavenging of peroxynitrite [4–6]. Redox reactions take place at the aromatic B-ring of flavonoids, which contains, depending on the flavonoid, one, two, or three OH groups. Peroxynitrous acid ($pK_a = 6.8$) is a strongly oxidizing agent [7], and nitrates and hydroxylates phenolic compounds under physiological conditions [8–12] with yields of *ca.* 8%, relative to peroxynitrite. These reactions are first-order in peroxynitrite and

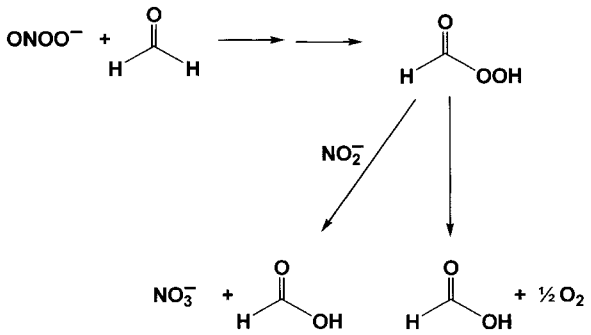
[1]) Some of these results have been presented at the 2nd International Conference on the Biology and Chemistry of Peroxynitrite, Crete, May 1999 [23].

zero-order in the phenolic compound, and therefore these compounds are unlikely to act as scavengers. Further, the concentration of free peroxynitrite *in vivo* may be very small due to its reaction with carbon dioxide. The rate constant of this reaction has been reported as 3×10^4 M^{-1}s^{-1} [13] at 25° and 5.6×10^4 M^{-1}s^{-1} at 37° [14], and the intracellular carbon dioxide concentration is *ca.* 1 mM. It can be estimated that most of the peroxynitrite anion formed *in vivo* reacts with carbon dioxide to form the unstable adduct 1-carboxylato-2-nitrosodioxidane anion, $ONOOCO_2^-$, while the remainder oxidizes thiols, selenoproteins and metalloproteins. In the absence of oxidizable substrates, the adduct decays to form NO_3^- and CO_2 [15]. The possibility exists, therefore, that flavonoids scavenge this adduct rather than peroxynitrite. In the case of the reaction of peroxynitrite with phenol or tyrosine, carbon dioxide reduces formation of hydroxylated products, and enhances the yield of nitration by a factor of two to three [11]. As it also diminishes methionine oxidation, carbon dioxide truly redirects the reactivity of peroxynitrite [16]. It has been proposed that $ONOOCO_2^-$, or its homolysis products nitrogen dioxide ($NO_2^{\bullet}$) and trioxocarbonate ($^{\bullet}1-$) ($CO_3^{\bullet -}$), are responsible for the nitration of tyrosine residues [17][18]. The requirements for effective scavenging of peroxynitrite can be deduced from the lifetime of $ONOOCO_2^-$ and the reactivity of the proposed homolysis products $NO_2^{\bullet}$ and $CO_3^{\bullet}$. These radicals react with rate constants of 10^7 to 10^9 M^{-1}s^{-1} with biomolecules. To prevent these reactions significantly (> 80%), a scavenger that reacts with peroxynitrite with a rate constant of *ca.* 10^9 M^{-1}s^{-1} (near the diffusion-controlled limit) must be present at concentration of 10 to 100 mM, which is not feasible *in vivo*. An alternative approach is to attempt to scavenge the adduct, $ONOOCO_2^-$. Given a rate of decay of *ca.* 400 s^{-1} [19] and possible physiological concentrations of the scavenger in the micromolar range, its rate constant would have to be on the order of 10^9 M^{-1}s^{-1} to accelerate the decay of $ONOOCO_2^-$ by a factor of *ca.* 10. We studied the reaction of peroxynitrite with the flavonoid monoHER, and examined as model compounds phenol, catechol, and hydroquinone in the presence and absence of carbon dioxide.



Adducts similar to the 1-carboxylato-2-nitrosodioxidane anion have been proposed for aldehydes [20] and other carbonyl compounds [21], and could similarly enhance formation of nitrated products. That peroxynitrite reacts with formaldehyde was first reported in 1929 (*Scheme 1*). *Gleu* and *Roell* [22] proposed that peroxynitrite oxidizes formaldehyde to peroxyformic acid, which then reacts with nitrite to form formic acid and nitrate. We investigated the reaction of peroxynitrite with *N*,*N*-dimethylacetamide, the dipeptide L-alanylalanine, methyl acetate, and acetone.

Scheme 1. *Reaction Mechanism for the Oxidation of Formaldehyde by Peroxynitrite* (according to *Gleu* and *Roell* [22])



2. Results and Discussion. – *Phenolic Compounds.* Aqueous solutions of catechol (benzene-1,2-diol) and hydroquinone (benzene-1,4-diol) were mixed with peroxynitrite in the presence and absence of carbon dioxide, and spectra were measured as a function of time. We verified by stopped-flow spectrophotometry that the reaction of peroxynitrite with phenol, catechol, hydroquinone, and the flavonoid monoHER is first-order in peroxynitrite and zero-order in the phenolic compounds, both in the presence and absence of carbon dioxide. In the presence of carbon dioxide, absorption changes were also measured at 640 nm, the maximum of the spectrum of 1-carboxylato-2-nitrosodioxidane anion [19].

Hydroquinone (Benzene-1,4-diol). The oxidation of hydroquinone by peroxynitrous acid at pH 5.6 and 20° in the absence (*Fig. 1,a*) and presence (*Fig. 1,b*) of 18 mM carbon dioxide resulted in the formation of quinone, which absorbs with a maximum at 246 nm ($\varepsilon_{246} = 20.5 \times 10^3$ M^{-1}cm^{-1}). The concentration of peroxynitrite was varied from 23 to 109 μM, and that of hydroquinone from 10 to 1 mM. The rate constants are 0.6 s^{-1} in the absence and 3.9×10^2 s^{-1} in the presence of 18 mM carbon dioxide. These rate constants are the same as those found for the isomerization of peroxynitrous acid in the absence of hydroquinone. The extinction coefficient of the oxidation product quinone is quite large, and hydroquinone ($\lambda_{max} = 288$ nm; $\varepsilon_{288} = 2.65 \times 10^3$ M^{-1}cm^{-1}) does not absorb at 246 nm, which facilitates the accurate determination of even small yields. The yields of quinone products, and the results of nitrite and nitrate determinations for the experiments carried out in the presence of carbon dioxide are given in the *Table*. The average yield of quinone was (30 ± 3)%, relative to peroxynitrite, and for every mole of quinone *ca*. one of nitrite was formed. Similar experiments, carried out in the absence of carbon dioxide with 10 to 500 μM hydroquinone and 1.5 to 120 μM peroxynitrite, but under otherwise identical conditions, resulted in a 10% yield of quinone relative to peroxynitrite. The absolute amount of product in the absence and presence of carbon dioxide remained unchanged, when the concentration of hydroquinone was varied from 10 to 500 μM. As a control experiment, we determined whether peroxynitrite and hydroquinone react within the mixing time of the stopped-flow instrument by using highly diluted peroxynitrite solutions (1.4 μM peroxynitrite, 10 μM hydroquinone, pH 6.3, 20°). Under these conditions, a bimolecular reaction would proceed correspondingly slower and could allow us to observe an initial step. However, the kinetics

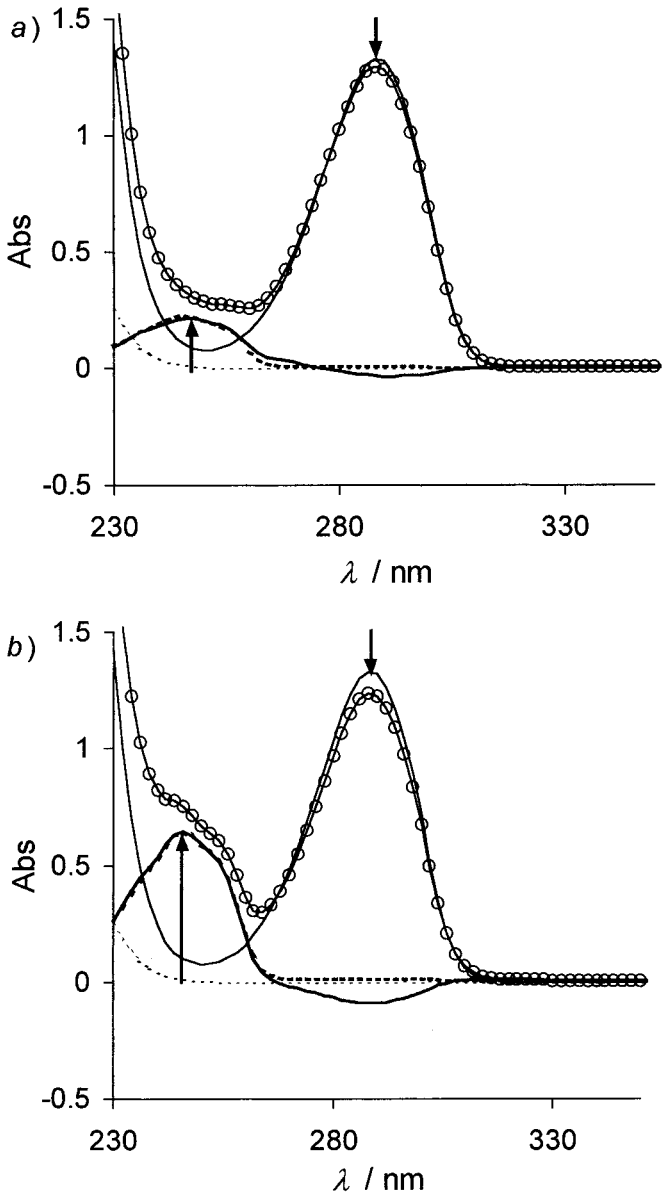


Fig. 1. *Spectra before and after the reaction of peroxynitrite with hydroquinone in* a) *the absence and* b) *the presence of carbon dioxide* (in 0.1M phosphate buffer at pH 5.6). (–): Hydroquinone, (– – –): isomerized peroxynitrite, (○): spectrum after mixing, (—) difference between the spectra of the products and reactants, (- - -) *para*-benzoquinone for comparison. *a*) 0.1 mM peroxynitrite, 0.5 mM hydroquinone; 0.01 mM *para*-benzoquinone. *b*) 0.08 mM peroxynitrite, 0.5 mM hydroquinone, 18 mM carbon dioxide; 0.03 mM *para*-benzoquinone.

Table. *Products from the Reaction of Peroxynitrite* ($ONOO^-$) *and Hydroquinone* (HQ) *in the Presence of 18 mM Carbon Dioxide* (in 10 mM phosphate buffer at pH 5.6 and 20°)

Initial conc. [μM]		Products (yields in μM or relative to starting $ONOO^-$ [%])				
$ONOO^-$	HQ	BQ[a])	BQ[a]) (rel. to $ONOO^-$)	NO_2^-[b])	NO_3^-[b])	NO_x^-
94	1000	32	34	36	56	92
94	500	28	30	28	68	96
79	200	25	32	28	58	86
79	100	24	30	26	58	84
98	0	0	0	0	96	96
47	500	16	34	–	–	–
47	100	14	30	–	–	–
47	50	13	28	–	–	–
47	10	10	21[c]	–	–	–
90	500	30	33	–	–	–
23	100	6	26	–	–	–
45	100	13	29	–	–	–
109	100	30	28	–	–	–

[a]) *para*-Benzoquinone (BQ) was determined by UV absorbance (246 nm, $\varepsilon = 20.5 \times 10^3\ M^{-1}cm^{-1}$. [b]) NO_2^- and NO_3^- were determined at four different hydroquinone concentrations. [c]) Due to the excess of peroxynitrite (47 μM) and the resulting quantitative reaction of hydroquinone (10 μM) to *para*-benzoquinone (10 μM), the yield relative to peroxynitrite cannot be higher than 21%.

were still first-order in peroxynitrite and zero-order in hydroquinone, and proceeded as fast as the isomerization of peroxynitrous acid to nitrate in 10 mM phosphate buffer ($0.4\ s^{-1}$). Furthermore, the absorbance recorded at the beginning of the experiment (*Fig. 2*) accounts for all of the peroxynitrite. We also carried out such controls with phenol present and determined the absorbance at 325 nm of 0.2 mM peroxynitrite with and without 0.25 mM phenol at pH 2.3. Here, too, the measured absorbances were exactly the same and comparable to that expected for peroxynitrous acid at that wavelength [23]. Even in the presence of 18 mM carbon dioxide, no difference in absorbance was found.

Catechol (Benzene-1,2-diol). The kinetics of the reactions of peroxynitrite with catechol in the absence and presence of 18 mM carbon dioxide were studied by rapid-scanning stopped-flow spectroscopy at pH 6.7 and 5.8 at 20°. The reaction is first-order in peroxynitrite and zero-order in catechol. The rate of formation at pH 6.7 and 20° in the absence of carbon dioxide is $0.5\ s^{-1}$, which corresponds to the rate of decay of peroxynitrite at that pH. The yield of the reaction between 0.14 mM peroxynitrite and 0.10 mM catechol was 12%. The yield in the presence of 18 mM carbon dioxide at pH 5.8 was 28%, and the rate is also independent of the catechol concentration. After the oxidation of catechol to *ortho*-benzoquinone, we observed spectral changes over a period of a few minutes during which a product that absorbs at 350 nm was formed. These changes could not have been caused by peroxynitrite, because by that time it had already completely decayed. To determine the kinetics of the formation of this product, we took the spectrum of *ortho*-benzoquinone as the baseline. The decrease at 276 nm (*Fig. 3,a*) shows the disappearance of catechol. This process is followed by the increase in absorption at 350 nm, which could indicate the formation of 1,2-dihydroxy-4-nitrobenzene. To verify this, we studied the reaction of *ortho*-benzoquinone with

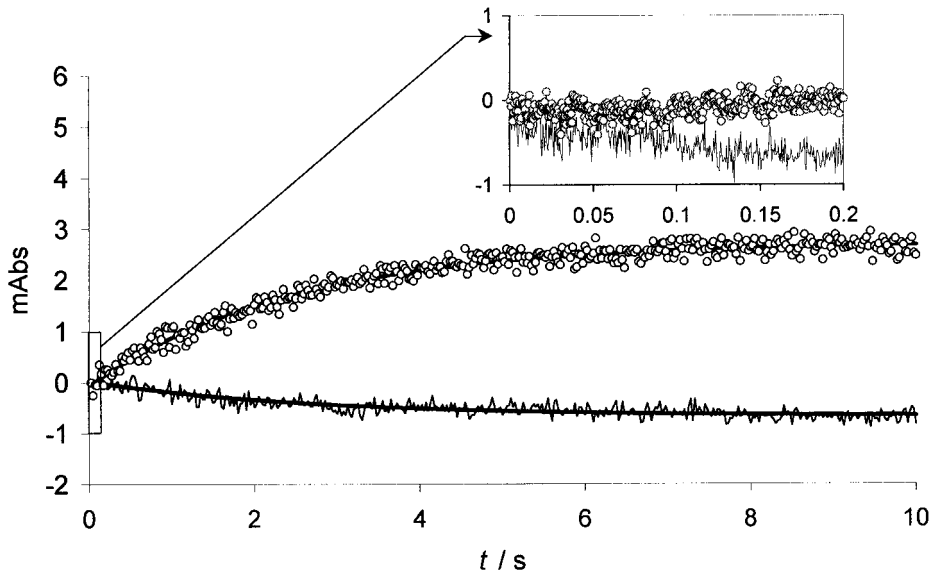


Fig. 2. *Reaction of* (○) *1.4* μM *peroxynitrite and 10* μM *hydroquinone* ($k = 0.4\ s^{-1}$) *and* (–) *isomerization of 1.4* μM *peroxynitrite* ($k = 0.4\ s^{-1}$; 10 mM phosphate buffer, pH 6.3, 20°, 250 nm). The absorption at $t = 0$ shows that no peroxynitrite was consumed in reactions during the mixing.

decayed peroxynitrite and nitrite at pH 6.7 and room temperature. *ortho*-Benzoquinone is unstable in aqueous solution and was synthesized at room temperature from 0.10 mM catechol and excess PbO_2 in 20 mM phosphate buffer. Immediately after the reaction, PbO_2 was removed by filtration, and, to 1 ml of the filtrate, 1 ml of 75 μM decayed peroxynitrite or 1.2 mM nitrite (*Fig. 3,b*) was added; in both cases 1,2-dihydroxy-4-nitrobenzene was formed.

The observation of the first reaction product, *ortho*-benzoquinone, is in agreement with the results reported for caffeic acid [24], but, in that study, formation of a nitrated compound was ruled out because the characteristic absorption of nitrated phenolics at 430 nm was not observed. However, we expect that such a nitrated product, being structurally similar to 1,2-dihydroxy-4-nitrobenzene, would absorb near 350 nm. Thus, formation of a nitrated product in a two-step reaction as described above is possible and even likely given that most peroxynitrite preparations contain substantial amounts of contaminating nitrite.

Flavonoid. Stopped-flow experiments were carried out at 20° in the absence and presence of carbon dioxide. The rate constant for the reaction of 60 μM peroxynitrite with 50 μM monoHER is $0.8\ s^{-1}$ and $350\ s^{-1}$ in the absence and presence of 18 mM carbon dioxide, respectively. Again, these rate constants are identical to the rate constant for disappearance of peroxynitrite in the absence of the flavonoid. As in case of the oxidation of hydroquinone by peroxynitrite, no reaction was observed during the mixing time. The yield of the reaction could not be determined due to subsequent reactions that gave an intractable mixture of secondary products.

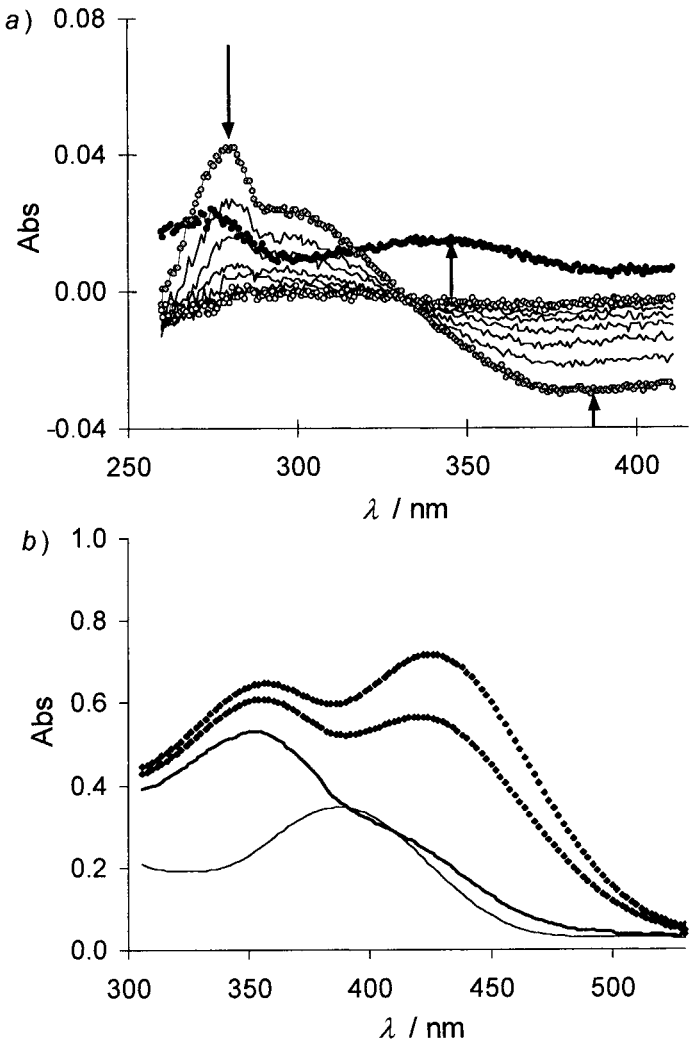


Fig. 3. a) *Reaction of 140* μM *peroxynitrite with 100* μM *catechol to give* ca. *17* μM ortho-*benzoquinone and 1,2-dihydroxy-4-nitrobenzene* (10 mM phosphate buffer, pH 6.7, and 20°). To separate the spectra of the two products, the spectrum of the first product, *ortho*-benzoquinone, was subtracted as a baseline: the decrease in absorption at 276 nm and the increase at 390 nm show the disappearance of 17 μM catechol and the formation of *ortho*-benzoquinone; the increase at 350 nm the formation of 1,2-dihydroxy-4-nitrobenzene over *ca.* 2 min. b) *Reaction of* ortho-*benzoquinone with nitrite at pH 6.7 and 20°* (–). *ortho*-Benzoquinone was synthesized by the oxidation of 0.30 mM catechol by excess PbO_2 in 20 mM phosphate buffer. Remaining PbO_2 was removed by filtration, and 1.2 mM nitrite was added. The spectrum of the product (–) 1,2-dihydroxy-4-nitrobenzene was obtained as the difference between the spectrum of the reactant and that of the product; (- - -) formation (lower trace) of a decomposition product at 430 nm that accumulates over several min (upper trace).

To determine the importance of the OH groups, we compared qualitatively the yields of the reaction of 1 mM peroxynitrite with 0.1 mM gallic acid and with 0.1 mM trimethoxygallic acid at pH 6.0. In contrast to the gallic acid, the triether compound showed nearly no change in absorption. These results are in agreement with those obtained by *Daiber et al.* [25] for the reaction of peroxynitrite with phenol and anisole.

Carbonyl Compounds. The reactions of peroxynitrite with *N*,*N*-dimethylacetamide, the dipeptide L-alanylalanine, methyl acetate, and acetone were studied by stopped-flow spectroscopy at 20°. Nitrite and nitrate from the reaction of 0.25 mM peroxynitrite with 0–0.5M acetone at pH 5.6 and 9.8 were determined by anion chromatography. In the pH range 3–12 with excess acetone the reaction is first-order in both peroxynitrite (*Fig. 4*,*a*) and in acetone (*Fig. 4*,*b*). The reaction of peroxynitrite with acetone has a rate constant of 1.8 $M^{-1}cm^{-1}$ at neutral pH and at 20°, which decreases at lower and higher pH values (*Fig. 5*). This indicates, in analogy to the peroxynitrite-aldehyde reaction [20], that it is the peroxynitrite anion that reacts with acetone. A similar reaction occurs between hydrogen trioxoperoxosulfate(1–) and ketones (*Scheme 2*) [26][27]. The decrease at higher pH suggests that at least one step in the reaction of peroxynitrite and acetone is catalyzed by acid. A similar pH dependence was observed for the reaction of aldehydes with H_2O_2 [28]. For the reaction of peroxynitrite and acetone, we proposed the formation of an adduct as intermediate (*Scheme 3*) [29]. In 1999, it was reported that activated ketones can act as catalysts for the isomerization of peroxynitrite, and dioxiranes were suggested as intermediates [21].

At alkaline pH peroxynitrite isomerizes to nitrate and also decomposes to nitrite and dioxygen [30]. As the reaction with acetone accelerates isomerization, the nitrate yield should increase with increasing acetone concentration. This is indeed the case, as shown in *Fig. 6* at pH 9.8. These results are in agreement with the observation of *Yang et al.* [21] that nitrite is oxidized quantitatively by the intermediate dimethyldioxirane. These authors studied the reaction of 0.32 mM peroxynitrite with 0–3.22 mM of the activated ketones methyl pyruvate and butanedione at pH 7.4. When the concentration of the ketones was increased, they found decreasing nitrate yields and suggested that this was caused by the formation of nitrite and oxygen from a secondary reaction of peroxynitrite with the dioxiranes.

To determine the nitrite and nitrate yields without these complications, we used a more than hundredfold higher concentration of acetone, up to 0.5M, and found that only nitrate was formed. As expected, peroxynitrite does not react with the deactivated carbonyl compounds *N*,*N*-dimethylacetamide, L-alanylalanine, and methyl acetate. With more electrophilic compounds like propionaldehyde or methyl pyruvate, the reaction proceeds faster ($530\ M^{-1}s^{-1}$ [20] and $1.3 \times 10^4\ M^{-1}s^{-1}$ [21], resp.). Thus, although peroxynitrite reacts with carbonyl compounds, these reactions, unlike that with carbon dioxide, cannot be relevant under physiological conditions.

3. Conclusions. – The results presented here for the reaction of peroxynitrite with carbonyl compounds imply that these reactions are too slow to play a role under physiological conditions. Thus, unlike carbon dioxide, carbonyl compounds do not modify the reactivity of peroxynitrite. As the reactions of peroxynitrite with phenol, catechol, hydroquinone, and the flavonoid monoHER are zero-order with respect to the phenolic compounds in the presence and absence of carbon dioxide, we must

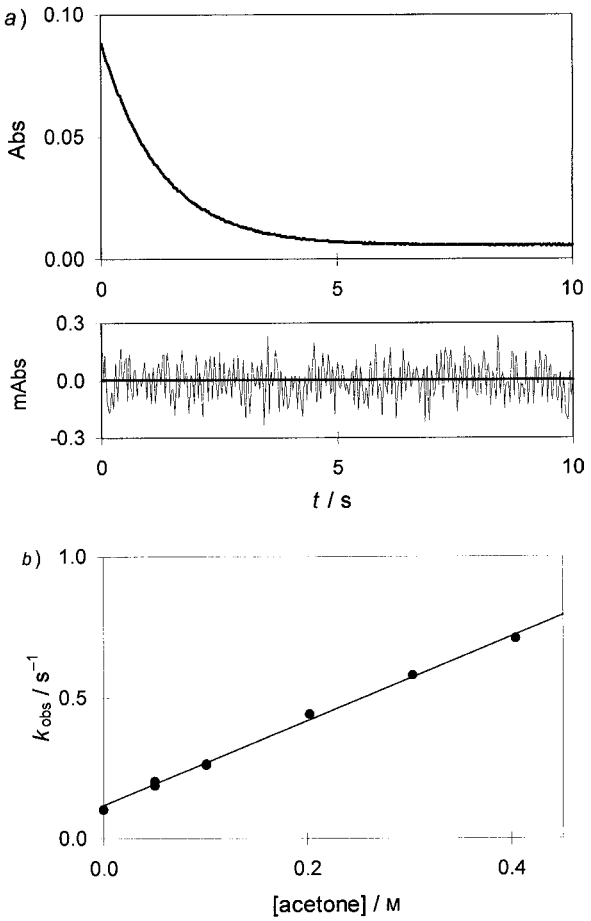


Fig. 4. a) *Loss of absorbance at 340 nm as a result of the pseudo-first-order reaction of 0.12* mM *peroxynitrite with 1.0*M *acetone* (pH 7.6 and 20° in 100 mM phosphate buffer; the residuals are given in the lower plot). b) *Pseudo-first-order rate constants* (k_{obs}) *for the reaction of 122* μM *peroxynitrite with 0–0.5*M *acetone at pH 7.6* (100 mM phosphate buffer at 20°; the slope yields a second-order rate constant of 1.5 $M^{-1}s^{-1}$).

conclude that phenolic compounds do not scavenge peroxynitrite or peroxynitrous acid. Furthermore, the observation of nitrate products from phenols is not necessarily evidence for the involvement of peroxynitrite *in vivo*, as the reaction of the *ortho*-benzoquinone, which is formed upon reaction of catechol with any suitable oxidizing species, with nitrite leads to nitrated product. In the case of flavonoids, the relevance of this reaction, which may also be limited due to steric hindrance, needs to be established.

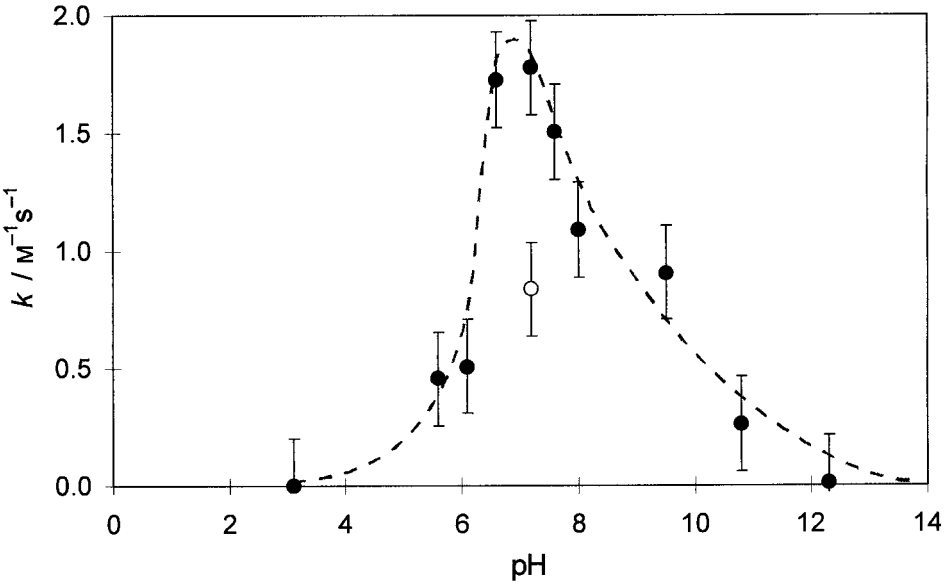
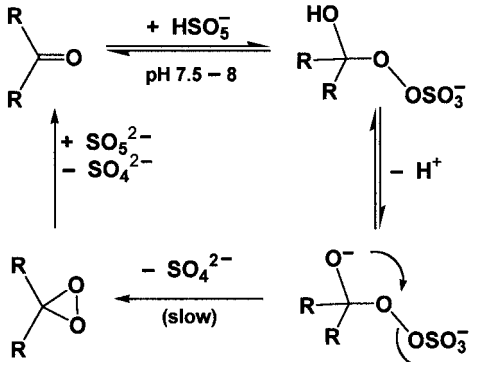


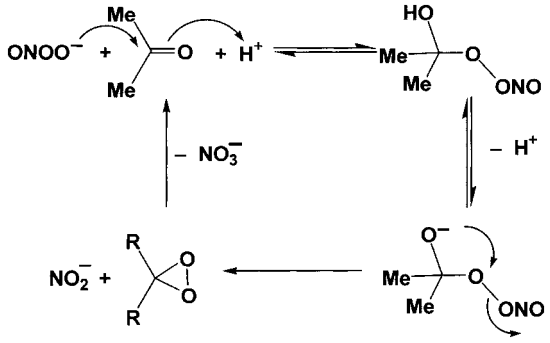
Fig. 5. *pH Dependence of the second-order rate constant* (*k*) *for the reaction of 100–120* μM *peroxynitrite with 0–0.5*M *acetone* (in (○) 5 mM or (●) 50–100 mM phosphate buffer at 20°; to obtain pH 3.1, 20 mM phosphoric acid was used). The rate constant exhibits a strong dependence on the buffer concentration.

Scheme 2. *Adduct Formation of Carbonyl Compounds with Hydrogen Trioxoperoxosulfate(1–)* [26][27]



Recently, it has been shown that DNA strand breaks induced by ionizing radiation were rapidly repaired by flavonoids [31]. This observation supports the notion that dietary flavonoids protect by repairing damage, as vitamin C does, rather than by direct scavenging of peroxynitrous acid.

Scheme 3. *Proposed Mechanism for the Reaction of Peroxynitrite with Acetone* (formation of an adduct analogous to that in the reaction of hydrogen trioxoperoxosulfate(1 –) with ketones [26] and regeneration of the carbonyl compound by the reaction with nitrite)



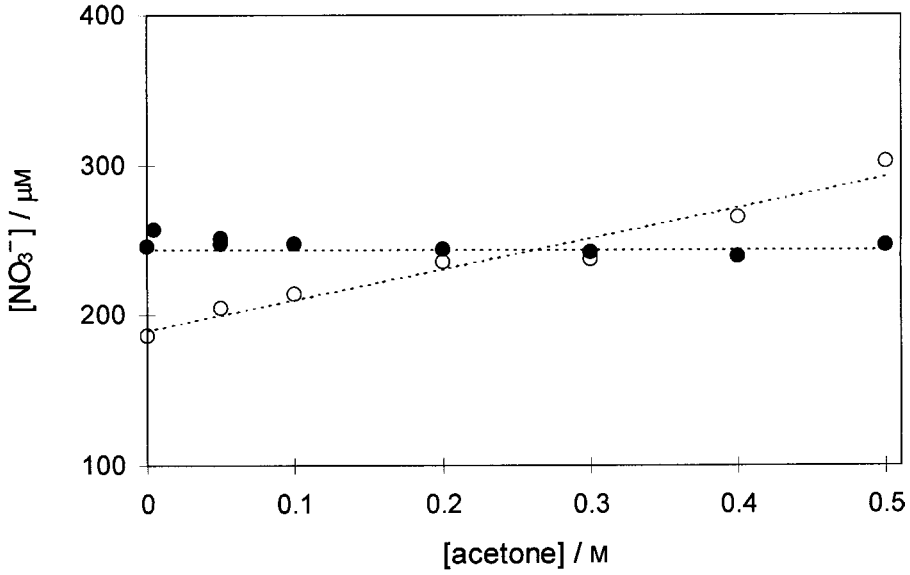


Fig. 6. *Product analysis by ion chromatography for the reaction of 0.25 mM peroxynitrite with 0–0.5M acetone* (at (●) pH 5.6 and (○) pH 9.8 at 20°). At pH 5.6, the product of the reaction of peroxynitrous acid with acetone is the same as that of the isomerization of peroxynitrous acid in the absence of acetone, *i.e.*, nitrate (and H^+). At pH 9.8, however, peroxynitrite forms nitrate *via* isomerization, and nitrite and dioxygen *via* decomposition. The reaction with acetone suppresses the decomposition of peroxynitrite, and the nitrate yield increases with increasing acetone concentration.

Experimental Part

General. Peroxynitrite was synthesized by the reaction of solid KO_2 with gaseous nitrogen monoxide [32]. The concentration was determined by UV/VIS absorption at 302 nm calculated from the extinction coefficient of 1700 $M^{-1}cm^{-1}$ [33]. All other reagents were purchased and were of the highest grade available.

Reactions with Phenolic Compounds. The kinetics were followed with an *Applied Photophysics SX17MV* single-wavelength stopped-flow spectrophotometer and with an *On-Line Instrument Systems, Inc.* stopped-flow instrument equipped with an *OLIS RSM 1000* rapid-scanning spectrophotometer. Eight to twelve traces per wavelength were averaged. Spectra used for product analysis were recorded on an *Uvikon* spectrophotometer; absorption changes were measured between 200 and 700 nm at 20°. Experiments with carbon dioxide were carried out as described in [19]. Due to the slow hydrolysis of carbon dioxide, the pH directly after mixing can be estimated from the amount of base present in the peroxynitrite preparation, and the pH and composition of the buffer. Nitrite and nitrate were determined by anion chromatography and conductivity detection (*ICSeparation Center 733*, *Anion SUPER-SEP column*, *ICDetector 732* and IC 709 pump; *Metrohm AG*). A phthalic acid soln. (2.5 mM phthalic acid, 5% MeCN, pH 4.6, *Tris*) was used as eluant. For calibration, 10 mM solns. of nitrite and nitrate were prepared and diluted to appropriate concentrations.

Reaction with Carbonyl Compounds. The kinetics of these reactions were followed with an *Applied Photophysics SX17MV* single-wavelength stopped-flow instrument. Absorption changes were measured at 340 nm with the carbonyl compounds in excess at 20°. Traces from eight to twelve experiments were averaged.

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