# $^{15}$N Chemically Induced Dynamic Nuclear Polarization ($^{15}$N-CIDNP) Investigations of the Peroxynitrite Decay and Nitration of *N*-Acetyl-L-tyrosine

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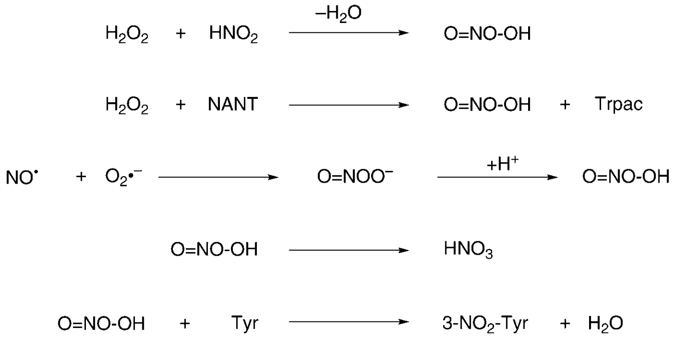
In Erinnerung an *Hanns Fischer*

During the decay of ($^{15}$N)peroxynitrite (O=$^{15}$NOO$^-$) in the presence of *N*-acetyl-L-tyrosine (Tyrac) in neutral solution and at 268 K, the $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ show emission (*E*) and enhanced absorption (*A*) as it has already been observed by *Butler* and co-workers in the presence of L-tyrosine (Tyr). The effects are built up in radical pairs [$CO_3^{\bullet-}$, $^{15}NO_2^{\bullet}$]$^S$ formed by O–O bond scission of the ($^{15}$N)peroxynitrite–CO$_2$ adduct (O=$^{15}$NO–OCO$_2^-$). In the absence of Tyrac and Tyr, the peroxynitrite decay rate is enhanced, and $^{15}$N-CIDNP does not occur. This is explained by a chain reaction during the peroxynitrite decay involving $N_2O_3$ and radicals NO$^\bullet$ and $NO_2^\bullet$. The interpretation is supported by $^{15}$N-CIDNP observed with ($^{15}$N)peroxynitrite generated *in situ* during reaction of $H_2O_2$ with *N*-acetyl-*N*-($^{15}$N)nitroso-DL-tryptophan (($^{15}$N)NANT) at 298 K and pH 7.5. In the presence of Na$^{15}$NO$_2$ at pH 7.5 and in acidic solution, $^{15}$N-CIDNP appears in the nitration products of Tyrac, 1-($^{15}$N)nitro-*N*-acetyl-L-tyrosine (1-$^{15}$NO$_2$-Tyrac) and 3-($^{15}$N)nitro-*N*-acetyl-L-tyrosine (3-$^{15}$NO$_2$-Tyrac). The effects are built up in radical pairs [Tyrac$^\bullet$, $^{15}NO_2^\bullet$]$^F$ formed by encounters of independently generated radicals Tyrac$^\bullet$ and $^{15}NO_2^\bullet$. Quantitative $^{15}$N-CIDNP studies show that nitrogen dioxide dependent reactions are the main if not the only pathways for yielding both nitrate and nitrated products.

**1. Introduction.** – Peroxynitrous acid (O=NO–OH, p*K* 6.5–6.8) is known since about one century as an instable intermediate during reaction of hydrogen peroxide ($H_2O_2$) with nitrous acid ($HNO_2$) [1] which slowly decays to the isomeric nitric acid and has nitrating properties as well [2] *(Scheme 1)*. It is also formed by reaction of $H_2O_2$ with nitroso compounds like *N*-acetyl-*N*-nitroso-DL-tryptophan (NANT) [3]. In recent years, peroxynitrite – this term is commonly used for the acid–base equilibrium mixture of peroxynitrous acid (O=NO–OH) and peroxynitrite anion (O=NOO$^-$) – has received considerable attention as a putative cytotoxic agent in living organisms where it may be produced by recombination of endogeneous nitric oxide (NO$^\bullet$) and superoxide anion ($O_2^{\bullet-}$) [4]. Its participation within more than 80 diseases has been proposed [5]. It may react with L-tyrosine (Tyr) and tyrosine residues in proteins to form 3-nitrotyrosine (3-NO$_2$-Tyr) and derivatives which might be useful markers of oxidants like peroxynitrite [6].

The mechanism of the peroxynitrite isomerization and nitration reactions was in discussion for a long time. A radical mechanism is generally accepted now (*Scheme 2*) [7]. The first step is an O–O bond homolysis giving hydroxyl radicals ($^\bullet$OH) and nitrogen dioxide ($NO_2^\bullet$). In the presence of $CO_2$, a decay reaction *via* a peroxyni-

Scheme 1. *Peroxynitrite Formation, Decay, and Nitration Reactions.* NANT = *N*-Acetyl-*N*-nitroso-DL-tryptophan.



$$H_2O_2 + HNO_2 \xrightarrow{-H_2O} O{=}NO{-}OH$$

$$H_2O_2 + \text{NANT} \longrightarrow O{=}NO{-}OH + \text{Trpac}$$

$$NO^\bullet + O_2^{\bullet -} \longrightarrow O{=}NOO^- \xrightarrow{+H^+} O{=}NO{-}OH$$

$$O{=}NO{-}OH \longrightarrow HNO_3$$

$$O{=}NO{-}OH + \text{Tyr} \longrightarrow 3\text{-}NO_2\text{-Tyr} + H_2O$$

trite–$CO_2$ adduct ($O{=}NO{-}OCO_2^-$) has been proposed leading to both $NO_2^\bullet$ and $CO_3^{\bullet -}$ [8]. In a second step, the radicals react by $O^-$ transfer to $NO_3^-$ or by H abstraction from Tyr to tyrosinyl radicals Tyr$^\bullet$. The 3-$NO_2$-Tyr is then formed by recombination of radicals $NO_2^\bullet$ and Tyr$^\bullet$ (*Scheme 2*).

The occurrence of radicals has been shown by ESR spectroscopy [9–11]. *Solvent cages* have been postulated because of radical scavenging experiments leading to a yield of *ca.* 30% hydroxyl radicals and nitrogen dioxide during the peroxynitrite decay [2][12] and of 30–33% $NO_2^\bullet$ and $CO_3^{\bullet -}$ radicals from the decomposition of the peroxynitrite–$CO_2$ adduct [11][13][14]. *Butler* and co-workers were able to establish the existence of *solvent cages* by the observation of $^{15}$N-CIDNP in the $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ during decay of ($^{15}$N)peroxynitrite$^1$) in the presence of Tyr in neutral and basic solution [15]. However, there are two puzzling observations which cannot be explained by using *Scheme 2* without further assumptions. The first one is the missing of 3-$NO_2$-Tyr formation. The second one is the lack of $^{15}$N-CIDNP effects in the absence of Tyr. For explaining these results, it has to be considered that the validity of *Scheme 2* has been proven under physiological conditions. Reaction conditions applied during the $^{15}$N-CIDNP experiments – mainly temperature and educt concentrations – are different from those. Therefore, the details of the peroxynitrite decay are more complex than described in *Scheme 2*. A recent kinetic simulation took into account 117 elementary reactions [16]. It seems very likely that, for understanding the $^{15}$N-CIDNP observations, additional reactions have to be taken into account.

The purpose of this report is to get a better understanding of the $^{15}$N-CIDNP results of *Butler* and co-workers [15] by describing the reaction mechanisms of peroxynitrite decay and nitration under $^{15}$N-CIDNP conditions. For doing this, *N*-acetyl-L-tyrosine (Tyrac) is used as an educt instead of L-tyrosine as its solubility in $H_2O$ is better than that of L-tyrosine. A few results have already been obtained with ($^{15}$N)peroxynitrite generated *in situ* during reactions of $H_2O_2$ with *N*-acetyl-*N*-($^{15}$N)nitroso-DL-tryptophan (($^{15}$N)NANT) and of $H_2O_2$ with Na$^{15}NO_2$ (*cf. Scheme 1*) [3][17][18]. They are summarized in *Sect. 2*. By using these precursors, $^{15}$N-CIDNP effects have been observed in

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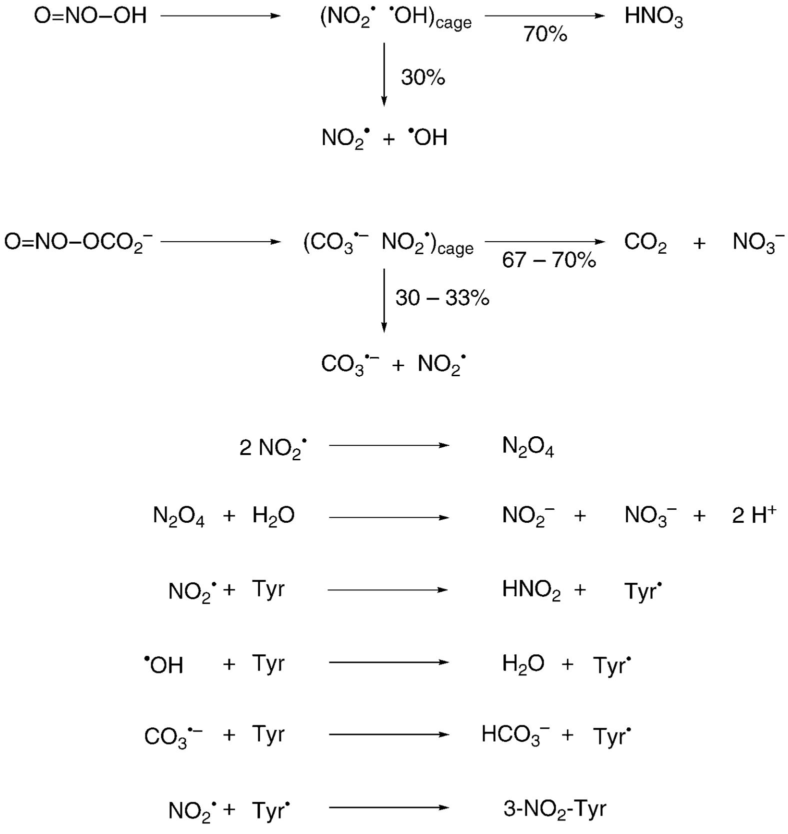
$^1$) For convenience, we use the IUPAC-recommended conventions for isotopically substituted species also for isotopically labeled species.

Scheme 2. *Mechanism of Peroxynitrite Isomerization and Nitration*

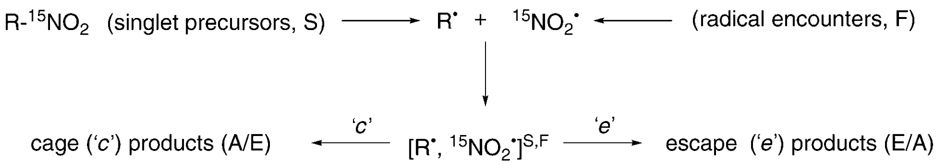


acidic solution during ($^{15}$N)peroxynitrite decay as well as during Tyrac nitration [3] [18]. It has been shown by quantitative $^{15}$N-CIDNP experiments that the radical mechanism is the main if not the only nitration pathway during reaction of $H_2O_2$ with Na$^{15}$NO$_2$ [18]. The $H_2O_2$/($^{15}$N)NANT system shows the expected $^{15}$N-CIDNP effects in the $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ at room temperature in neutral solution, too [3], in contrast to the results during decay of authentic ($^{15}$N)peroxynitrite at lower temperatures [15]. In *Sect. 4*, detailed $^{15}$N-CIDNP investigations will be described, performed with authentic ($^{15}$N)peroxynitrite at pH 7.5 at low temperatures, and explained by reaction of peroxynitrite with $N_2O_3$ and by decay of peroxynitric acid. $N_2O_3$ as well as peroxynitric acid are unstable reaction intermediates during the decomposition of peroxynitrite. The occurrence of hydroxyl radicals will be demonstrated by reaction with nitrite during unlabeled peroxynitrite decay in the presence of $^{15}NO_2^-$. The influence of $CO_2$ on the $^{15}$N-CIDNP effects will be shown during decay of ($^{15}$N)peroxynitrite generated *in situ* by reaction of Na$^{15}$NO$_2$ with $H_2O_2$ in the presence of $NaHCO_3$. Furthermore, quantitative $^{15}$N-CIDNP experiments were performed during formation of nitrate and nitrated products of *N*-acetyl-L-tyrosine by using different sources of ($^{15}$N)peroxynitrite. They will be discussed in terms of *Pedersen*'s quantitative treatment of the radical-pair theory.

**2. Application of CIDNP to Mechanistic Studies.** – 2.1. *The CIDNP Effect.* In 1967, *Bargon, Fischer*, and *Johnsen* observed emission and enhanced absorption signals in NMR spectra during decay reactions of organic peroxides in the probe of an NMR spectrometer [19]. The unusual signal intensities indicate the appearance of nuclear polarizations in the products of radical reactions (CIDNP). Following the radical-pair theory first proposed by *Closs* and *Kaptein*, nuclear polarizations are built up in radical pairs which are formed by homolytic O–O bond scission of peroxides from singlet states (S pairs). They might also be generated in radical pairs formed by diffusive encounters of independently generated radicals (F pairs) [20]. The appearance of CIDNP is used to establish the radical character of chemical reactions, particularly the product formation *via* radical pairs. If radicals are formed during peroxynitrite decay and nitration reactions, the formation of S as well as of F pairs is expected (*Scheme 2*) leading to CIDNP. Nuclear polarizations are not generated in radical pairs containing radicals with axial symmetry and degenerated orbitals like $\mathrm{HO}^\bullet$, $\mathrm{O}_2^{\bullet-}$, or $\mathrm{NO}^\bullet$ [21]. Because of this, radical pairs $[\mathrm{HO}^\bullet, \mathrm{NO}_2^\bullet]^{\mathrm{S}}$ formed by O–O bond scission of peroxynitrite should not lead to CIDNP effects.

N-Containing radicals and reaction products are involved during peroxynitrite decay and nitration reactions. Therefore, it is convenient to apply $^{15}$N-NMR spectroscopy. This requires the use of $^{15}$N-enriched compounds. $^{15}$N-CIDNP effects which are expected from thermal reactions of $^{15}\mathrm{NO}_2^\bullet$ and free radicals $\mathrm{R}^\bullet$ with $g(\mathrm{R}^\bullet) > g(^{15}\mathrm{NO}_2^\bullet)$ are exemplified in *Scheme 3* [20][22].

Scheme 3. *$^{15}$N-CIDNP Effects from Reactions of $^{15}\mathrm{NO}_2^\bullet$ with Radicals $\mathrm{R}^\bullet$ assuming* $g(\mathrm{R}^\bullet) > g(^{15}\mathrm{NO}_2^\bullet)$. E = emission, A = enhanced absorption.



Radicals which react within the radical pairs give cage ('*c*') products showing A(E) if arising from S(F) pairs (A = enhanced absorption, E = emission). This allows to distinguish between S and F precursors. Radicals which do not react within the pairs form escape ('*e*') products giving CIDNP patterns of opposite signs. The analysis of CIDNP effects allows a differentiation between product formation *via* '*c*' or '*e*' reactions, too. CIDNP Intensities are dependent on magnetic and diffusive properties of the radicals and the reaction products. The appearance of CIDNP, therefore, does not allow a simple answer to the question as to whether a radical path is the main reaction or only a side reaction. For judging this, CIDNP intensities have to be compared with those observed during well-known radical reactions or those calculated by quantitative treatments of the radical-pair theory. Both ways will be used in the following.

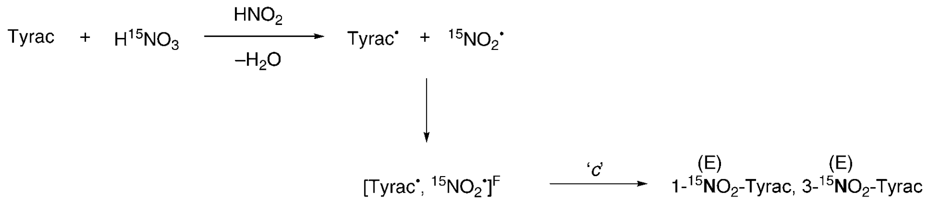
For a quantitative description of CIDNP, an enhancement factor $E$ has been introduced [20] which is the ratio between the intensity of the NMR signal $I$ immediately after formation of the polarized product within $t \ll T_1$ and the NMR signal $I_o$ after stopping the reaction taken at $t \gg T_1$ ($t$: reaction time; $T_1$: longitudinal nuclear relaxation time) (*Eqn. 1*). If the reaction time $t$ is much longer than $T_1$, $E$ is determined from *Eqn. 2* [23]. $I_i$ is the signal intensity during the $i^{th}$ measurement, $t(i,i+1)$ the time interval between the $i^{th}$ and the $(i+1)^{th}$ pulse. The summation is carried out as long as the CIDNP effects are observed. *Eqn. 2* is an extension of the relation $E = It/I_oT_1$ which has been used for $0^{th}$-order reactions [24]. This approximation is valid if $t \gg T_1$ and $I \gg I_o$.

$$E = I/I_o \tag{1}$$

$$E = \Sigma\, I_i\, \Delta\, t(i,i+1)/I_oT_1 \tag{2}$$

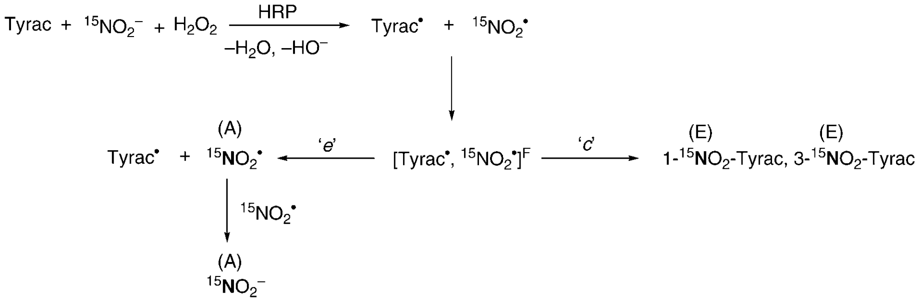
2.2. *Nitration of Tyrac with $H^{15}NO_3$ in Acidic Medium and with the System $^{15}NO_2^-$/$H_2O_2$/Horseradish Peroxidase (HRP) in Neutral Solution.* For comparison, Tyrac has been nitrated by systems different from ($^{15}$N)peroxynitrite. In acidic solution, ($^{15}$N)nitric acid has been applied as nitrating agent [15] [18]. The nitrous acid catalyzed nitration of phenolic compounds with nitric acid is known to be a radical reaction which has been confirmed by $^{15}$N-CIDNP [22] [23]. For instance, the $^{15}$N-NMR signals of 2-($^{15}$N)nitrophenol and 4-($^{15}$N)nitrophenol appear in emission during nitration of phenol with $H^{15}NO_3$ in AcOH [23]. An $E$ value of $-1180$ has been determined for 2-($^{15}$N)nitrophenol (see below, *Table 7*). During reaction of *N*-acetyl-L-tyrosine (Tyrac) with $H^{15}NO_3$, 3-($^{15}$N)nitro-*N*-acetyl-L-tyrosine (3-$^{15}NO_2$-Tyrac) and 1-($^{15}$N)nitro-*N*-acetyl-L-tyrosine (1-$^{15}NO_2$-Tyrac) are formed; the $^{15}$N-NMR signals appear in emission, too. The $^{15}$N-CIDNP effects are built up in radical pairs [Tyrac$^\bullet$, $^{15}NO_2^\bullet$]$^F$ formed by encounters of the radicals Tyrac$^\bullet$ and $^{15}NO_2^\bullet$ (*Schemes 3* and *4*). An $E$ value of $-1440$ has been determined for the $^{15}$N-NMR signal of 3-$^{15}NO_2$-Tyrac in AcOH which is similar to that observed in 2-($^{15}$N)nitrophenol during nitration of phenol with ($^{15}$N)nitric acid (see below, *Table 7*) [18].

Scheme 4. *$^{15}$N-CIDNP in Nitration Products of Tyrac during Nitrous Acid Catalyzed Reaction with $H^{15}NO_3$.* E = emission.



In neutral solution, Tyrac has been nitrated by the system $^{15}NO_2^-/H_2O_2$/HRP [18]. During the reaction, the $^{15}$N-NMR signals of 1-$^{15}NO_2$-Tyrac and 3-$^{15}NO_2$-Tyrac appear in emission indicating their formation by recombination of the radicals Tyrac$^\bullet$ and $^{15}NO_2^\bullet$, too. Additionally, the $^{15}$N-NMR signal of $^{15}NO_2^-$ appears in enhanced absorption which is an '*e*' type effect built up in radical pairs [Tyrac$^\bullet$, $^{15}NO_2^\bullet$]$^F$ and transferred to $^{15}NO_2^-$ by reactions between the radicals $^{15}NO_2^\bullet$ (*Schemes 2* and *5*). Because of long reaction times (*ca.* 10 h), an enhancement factor was not determined.

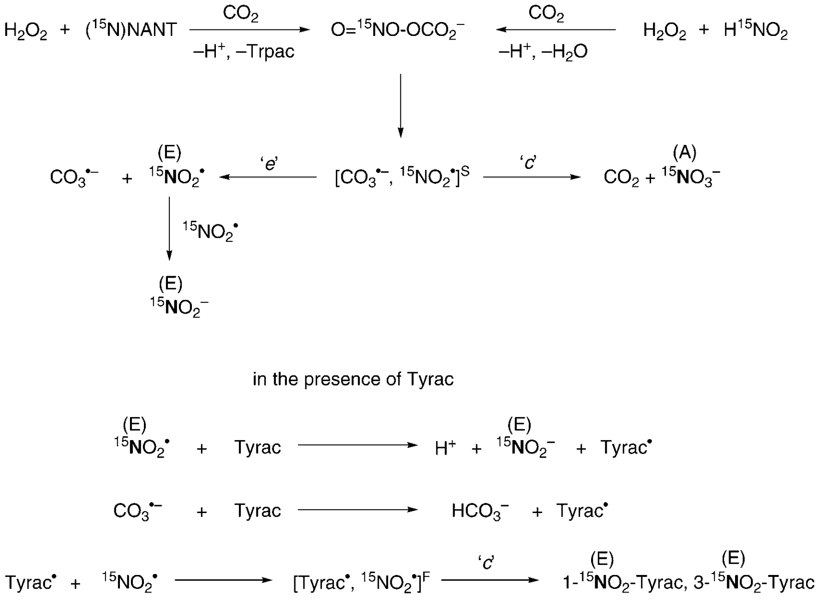
Scheme 5. *$^{15}$N-CIDNP in Nitration Products of Tyrac and in $^{15}NO_2^-$ during Reaction with $^{15}NO_2^-$/$H_2O_2$/Horseradish Peroxidase* (HRP) *at pH 7.* E = emission, A = enhanced absorption.



2.3. *Nitration of Tyrac with ($^{15}$N)Peroxynitrite at pH* ca. *5.* Peroxynitrite decay and nitration of Tyrac have been studied at pH *ca.* 5 by using *in situ* generated ($^{15}$N)peroxynitrite in the presence of $NaHCO_3$ (*Scheme 1*) [3][17][18]. During decay of ($^{15}$N)peroxynitrite, the $^{15}$N-NMR signals of $^{15}NO_3^-$ and of $^{15}NO_2^-$ show enhanced absorption and emission. In the presence of Tyrac, 1-$^{15}NO_2$-Tyrac and 3-$^{15}NO_2$-Tyrac appear in emission, too. The nuclear polarizations are built up in radical pairs formed by O–O bond scission of the ($^{15}$N)peroxynitrite–$CO_2$ adduct, [$CO_3^{\bullet-}$, $^{15}NO_2^\bullet$]$^S$, and by encounters of the radicals Tyrac$^\bullet$ and $^{15}NO_2^\bullet$, [Tyrac$^\bullet$, $^{15}NO_2^\bullet$]$^F$. The results are listed in *Scheme 6* and correspond to the radical mechanism of peroxynitrite decay and nitration (*Scheme 2*). An enhancement factor *E* of – 1520 has been determined for the $^{15}$N-NMR signal of 3-$^{15}NO_2$-Tyrac during reaction of $H_2O_2$ with $Na^{15}NO_2$ in the presence of Tyrac and $NaHCO_3$ (see below, *Table 7*) [18]. This value corresponds to that obtained during nitration of Tyrac with $H^{15}NO_3$ (– 1440) establishing the radical character of the peroxynitrite nitration.

2.4. *Calculation of CIDNP enhancement factors* $E_{calc}$. For determining theoretical enhancement factors $E_{calc}$ for $^{15}$N-NMR signals of 3-$^{15}NO_2$-Tyrac formed in F pairs, *Pedersen*'s treatment of the radical-pair theory has been applied [23–26]. It encloses quantities characterizing the magnetic properties and diffusion behavior of radicals and specific parameters describing the radical pair. *g* Values and hyperfine coupling constants *a* are known from ESR investigations [27] as well as transversal relaxation times $T_2$ of the radical electrons which are obtained from the linewidths of ESR signals. The diffusional behavior is described by diffusion constants $D = kT/6\pi\eta r$, with the viscosity $\eta$ of the solvent and the radius *r* of the radical, which might be estimated from radical models. *Pedersen*'s model-specific parameters are $\lambda$, the probability of reaction per radical encounter, *p*, the probability of at least one encounter, *d*, the distance of closest approach during an encounter, and $\Lambda$, the spin-independent probability of reaction of singlets for the entire collision, including all spin-independent re-encounters. $\lambda = 0.46$, $p = 0.32$, $d = 4$ Å $= 2r$, and $\Lambda = 0.5$ have been taken for calculating H-CIDNP effects in recombination products of the free-diffusing C-centered radicals $^\bullet$Me, $^\bullet CHCl_2$, $^\bullet CH_2COMe$ and $^\bullet CHClCO_2H$ [24][26]. They are also suitable for describing $^{119}$Sn-CIDNP in $Me_6Sn_2$ formed by recombination of trimethylstannyl radicals [26]. They have been applied for describing $^{15}$N-CIDNP in nitration products of phenol in AcOH [23]. By using $T_2$ ($NO_2^\bullet$) $= 5 \cdot 10^{-10}$ s [28], $a_N$ ($^{15}NO_2^\bullet$) $= 76.4$ G [28], *g* ($NO_2^\bullet$) $= 2.000$ [28], *g*

Scheme 6. *$^{15}$N-CIDNP during Decay of ($^{15}$N)Peroxynitrite* in situ *Generated at pH* ca. *5 in the Presence of NaHCO$_3$*. E = emission, A = enhanced absorption.



(PhO$^\bullet$) = 2.0046 [27], and $\eta$ (AcOH) = 1.056 mPa·s, $E_{\mathrm{calc}} = -1260$ was obtained which agrees well with the experimental value for 2-($^{15}$N)nitrophenol ($-1180$; see below, *Table 7*) [23]. The same $E_{\mathrm{calc}}$ value has been accepted for the $^{15}$N-NMR signal of 3-$^{15}$NO$_2$-Tyrac during nitration of Tyrac with H$^{15}$NO$_3$ in AcOH because of $g$(Tyrac$^\bullet$) = $g$(PhO$^\bullet$) [29].

**3. Experimental.** – 3.1. *$^{15}$N-CIDNP Experiments with Authentic ($^{15}$N)Peroxynitrite.* Stock solns. of ($^{15}$N)peroxynitrite (0.71 ± 0.02M O=NOO$^-$/0.435 ± 0.01M O=$^{15}$NOO$^-$) were prepared by reaction of isoamyl ($^{15}$N)nitrite (= 3-methylbutyl ($^{15}$N)nitrite; 0.12 mol/0.0024 mol) with hydrogen peroxide (100 ml $H_2O_2$/2 ml $H_2O_2$, 1M each) in solns. free of diethylenetriaminepentaacetic acid (= *N*,*N*-bis{2-[bis(carboxymethyl)amino]ethyl}glycine) and purified (6 × solvent extraction with hexane, removal of excess $H_2O_2$ by passing over MnO$_2$, and N$_2$ purging) as described by *Uppu* and *Pryor* [30], divided into 200-μl aliquots in 1-ml *Eppendorf* vials, and stored at $-79°$ in the dark. The reaction mixtures were prepared in 10-mm NMR tubes by adding ($^{15}$N)peroxynitrite to the frozen solvent ($H_2O/D_2O$ 9 : 1) containing phosphate buffer (0.3M) and, if required, further additives like NaHCO$_3$ (0.05M), Tyrac (0.05M), or Na$^{15}$NO$_2$ (2M; for reactions with peroxynitrite O=NOO$^-$) at 268 K. The final pH after mixing was 7.5. The tubes were quickly transferred into the probe of the $^{15}$N-NMR spectrometer (*Bruker DPX-300*) and locked (internal lock: D$_2$O). One minute after mixing of the reactants, the first $^{15}$N-NMR spectrum was taken by using single pulses with pulse angles of 90°. This procedure was repeated every minute until the reaction was completed. For detecting reaction products, $^{15}$N-NMR spectra were taken with several pulses at room temperature.

3.2. *$^{15}$N-CIDNP Experiments with* in situ *Generation of ($^{15}$N)Peroxynitrite.* A soln. of *N*-acetyl-*N*-($^{15}$N)nitroso-DL-tryptophan (0.1M) or Na$^{15}$NO$_2$ (0.05M) in $H_2O/D_2O$ 9 : 1, containing phosphate buffer (0.3M) and, when needed, NaHCO$_3$ (0.05M) or Tyrac (0.05M), was prepared in 10-ml tubes, and a single

$^{15}$N-NMR spectrum was taken by using a 90° pulse. After that, the tube was replaced, and $H_2O_2$ (1M or 3M) was added to the soln. $^{15}$N-NMR Spectra were then taken every 1–5 min until the reaction was completed.

3.3. *Determination of* I *and* $T_1$. $^{15}$N-NMR Intensities $I$ were directly taken from the signal-to-noise ratios by using single 90° pulses. During single runs, signal intensities are proportional to concentrations within *ca.* 5%. $I$ Values taken during different runs differ within *ca.* 20%. $T_1$ Values were determined after completion of the reactions under the conditions of the experiment applying $\pi$-$\pi/2$ pulse sequences.

3.4. *Materials and Solutions.* It was taken care to exclude possible contamination by $CO_2$ and transition-metal ions. The following procedures were performed under $N_2$ and at reduced incident light. Doubly distilled $H_2O$ was bubbled (2 l min$^{-1}$) with $N_2$ (5.0) at r.t. for 20 min and was then treated with the heavy-metal-ion scavenger resin *Chelex 100* (0.5 g in 10 ml) by gently shaking for 18 h in the dark. After separation from the resin by low-speed centrifugation for 5 min and careful decanting, the $H_2O$ was again bubbled with $N_2$ for 20 min.

*N*-Acetyl-*N*-($^{15}$N)nitroso-DL-tryptophan (($^{15}$N)NANT) was prepared according to a procedure described by *Bonnett* and *Holleyhead* and improved by us [31]. Na$^{15}$NO$_2$ and isoamyl ($^{15}$N)nitrit were purchased from *Aldrich/Isotec Inc.* (Taufkirchen, Germany). All other chemicals were from *Sigma* (Deisenhofen, Germany) and were of the highest purity available.

## 4. Results and Discussion. – 4.1. *$^{15}$N-CIDNP Experiments with Authentic ($^{15}$N)Peroxynitrite at pH 7.5.*

The protonated form of peroxynitrite decays with a rate of 1.3 s$^{-1}$ at 298 K [32]. Therefore, $^{15}$N-CIDNP experiments were performed at low temperatures. At pH 7.5, the ($^{15}$N)peroxynitrite decay was studied at 268 K in $CO_2$-free solutions as well as in the presence of $NaHCO_3$. During the reactions, $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ appear within 3 min indicating the end of the reaction at that time. $^{15}$N-CIDNP Effects are not observed. Some further details are given in *Table 1*. An apparent decay rate constant $k > 0.01$ s$^{-1}$ follows from the time dependence of the $^{15}$N-NMR signal intensities $I$ listed in *Table 2,a*. The results agree with observations reported by *Butler* and co-workers [15]. In addition to this, *Short* reports that $^{15}NO_3^-$ and $^{15}NO_2^-$ might show weak emission signals during the reaction [33].

The missing of $^{15}$N-CIDNP in the absence of $CO_2$ is not in contradiction to the general radical-decay mechanism of peroxynitrite (*Scheme 2*). Radical pairs $[^{15}NO_2^{\bullet}, {}^{\bullet}OH]^S$ formed by O–O bond scission of ($^{15}$N)peroxynitrite are not capable to build up nuclear polarizations. The weak emission-type effects observed in the $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ show that, if any, only a few radical pairs are present during the ($^{15}$N)peroxynitrite decay which are capable to generate $^{15}$N-CIDNP. They might be formed by a side reaction during the ($^{15}$N)peroxynitrite decay which will be discussed later. The missing of $^{15}$N-CIDNP in the presence of $NaHCO_3$ shows that the ($^{15}$N)peroxynitrite–$CO_2$ adduct is not formed under the reaction conditions or, if present, does not decay. It is obvious that *Scheme 6* is not valid for describing the decay of ($^{15}$N)peroxynitrite. We think that free $CO_2$ is not present which will be discussed in detail, too.

In the presence of Tyrac and of $NaHCO_3$, the $^{15}$N-NMR signal of $^{15}NO_2^-$ shows emission and the signal of $^{15}NO_3^-$ enhanced absorption for 6–10 min. Nitration products are not formed. This surprising result is in accordance with observations of *Butler* and co-workers during decay of ($^{15}$N)peroxynitrite in the presence of L-tyrosine [15]. Details of the reaction are listed in *Table 1*, signal intensities $I$ in *Table 2,b*. A spectrum taken 3 min after preparing the reaction solution is given in *Fig. 1,a*. The time dependence of the signal intensities is complex, as the conditions drastically change during the reac-

Table 1. *$^{15}$N-CIDNP During Peroxynitrite Decay and Nitration of* L-*Tyrosine* [15] *and* N-*Acetyl*-L-*tyrosine*

| System | $^{15}$N-NMR Signals[a]) | CIDNP[b]) | Yield[c]) | *E*[d]) |
|---|---|---|---|---|
| ($^{15}$N)Peroxynitrite (0.03M), *T* 268 K, $NaHCO_3$ (0.05M), pH 7.5 (*Table 2,a*)[e]) | 8.8 ($^{15}NO_3^-$) | N | 4.0 | |
| | 241.9 ($^{15}NO_2^-$) | N | 1.8 | |
| ($^{15}$N)Peroxynitrite (0.1M), *T ca.* 273 K, pH 7.6 [15][33] | 5.6 ($^{15}NO_3^-$) | N (E ?) | | |
| | 238.8 ($^{15}NO_2^-$) | N (E ?) | | |
| ($^{15}$N)Peroxynitrite (0.03M), *T* 268 K, Tyrac (0.05M), $NaHCO_3$ (0.05M), pH 7.5 (*Fig. 1, Table 2,b*) | *ca.* 7 (3-$^{15}NO_2$-Tyrac) | – | $< 0.03$ | |
| | 8.8 ($^{15}NO_3^-$) | A | 4.2 | 60 |
| | 241.9 ($^{15}NO_2^-$) | E | 2.2 | |
| Peroxynitrite (0.2M), *T* 268 K, Tyrac (0.1M), $Na^{15}NO_2$ (2M), $NaHCO_3$ (0.05M), pH 7.5 (*Fig. 2,a* and *b*, *Table 2,c*) | 7.2 (3-$^{15}NO_2$-Tyrac) | E | 0.32 | −400 |
| | 9.0 ($^{15}NO_3^-$) | E | 2.0 | −10 |
| | 21.2 (1-$^{15}NO_2$-Tyrac) | E | | |
| | 241.9 ($^{15}NO_2^-$) | N | 130 | |
| Peroxynitrite (0.2M), *T* 268 K, $Na^{15}NO_2$ (2M), $NaHCO_3$ (0.05M), pH 7,5 (*Table 2,d*) | 9.0 ($^{15}NO_3^-$) | N | 6 | |
| | 241.9 ($^{15}NO_2^-$) | N | 200 | |
| ($^{15}$N)Peroxynitrite (0.1M), *T* 298 K, Tyr (0.025M), pH 12.5 [15][f]) | 5.6 ($^{15}NO_3^-$) | A | | 110 |
| | 238.8 ($^{15}NO_2^-$) | E | | |

[a]) $\delta$ Values against Ph-$^{15}NO_2$, positive $\delta$ values downfield. [b]) E = emission, A = enhanced absorption, N = no CIDNP. [c]) $^{15}$N-NMR Intensities after reaction. [d]) *E* = enhancement factor determined by *Eqn. 1* and *2* from the data given in *Table 2* and in [15]. [e]) No changes by using $CO_2$-free solvents. [f]) Yield of 3-$NO_2$-Tyr 0.45%, determined by UV/VIS spectral studies. Similar $^{15}$N-CIDNP spectra were observed at pH 7.6 and *T ca.* 273 K [15].

tion. The highest intensities are observed 2 min after starting the reaction, when the solution is homogeneous and the reactants are completely mixed. During the next 4 min, the intensities decay by a factor of 2 indicating a decay rate constant $k = 0.006\ s^{-1}$. After that, the decay accelerates when the probe is warming. A spectrum taken after reaction is given in *Fig. 1,b*. It shows $^{15}$N-NMR signals of $^{15}NO_2^-$ and $^{15}NO_3^-$ with intensities corresponding to thermal equilibrium of the nuclear spin states. For describing the intensity of the $^{15}$N-CIDNP effect in $^{15}NO_3^-$, an enhancement factor *E* of 60 is determined by using *Eqn. 2* with $T_1 = 90$ s ($T_1 = 140$ s is given in [34]).

For explaining the results, the observed decay rates at pH 7.5 will be discussed first. Peroxynitrite decay rates are known at pH 7.4 in phosphate buffer (0.5M) in the temperature range $278\ K < T < 323\ K$ determined with an initial peroxynitrite concentration of 0.6 mM [35]. An extrapolation to 268 K leads to a decay rate constant $k = 0.0025\ s^{-1}$ which is similar to the decay rate found in the presence of $NaHCO_3$ and Tyrac ($k = 0.006\ s^{-1}$) and without Tyrac ($k > 0.01\ s^{-1}$).

The $^{15}$N-CIDNP effects observed in the presence of $NaHCO_3$ and Tyrac are explained as described (*Scheme 6*) [18]. Radical pairs $[CO_3^{\bullet -}, {}^{15}NO_2^{\bullet}]^S$ generated by O−O bond scission of the ($^{15}$N)peroxynitrite−$CO_2$ adduct, $O{=}^{15}NO{-}OCO_2^-$, are responsible for the $^{15}$N-CIDNP effects. Nitration products are not formed because of the high concentration of Tyrac which effectively scavenges the radicals $^{15}NO_2^{\bullet}$ and, therefore, prevents the recombination of $^{15}NO_2^{\bullet}$ and the radicals Tyrac$^{\bullet}$ [7][14][36].

The missing of $^{15}$N-CIDNP effects in the absence of Tyrac points to a lack of $CO_2$ under the applied reaction conditions which prevents the formation of the peroxynitrite−$CO_2$ adduct [37] (*Schemes 2* and *7*). Furthermore, there is a fast reaction without

Table 2. *$^{15}$N-NMR Intensities* I a) *during Decay* a) *of ($^{15}$N)Peroxynitrite* (0.03M), b) *of ($^{15}$N)Peroxynitrite* (0.03M) *with Tyrac* (0.05M), c) *of Peroxynitrite* (0.2M) *with Tyrac* (0.1M) *and Na$^{15}$NO$_2$* (2M), *and* d) *of Peroxynitrite* (0.2M) *and Na$^{15}$NO$_2$* (2M) *in the Presence of NaHCO$_3$* (50 mM) *at pH 7.5 and 268 K*

*a*) ($^{15}$N)Peroxynitrite/NaHCO$_3$ system

| $t$ b) | 1 | 2 | 3 | 10 | 100 |
|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | – | – | 1 | 2 | 1.8 c) |
| $I(^{15}NO_3^-)$ | – | – | 2 | 4 | 4.0 c) |

*b*) ($^{15}$N)Peroxynitrite/Tyrac/NaHCO$_3$ system

| $t$ b) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | –15 | –20 | –15 | –10 | –20 | –10 | 2 | 2 | 2 | 2 | 2.2 d) |
| $I(^{15}NO_3^-)$20 |  | 40 | 30 | 25 | 100 | 100 | 40 | 10 | 6 | 5 | 4.2 d) |

*c*) Peroxynitrite/Tyrac/Na$^{15}$NO$_2$/NaHCO$_3$ system

| $t$ b) | 1 | 2 | 3 | 100 |
|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | 180 | 130 | 160 | 130 e) |
| $I(^{15}NO_3^-)$ | –7 | – | – | 2.0 e) |
| $I$(3-$^{15}$NO$_2$-Tyrac) | –60 | –3 | – | 0.32 e) |
| $I$(1-$^{15}$NO$_2$-Tyrac) | –25 | –2 | – | – |

*d*) Peroxynitrite/Na$^{15}$NO$_2$/NaHCO$_3$ system

| $t$ b) | 1 | 2 | 3 | 100 |
|---|---|---|---|---|
|  | 100 | 200 | 200 | 200 |
|  | 3 | 6 | 6 | 6 |

a) Determined from the signal-to-noise ratios by using single 90° pulses. b) $t$ = Time [min] after mixing the reactants and putting the tube into the probe of the $^{15}$N-NMR spectrometer. c) From $^{15}$N-NMR spectra taken after reaction (16 scans, delay time 3 min). d) From $^{15}$N-NMR spectra taken after reaction (10 scans, delay time 3 min). Yield (3-$^{15}$NO$_2$-Tyrac) < 0.03 (910 scans, delay time 3 min). e) From $^{15}$N-NMR spectra taken after reaction (235 scans, delay time 5 min).

involvement of CO$_2$ which is inhibited by addition of Tyrac. We propose that this is a N$_2$O$_3$-induced decomposition of peroxynitrite under participation of free radicals $^{\bullet}$OH, NO$^{\bullet}$, and NO$_2^{\bullet}$ [38–40] (*Scheme 7*). If CO$_2$ is present at the beginning of the reaction, it reacts to CO$_3^{2-}$ and HCO$_3^-$ during the first seconds. At low temperatures, HCO$_3^-$ ions decompose to CO$_2$ only slowly as will be discussed now. From the half-life values of HCO$_3^-$ at 298 K (26 s at pH 7 and 25 s at pH 8) and at 273 K (240 s at pH 7 and 300 s at pH 8) [37], half-lives of 380 s at pH 7 and of 500 s at pH 8 are extrapolated at 268 K. In the presence of Tyrac, the N$_2$O$_3$-induced decomposition of peroxynitrite is inhibited as $^{\bullet}$OH and NO$_2^{\bullet}$ are effectively scavenged by Tyrac (*Schemes 2* and *6*). The stationary concentration of CO$_2$ is high enough to form the peroxynitrite–CO$_2$ adduct in the presence of Tyrac but not in Tyrac-free solutions.

The weak emission in the $^{15}$N-NMR signals of $^{15}$NO$_2^-$ and $^{15}$NO$_3^-$ in the absence of Tyrac is explained by reactions of ($^{15}$N)peroxynitric acid with its anion (*Scheme 8*). ($^{15}$N)Peroxynitric acid (O$_2$$^{15}$NOOH/O$_2$$^{15}$NOO$^-$: *p$K_a$* 5.9) is known to be formed *via* recombination of superoxide anions O$_2^{\bullet-}$ (HO$_2^{\bullet}$/O$_2^{\bullet-}$: p$K_a$ 4.8) and $^{15}$NO$_2^{\bullet}$ during decay of ($^{15}$N)peroxynitrite as a side reaction. This reaction does not lead to $^{15}$N-CIDNP effects in neutral medium. It has been observed during decay of ($^{15}$N)peroxynitric acid that the $^{15}$N-NMR signals of $^{15}$NO$_3^-$ and of $^{15}$NO$_2^-$ appear in emission [41] which has been explaind by reactions of the acid with its anion (*Scheme 8*).

The formation of 3-$^{15}$NO$_2$-Tyrac and of $^{15}$N$_2$O$_3$ is supported by high concentrations of $^{15}$NO$_2^{\bullet}$ (*Schemes 2*, *6*, and *7*). Addition of $^{15}$NO$_2^-$ to the reaction mixture should, there-

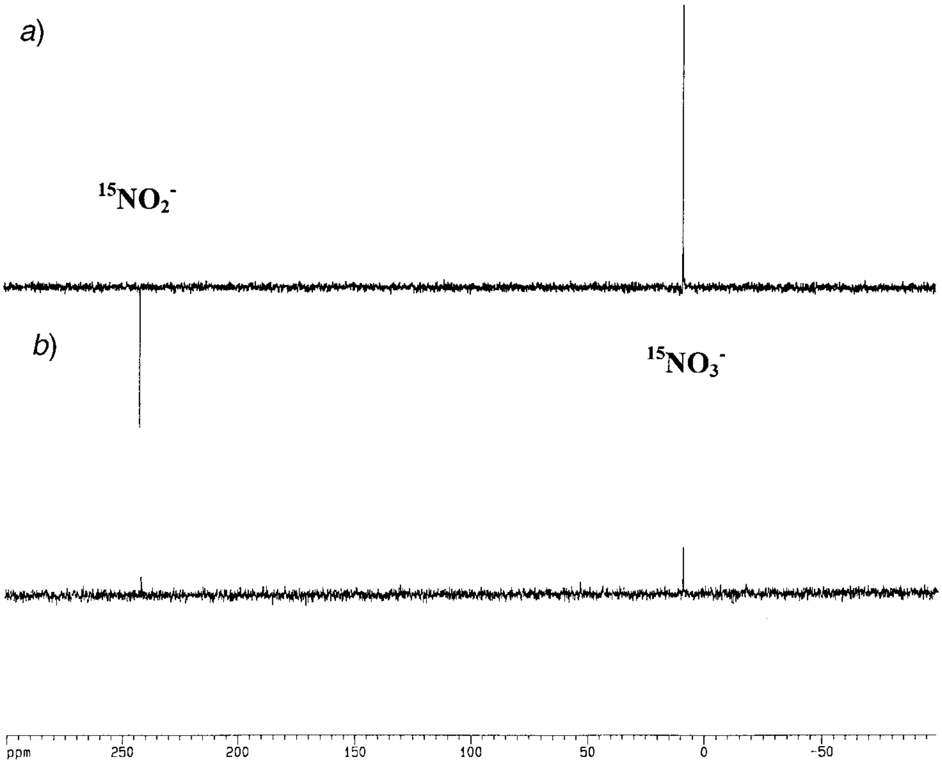
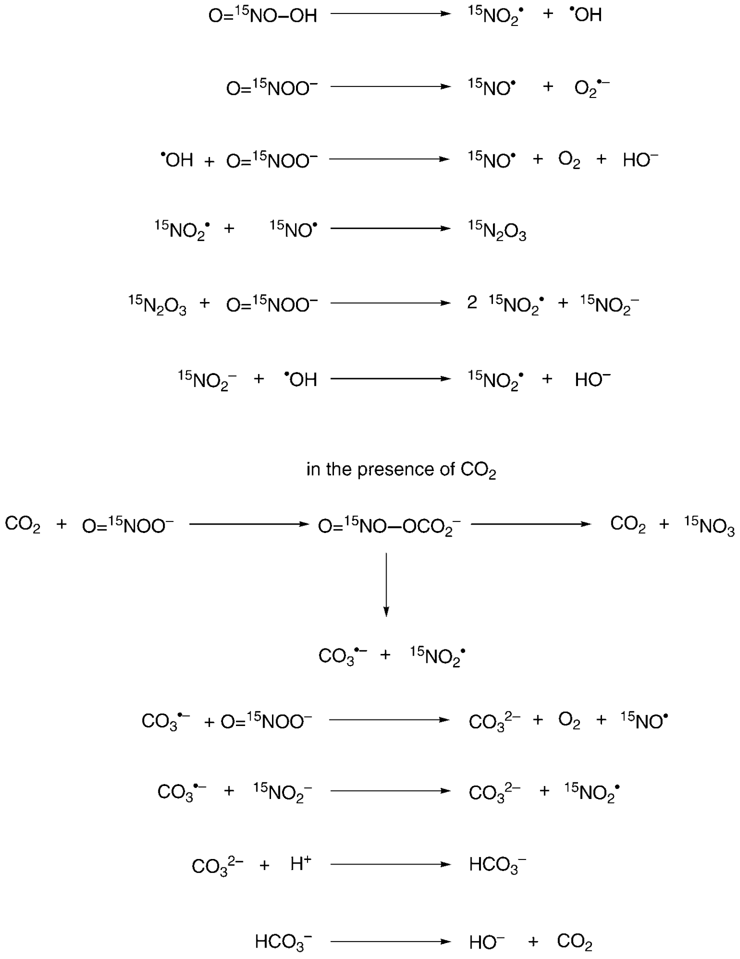


Fig. 1. *$^{15}$N-NMR Spectra of a solution of ($^{15}$N)peroxynitrite with Tyrac and $NaHCO_3$ at pH 7.5 and 268 K taken* a) *3 min and* b) *100 min after putting the tube into the probe* (1 scan)

fore, enhance the yield of 3-$^{15}NO_2$-Tyrac as well as the decay rate of peroxynitrite (*Scheme 7*). Both has indeed been observed, see *Tables 1* and *2,c* and *d* and *Fig. 2,a* and *b*. One minute after adding $Na^{15}NO_2$ (2M) to a solution of authentic unlabeled peroxynitrite and Tyrac at pH 7.5, strong emission signals are observed at $\delta$ 7.2, 9.0, and 21.2 which disappear 2 min later. After reaction, only the signals at $\delta$ 7.2 and $\delta$ 9.0 are observed which are assigned to $^{15}NO_3^-$ and 3-$^{15}NO_2$-Tyrac. The signal at $\delta$ 21.2 is due to the unstable cyclohexadienyl-like nitration product 1-$^{15}NO_2$-Tyrac [42]. The $^{15}$N-CIDNP effects in the signals of $^{15}NO_3^-$ and 3-$^{15}NO_2$-Tyrac are explained as described (*Schemes 6* and *8*). Enhancement factors $E = -10$ and $-400$ are deduced by *Eqn. 1* (*Table 1*). In the absence of Tyrac, $^{15}$N-CIDNP is not observed under the same conditions (*Table 1* and *2,d*) as the reaction time is shorter than the relaxation time ($T_1$ 90 s). The reaction is completed before putting the tube into the probe of the spectrometer.

At pH 12.5 and room temperature, $^{15}$N-CIDNP experiments have been performed during decay of ($^{15}$N)peroxynitrite in the presence of L-tyrosine [15]. The reaction is finished within $\frac{1}{2}$ h. The $^{15}$N-NMR signals of $^{15}NO_2^-$ and of $^{15}NO_3^-$ appear in emission and enhanced absorption as observed at pH 7.5 [15]. No precautions have been reported for removing $CO_2$. Therefore, we think that the effects might be explained as described

Scheme 7. *$^{15}N_2O_3^{\bullet -}$ Induced Decomposition of ($^{15}N$)Peroxynitrite*

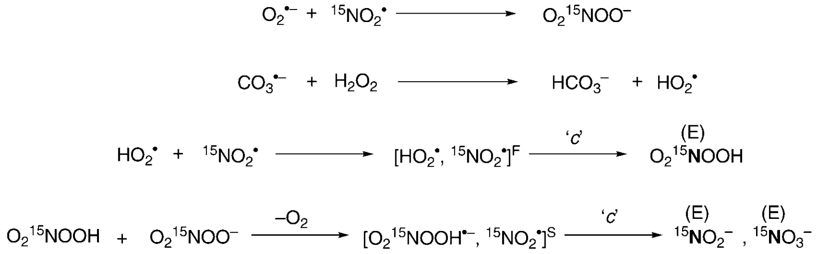


$$\mathrm{O{=}^{15}NO{-}OH \longrightarrow {}^{15}NO_2^{\bullet} + {}^{\bullet}OH}$$
$$\mathrm{O{=}^{15}NOO^- \longrightarrow {}^{15}NO^{\bullet} + O_2^{\bullet -}}$$
$$\mathrm{{}^{\bullet}OH + O{=}^{15}NOO^- \longrightarrow {}^{15}NO^{\bullet} + O_2 + HO^-}$$
$$\mathrm{{}^{15}NO_2^{\bullet} + {}^{15}NO^{\bullet} \longrightarrow {}^{15}N_2O_3}$$
$$\mathrm{{}^{15}N_2O_3 + O{=}^{15}NOO^- \longrightarrow 2\ {}^{15}NO_2^{\bullet} + {}^{15}NO_2^-}$$
$$\mathrm{{}^{15}NO_2^- + {}^{\bullet}OH \longrightarrow {}^{15}NO_2^{\bullet} + HO^-}$$

in the presence of $CO_2$

$$\mathrm{CO_2 + O{=}^{15}NOO^- \longrightarrow O{=}^{15}NO{-}OCO_2^- \longrightarrow CO_2 + {}^{15}NO_3}$$
$$\mathrm{O{=}^{15}NO{-}OCO_2^- \longrightarrow CO_3^{\bullet -} + {}^{15}NO_2^{\bullet}}$$
$$\mathrm{CO_3^{\bullet -} + O{=}^{15}NOO^- \longrightarrow CO_3^{2-} + O_2 + {}^{15}NO^{\bullet}}$$
$$\mathrm{CO_3^{\bullet -} + {}^{15}NO_2^- \longrightarrow CO_3^{2-} + {}^{15}NO_2^{\bullet}}$$
$$\mathrm{CO_3^{2-} + H^+ \longrightarrow HCO_3^-}$$
$$\mathrm{HCO_3^- \longrightarrow HO^- + CO_2}$$

Scheme 8. *$^{15}N$-CIDNP during Formation and Decay of ($^{15}N$)Peroxynitric Acid*



$$\mathrm{O_2^{\bullet -} + {}^{15}NO_2^{\bullet} \longrightarrow O_2{}^{15}NOO^-}$$
$$\mathrm{CO_3^{\bullet -} + H_2O_2 \longrightarrow HCO_3^- + HO_2^{\bullet}}$$
$$\mathrm{HO_2^{\bullet} + {}^{15}NO_2^{\bullet} \longrightarrow [HO_2^{\bullet}, {}^{15}NO_2^{\bullet}]^F \xrightarrow{\text{‘c’}} \overset{(E)}{O_2{}^{15}\mathbf{N}OOH}}$$
$$\mathrm{O_2{}^{15}NOOH + O_2{}^{15}NOO^- \xrightarrow{-O_2} [O_2{}^{15}NOOH^{\bullet -}, {}^{15}NO_2^{\bullet}]^S \xrightarrow{\text{‘c’}} \overset{(E)}{{}^{15}\mathbf{N}O_2^-}, \overset{(E)}{{}^{15}\mathbf{N}O_3^-}}$$

(*Scheme 6*). From the spectra given in [15], a slightly higher *E* value is deduced than that observed at pH 7.5 and listed in *Table 7* ($E = 110$, see below).

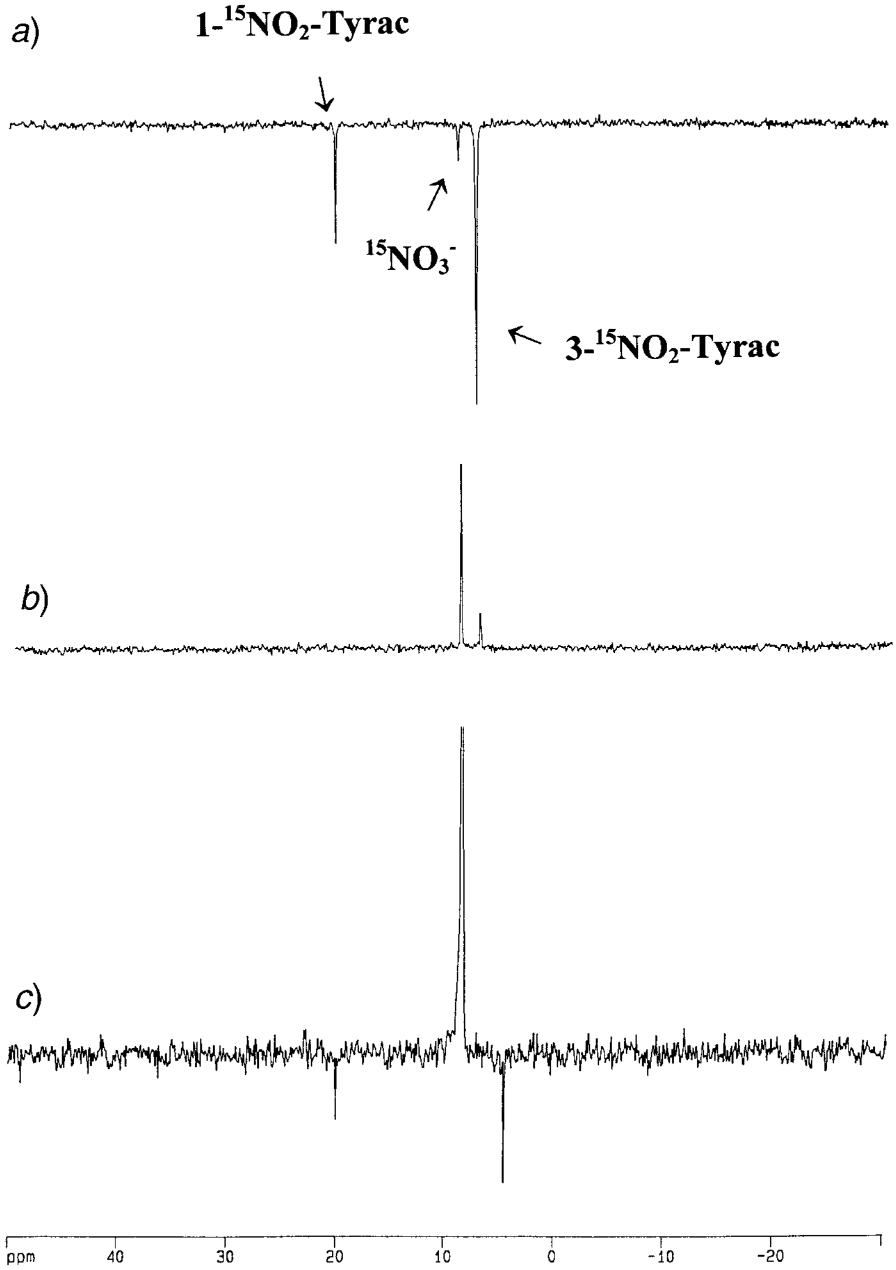


Fig. 2. *$^{15}$N-NMR Spectrum of a solution of peroxynitrite with Tyrac, Na$^{15}$NO$_2$, and NaHCO$_3$ at pH 7.5 and 268 K taken* a) *1 min* (1 scan) *and* b) *100 min after putting the tube into the probe* (235 scans). c) *$^{15}$N-NMR Spectrum of a solution of ($^{15}$N)NANT, Tyrac, and NaHCO$_3$ at pH 4.0 and 298 K taken 3 min after adding $H_2O_2$* (1 scan). NANT = *N*-Acetyl-*N*-nitroso-DL-tryptophan.

4.2. *${}^{15}$N-CIDNP Experiments with (${}^{15}$N)Peroxynitrite Generated* in situ. The observation of ${}^{15}$N-CIDNP effects during *in situ* generation of (${}^{15}$N)peroxynitrite by means of the reactions of $H_2O_2$ with *N*-acetyl-*N*-(${}^{15}$N)nitroso-DL-tryptophan ((${}^{15}$N)NANT) and with $Na^{15}NO_2$ (*Scheme 1*) has already been reported [3][17][18]. In the following, a few additional experiments will be described to complete these results.

For performing ${}^{15}$N-CIDNP experiments with (${}^{15}$N)peroxynitrite at pH *ca.* 7, the reaction of $H_2O_2$ with (${}^{15}$N)NANT is suitable (*Scheme 1*) [3]. During the reaction in the presence of $NaHCO_3$ with and without Tyrac, emission and enhanced absorption are observed in the ${}^{15}$N-NMR signals of ${}^{15}NO_2^-$ and ${}^{15}NO_3^-$ [3]. Quantitative results at pH 7.5 and pH 4.0 and room temperature were performed. The ${}^{15}$N-NMR spectrum shown in *Fig. 3,a* is taken from a solution of (${}^{15}$N)NANT at pH 7.5 before adding $H_2O_2$, the spectra in *Fig. 3,b* and *Fig. 3,c*, 18 min and 60 min after adding $H_2O_2$. Details of the results and the time dependence of the signal intensities *I* are listed in *Tables 3* and *4*. The emission signal of ${}^{15}NO_2^-$ is observed for *ca.* 34 min, the enhanced absorption of the ${}^{15}NO_3^-$ signal for 54 min. An enhancement factor $E = 110$ is found which is comparable to the values found during the decay of (${}^{15}$N)peroxynitrite in the presence of Tyrac ($E = 60$) and Tyr ($E = 110$).

The effects are generated during decay of the (${}^{15}$N)peroxynitrite$-CO_2$ adduct (*Scheme 6*) showing that the formation rate of $O{=}{}^{15}NO{-}OCO_2^-$ is higher than the decay rate of the (${}^{15}$N)peroxynitrite under the given conditions. Because of the low stationary (${}^{15}$N)peroxynitrite concentration, the ${}^{15}N_2O_3$-induced decomposition is of no importance. The enhanced absorption in the ${}^{15}$N-NMR signal of ${}^{15}NO_3^-$ is of the '*c*' type, the emission in the ${}^{15}$N-NMR signal of ${}^{15}NO_2^-$ of the '*e*' type which is transferred by reaction of two radicals ${}^{15}NO_2^{\bullet}$ to ${}^{15}NO_2^-$ (*Schemes 2* and *6*).

Table 3. *${}^{15}$N-CIDNP during Reaction of (${}^{15}$N)NANT with $H_2O_2$, Tyrac, and $NaHCO_3$ at 295 K.* NANT = *N*-Acetyl-*N*-nitroso-DL-tryptophan.

| System | ${}^{15}$N-NMR Signals[a]) | CIDNP[b]) | Yield[c]) | $E$[d]) |
|---|---|---|---|---|
| (${}^{15}$N)NANT (0.1M), $H_2O_2$ (3M), $NaHCO_3$ (0.05M), pH 7.5 (*Fig. 3*, *Table 4,a*) | 8.7 (${}^{15}NO_3^-$) | A | 5 | 110 |
| | 178.4 ((${}^{15}$N)NANT) | N | | |
| | 192.8 ((${}^{15}$N)NANT) | N | | |
| | 241.8 (${}^{15}NO_2^-$) | E | 1 | |
| (${}^{15}$N)NANT (0.1M), $H_2O_2$ (1M), $NaHCO_3$ (0.05M), Tyrac (0.1M), pH 7.5 | 8.7 (${}^{15}NO_3^-$) | A | | |
| | 178.4 ((${}^{15}$N)NANT) | N | | |
| | 192.8 ((${}^{15}$N)NANT) | N | | |
| | 241.8 (${}^{15}NO_2^-$) | E | | |
| (${}^{15}$N)NANT (0.1M), $H_2O_2$ (1M), $NaHCO_3$ (0.05M), Tyrac (0.1M), pH 4.0 (*Fig. 2,c*, *Table 4,b*) | 5.1 (3-${}^{15}NO_2$-Tyrac) | E | 0.077 | −1430 |
| | 8.5 (${}^{15}NO_3^-$) | E | 8.5 | 310 |
| | 30.1 (1-${}^{15}NO_2$-Tyrac) | E | | |
| | 178.4 ((${}^{15}$N)NANT) | N | | |
| | 192.7 ((${}^{15}$N)NANT) | N | | |
| | 240.5 (${}^{15}NO_2^-$) | E | | |

[a]) $\delta$ Values against Ph-${}^{15}NO_2$, positive $\delta$ valued downfield. [b]) E = emission, A = enhanced absorption, N = no CIDNP. [c]) ${}^{15}$N-NMR Intensities after reaction. [d]) $E$ = Enhancement factor determined by *Eqn. 2* from the data given in *Table 4*.

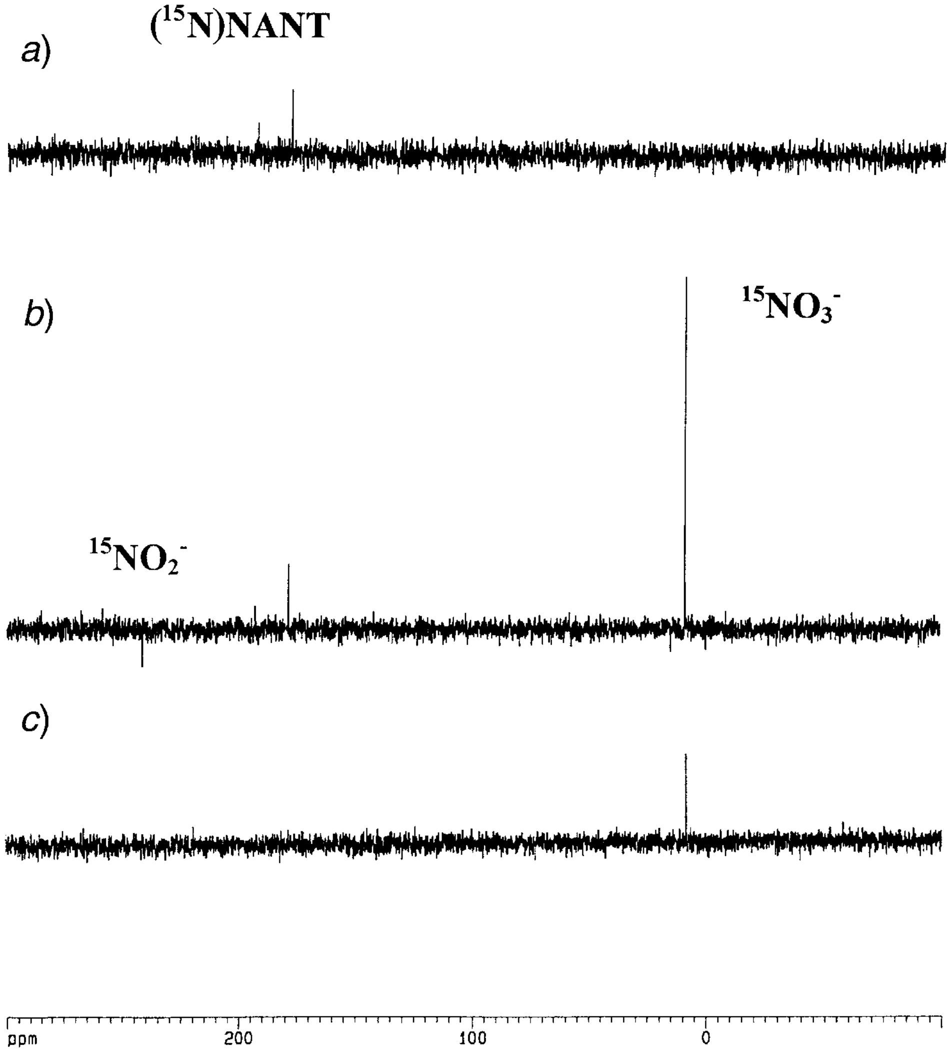


Fig. 3. *$^{15}$N-NMR Spectra of a solution of ($^{15}$N)NANT with $NaHCO_3$ at pH 7.5 and 298 K taken* a) *before adding $H_2O_2$,* b) *18 min, and* c) *60 min after adding $H_2O_2$* (1 scan). NANT = *N*-Acetyl-*N*-nitroso-DL-tryptophan

Identical spectra are observed during the reaction of the system $H_2O_2$/($^{15}$N)NANT with Tyrac at pH 7.5 indicating that nitration products of Tyrac are not formed either. In contrast to this, nitration products of Tyrac are formed at pH 4.0 which appear in emission [3] (*Fig. 2,c, Table 4,b*). This might be due to the lower H-abstraction rate of nitrogen dioxide from Tyrac observed at lower pH values [43] [44] which raises the stationary concentration of free radicals $^{15}NO_2^{\bullet}$. An enhancement factor $E = -1430$ is determined which is similar to that during nitrous acid catalyzed nitration of Tyrac with nitric acid ($E = -1440$; see below, *Table 7* [17]). An enhancement factor $E = 310$ is found for

Table 4. *$^{15}$N-NMR Intensities* I[a]) *during Reaction* a) *of ($^{15}$N)NANT* (0.1M) *with $H_2O_2$* (3M) *at pH 7.5, and* b) *of ($^{15}$N)NANT* (0.1M) *with $H_2O_2$* (1M) *and Tyrac* (50 mM) *at pH 4.0 in the presence of $NaHCO_3$* (50 mM) *at 295 K*

*a*) ($^{15}$N)NANT/$H_2O_2$/$NaHCO_3$ system at pH 7.5

| $t$[b]) | 0 | 1 | 6 | 10 | 18 | 34 | 54 | 60 |
|---|---|---|---|---|---|---|---|---|
| $I$(($^{15}$N)NANT) | 3/2 | 3/2 | 4/1 | 4/1 | 4/1 | 2/1 | – | – |
| $I(^{15}NO_2^-)$ | – | −2 | 0 | −1 | −2 | −2 | 0 | 1[c]) |
| $I(^{15}NO_3^-)$ | – | 12 | 14 | 15 | 20 | 10 | 8 | 5[c]) |

*b*) ($^{15}$N)NANT/$H_2O_2$/Tyrac/$NaHCO_3$ system at pH 4.0

| $t$[b]) | 0 | 1 | 3 | 5 | 7 | 11 | 14 | 18 | 25 | 38 | 56 | 70 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $I$(($^{15}$N)NANT) | 3/1 | 3/1 | 3/1 | 3/1 | 2/0 | 2/0 | 2/0 | – | – | – | – | – | – |
| $I(^{15}NO_2^-)$ | – | −4 | −4 | −4 | −3 | −3 | −3 | −2 | – | – | – | – | – |
| $I(^{15}NO_3^-)$ | – | 100 | 160 | 160 | 100 | 100 | 80 | 60 | 40 | 30 | 20 | 10 | 8.5[d]) |
| $I$(3-$^{15}NO_2$-Tyrac) | – | −8 | −6 | −4 | −2 | – | – | – | – | – | – | – | 0.077[d]) |
| $I$(1-$^{15}NO_2$-Tyrac) | – | −4 | −3 | −2 | −2 | – | – | – | – | – | – | – | – |

[a]) Determined from the signal-to-noise ratios by using single 90° pulses. [b]) $t$ = Time [min] after mixing the reactants and putting the tube into the probe of the $^{15}$N-NMR spectrometer [c]) From $^{15}$N-NMR spectra taken after reaction (9 scans, delay time 3 min). [d]) From $^{15}$N-NMR spectra taken after reaction (180 scans, delay time 3 min).

the $^{15}$N-NMR signal of $^{15}NO_3^-$ at pH 4.0 which is higher than the value deduced at pH 7.5 ($E = 110$). This might be due to a higher stationary concentration of $CO_2$ at pH 4.0 [37].

By using the system $H_2O_2$/$Na^{15}NO_2$ for generating ($^{15}$N)peroxynitrite at pH 5.25, analogous $^{15}$N-CIDNP effects have been found (*Tables 5* and *6*) [17][18]. From the $I$ values of $^{15}NO_3^-$ given in [17], $E = 290$ and $E = 320$ are determined in the absence and in the presence of additional $NaHCO_3$ (*Tables 5* and *7*). For getting these values, no special precautions had been performed to remove $CO_2$ from the solvents. Under these conditions, the $CO_2$ concentration seems to be high enough for allowing the formation of the ($^{15}$N)peroxynitrite−$CO_2$ adduct, even in the absence of $NaHCO_3$. For demonstrating the influence of $CO_2$ on the $^{15}$N-CIDNP effects in greater detail, the experiments were now repeated at pH 4.7 by using carefully degassed solvents (see *Sect. 3*, *Exper.*). The results are shown in *Fig. 4* and the *Tables 5* and *6,a*. For comparison, a spectrum obtained after adding $NaHCO_3$ to the solution is given in *Fig. 5,a*, the time dependence of $I$ values in *Table 6,b*. In the absence of $CO_2$, the $^{15}$N-NMR signal of $^{15}NO_3^-$ shows a weak enhanced absorption during the beginning of the reaction which is converted to emission later. The $^{15}$N-NMR signal of $^{15}NO_2^-$ appears in weak emission all the time, and a further signal is observed in emission at $\delta$ −19.5 which is assigned to $^{15}NO_4^-$ and/or its protonated form $H^{15}NO_4$. The enhanced absorption in the $^{15}$N-NMR signal of $^{15}NO_3^-$ is explained by decomposition of the ($^{15}$N)peroxynitrite−$CO_2$ adduct formed by traces of $CO_2$ which are still present (*Scheme 6*). The emission-type effect in the $^{15}$N-NMR signals of $^{15}NO_2^-$, $^{15}NO_3^-$, and $^{15}NO_4^-$ is caused by formation and decay of ($^{15}$N)peroxynitric acid as discussed [41][42] (*Scheme 8*). Encounters of the radicals $HO_2^{\bullet}$ and $^{15}NO_2^{\bullet}$ lead to radical pairs $[HO_2^{\bullet}, {}^{15}NO_2^{\bullet}]^F$ which are able to generate $^{15}$N-CIDNP effects (*Scheme 3*). This is not the case in radical pairs $[O_2^{\bullet -}, {}^{15}NO_2^{\bullet}]^F$ formed

Table 5. *$^{15}$N-CIDNP during Reaction of Na$^{15}$NO$_2$ with H$_2$O$_2$ and of Na$^{15}$NO$_2$ with H$_2$O$_2$and Tyrac at 295 K*

| System | $^{15}$N-NMR Signals[a]) | CIDNP[b]) | Yield[c]) | *E*[d]) |
|---|---|---|---|---|
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), AcONa (1M), pH 5.25 [17] | 8.8 ($^{15}$NO$_3^-$) | A | 5 | 290 |
| | 241.7 ($^{15}$NO$_2^-$) | N | | |
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), AcONa (1M), NaHCO$_3$ (0.05M), pH 5.25 [17] | 8.8 ($^{15}$NO$_3^-$) | A | 5 | 320 |
| | 241.7 ($^{15}$NO$_2^-$) | N | | |
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), pH 4.7 (*Fig. 4*, *Table 6,a*) | – 19.5 ($^{15}$NO$_4^-$) | E | | |
| | 8.7 ($^{15}$NO$_3^-$) | A → E | 8 | *ca.* 0 |
| | 240.1 ($^{15}$NO$_2^-$) | E | | |
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), NaHCO$_3$ (0.05M), pH 4.7 (*Fig. 5,a, Table 6,b*) | – 19.5 ($^{15}$NO$_4^-$) | E | | |
| | 8.7 ($^{15}$NO$_3^-$) | A | | 240 |
| 240.1 ($^{15}$NO$_2^-$) | | E | | |
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), Tyrac (0.05M), NaHCO$_3$ (0.05M), pH 4.7 (*Fig. 5,b, Table 6,c*) | – 19.5 ($^{15}$NO$_4^-$) | E | | |
| | 5.0 (3-$^{15}$NO$_2$-Tyrac) | E | 0.28 | – 1740 |
| | 8.6 ($^{15}$NO$_3^-$) | A | 6.0 | 260 |
| | 16.5 (1-$^{15}$NO$_2$-Tyrac) | E | | |
| | 240.1 ($^{15}$NO$_2^-$) | E | | |
| Na$^{15}$NO$_2$ (0.05M), H$_2$O$_2$ (1M), Tyrac (0.05M), NaHCO$_3$ (0.05M), pH 5.25 (*Table 6,d* [18]) | – 19.5 ($^{15}$NO$_4^-$) | E | | |
| | 5.0 (3-$^{15}$NO$_2$-Tyrac) | E | 0.7 | – 1520 |
| | 8.7 ($^{15}$NO$_3^-$) | A | 7 | 430 |
| | 16.5 (1-$^{15}$NO$_2$-Tyrac) | E | | |
| | 240.1 ($^{15}$NO$_2^-$) | E | | |

[a]) $\delta$ Values against Ph-$^{15}$NO$_3$, positive $\delta$ values downfield. [b]) E = emission, A = enhanced absorption, N = no CIDNP. [c]) $^{15}$N-NMR Intensities after reaction. [d]) *E* = enhancement factor determined by *Eqn. 2* from the data given in *Table 6* and in [17] and [18].

from the unprotonated superoxide anion O$_2^{\cdot-}$ and $^{15}$NO$_2^{\cdot}$ at pH *ca.* 7 [18]. In contrast to the results in CO$_2$-free solvents, a strong $^{15}$N-CIDNP effect in the $^{15}$N-NMR signal of $^{15}$NO$_3^-$ is observed in the presence of NaHCO$_3$ (*Fig. 5,a, Table 6,b*). The enhancement factor at pH 4.7 (*E* = 240) is similar to the values found at pH 5.25 (*Tables 5* and *7*) [17]. For completing the results, a $^{15}$N-NMR spectrum taken during reaction of H$_2$O$_2$ with Na$^{15}$NO$_2$ and Tyrac in the presence of NaHCO$_3$ at pH 4.7 is given in *Fig. 5,b*; details of the reaction, signal intensities *I* and enhancement factors *E* are listed in *Tables 5* and *6,c*. The $^{15}$N-CIDNP effects resemble those obtained at pH 5.25 (*Table 6,d*, [17]). The reaction rate is higher by a factor of 2 because of the pH dependence of the *Baeyer–Villiger* reaction [1].

4.3. *Calculation of $^{15}$N-CIDNP Enhancement Factors* E$_{calc}$. Enhancement factors *E* of $^{15}$N-NMR signals of the nitration product 3-$^{15}$NO$_2$-Tyrac and of $^{15}$NO$_3^-$ have similar values (*Table 7*) if various nitrating systems are used. It is concluded that $^{15}$N-CIDNP effects are built up in identical radical pairs, [Tyrac$^{\cdot}$, $^{15}$NO$_2^{\cdot}$]$^{F}$ and [CO$_3^{\cdot-}$, $^{15}$NO$_2^{\cdot}$]$^{S}$. This will be supported by calculations by means of the radical-pair model of CIDNP in *Pedersen*'s version [25] which has been performed for nitration reactions of phenolic compounds with ($^{15}$N)nitric acid in AcOH (*Table 7*) [23]. It has been shown that the calculations describe the observed *E* values sufficiently. Therefore, the model-specific parameters will be accepted here for calculating the radical pair [Tyrac$^{\cdot}$, $^{15}$NO$_2^{\cdot}$]$^{F}$. By using $g$ (Tyrac$^{\cdot}$) = 2.0046, $\eta$ (AcOH) = 1.056 mPa·s, $\eta$ (H$_2$O) = 0.894 mPa·s,

Table 6. *$^{15}$N-NMR Intensities* I[a] *during Reaction* a) *of Na$^{15}$NO$_2$* (0.05M) *with $H_2O_2$* (1M), b) *of Na$^{15}$NO$_2$* (0.05M) *with $H_2O_2$* (1M) *and $NaHCO_3$* (50 mM), *and* c) d) *of Na$^{15}$NO$_2$* (0.05M) *with $H_2O_2$* (1M), *Tyrac* (0.05M) *and $NaHCO_3$* (50 mM), c) *at pH 4.7 and* d) *at pH 5.25 and 295 K*

*a*) Na$^{15}$NO$_2$/H$_2$O$_2$ system

| $t$[b] | 0 | 1 | 2 | 4 | 5 | 6 | 7 | 10 |
|---|---|---|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | 7 | – | −5 | −7 | −7 | – | – | – |
| $I(^{15}NO_3^-)$ | – | 10 | 10 | 7 | – | −4 | 2 | 8 |
| $I(^{15}NO_4^-)$ | – | −4 | −4 | −7 | −4 | – | – | – |

*b*) Na$^{15}$NO$_2$/H$_2$O$_2$/NaHCO$_3$ system

| $t$[b] | 0 | 1 | 2 | 4 | 7 | 10 | 20 | 30 |
|---|---|---|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | 7 | −12 | −15 | −6 | −4 | −2 | – | – |
| $I(^{15}NO_3^-)$ | – | 320 | 320 | 220 | 120 | 40 | 10 | 7 |
| $I(^{15}NO_4^-)$ | – | −20 | −16 | −10 | −4 | −2 | – | – |

*c*) Na$^{15}$NO$_2$/H$_2$O$_2$/Tyrac/NaHCO$_3$ system at pH 4.7

| $t$[b] | 0 | 1 | 2 | 3 | 5 | 6 | 8 | 9 | 14 | 20 | 28 | 35 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | 2 | −16 | −8 | −8 | −8 | −6 | −3 | −2 | – | – | – | – | – |
| $I(^{15}NO_3^-)$ | – | 400 | 400 | 260 | 100 | 80 | 40 | 25 | 20 | 10 | 6 | 6 | 6.0[c] |
| $I(^{15}NO_4^-)$ | – | −8 | −5 | −2 | – | – | – | – | – | – | – | – | – |
| $I$(3-$^{15}$NO$_2$-Tyrac) | – | −50 | −40 | −15 | −12 | −5 | −3 | −1 | – | – | – | – | 0.28[c] |
| $I$(1-$^{15}$NO$_2$-Tyrac) | – | −15 | −12 | −5 | −5 | −2 | −1 | – | – | – | – | – | – |

*d*) Na$^{15}$NO$_2$/H$_2$O$_2$/Tyrac/NaHCO$_3$ system at pH 5.25

| $t$[b] | 0 | 2 | 7 | 12 | 17 | 22 | 27 | 32 | 42 | 52 | 62 | 72 | 100 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $I(^{15}NO_2^-)$ | 5 | −5 | −2 | −1 | −1 | – | – | – | – | – | – | – | – |
| $I(^{15}NO_3^-)$ | – | 40 | 80 | 130 | 100 | 100 | 100 | 80 | 50 | 30 | 20 | 10 | 7 |
| $I$(3-$^{15}$NO$_2$-Tyrac) | – | −6 | −7 | −15 | −14 | −10 | −9 | −6 | −4 | −2 | – | – | 0.7[d] |
| I(1-$^{15}$NO$_2$-Tyrac) | – | −2 | −2 | −3 | −3 | −3 | −3 | −2 | −2 | −1 | – | – | – |

[a] Determined from the signal-to-noise ratios by using single 90° pulses. [b] $t$ = Time [min] after mixing the reactants and putting the tube into the probe of the $^{15}$N-NMR spectrometer. [c] From $^{15}$N-NMR spectra taken after reaction (466 scans, delay time 3 min). [d] From $^{15}$N-NMR spectra taken after reaction (51 scans, delay time 5 min) [18].

$E_{calc} = -1260$ and $E_{calc} = -1280$ are obtained which describe the experimental values well. For calculating the radical pair $[CO_3^{\bullet-}, {}^{15}NO_2^{\bullet}]^S$, it is taken into account that the radicals react to '*c*' products with a yield of 70% demanding $\Lambda = 0.7$ which gives $\lambda = 0.66$ if all the other parameters are not changed. It follows $E_{calc} = 370$ which is in good agreement with the experimental data determined at pH 4.0, 4.7, and 5.25, respectively. Thus, it can be safely deduced that the peroxynitrite-dependent yield of nitrate is exclusively achieved *via* nitrogen dioxide mediated radical reactions in the presence of CO$_2$. *E* Values found at pH 7.5 and 7.6 are smaller. Because of lower CO$_2$ concentrations, peroxynitrite might in part decay without formation of the peroxynitrite–CO$_2$ adduct. *E* Values might also be reduced by superposition of the enhanced absorption with the emission-type $^{15}$N-CIDNP effect built up in radical pairs $[O_2{}^{15}NOOH^{\bullet-}, {}^{15}NO_2^{\bullet}]^S$ (*Scheme 8*) which was not quantified.

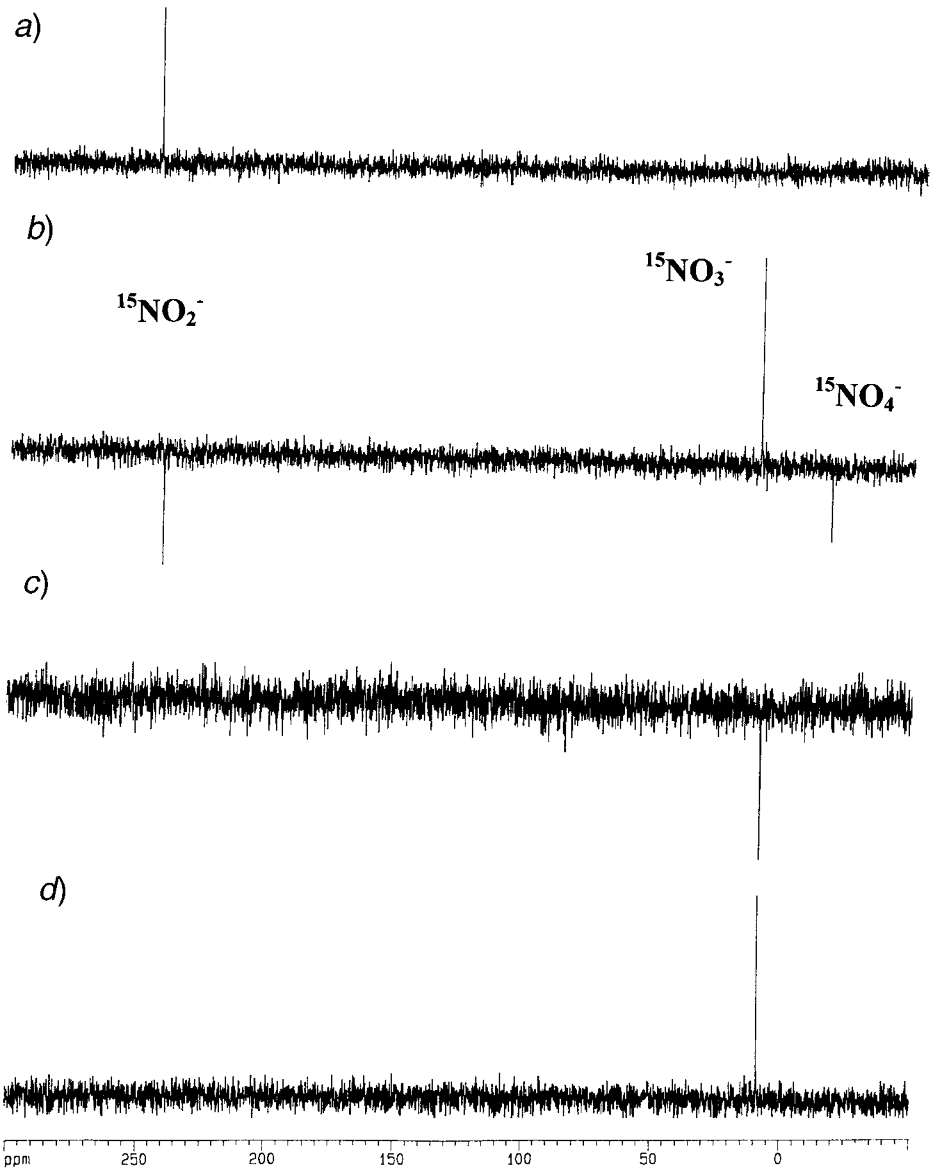


Fig. 4. *$^{15}$N-NMR Spectra of a solution of $Na^{15}NO_2$ at pH 4.7 and 298 K taken* a) *before adding $H_2O_2$,* b) *2 min,* c) *6 min, and* d) *10 min after adding $H_2O_2$* (1 scan)

It was concluded that the emission observed in the $^{15}$N-NMR signal of $^{15}NO_2^-$ might be an '*e*'-type effect built up in radical pairs $[CO_3^{\bullet-}, {}^{15}NO_2^{\bullet}]^S$ as well of '*c*' type generated in radical pairs $[O_2{}^{15}NOOH^{\bullet-}, {}^{15}NO_2^{\bullet}]^S$. It was not attempted to discriminate between the two different origins of $^{15}$N-CIDNP either. Furthermore, '*e*'-type polarizations

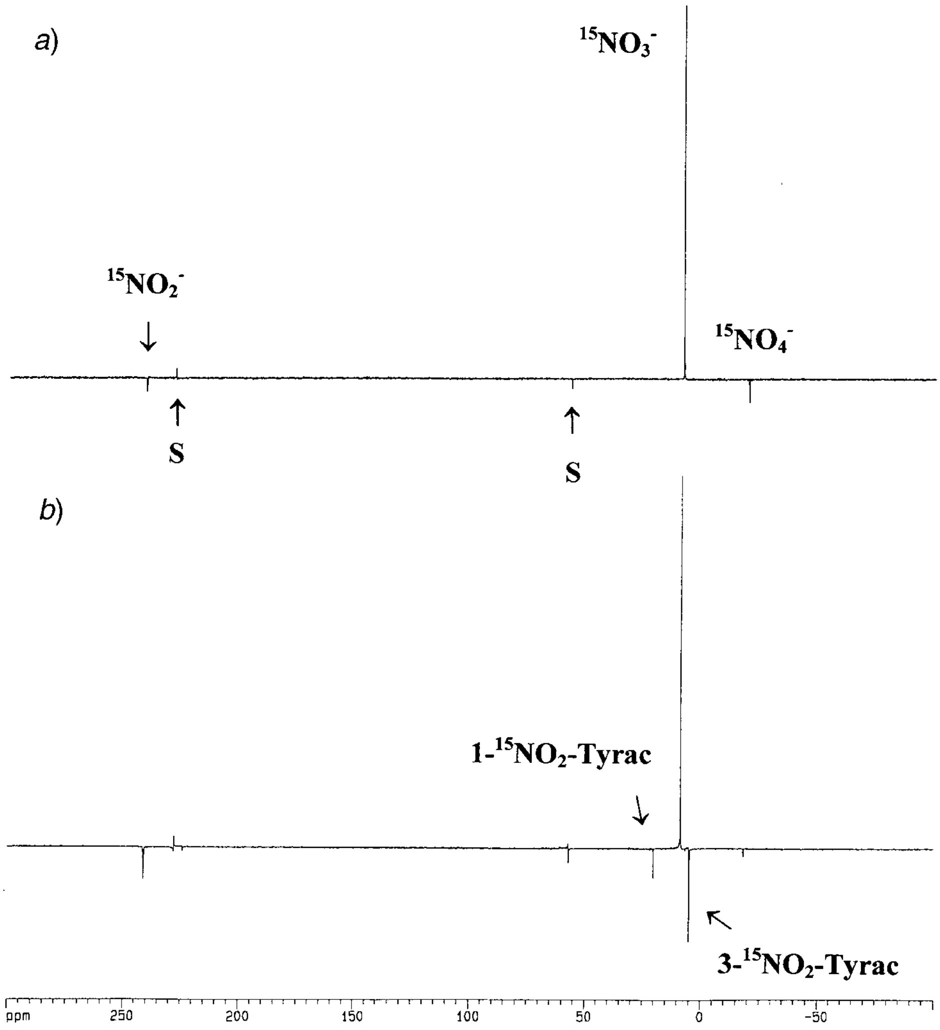


Fig. 5. *$^{15}N$-NMR Spectra of a solution of Na$^{15}NO_2$ and NaHCO$_3$ at pH 4.7 and 298 K* a) *1 min after adding $H_2O_2$, and* b) *with Tyrac 1 min after adding $H_2O_2$* (1 scan). S: Satellites.

might be lowered because of nuclear relaxation in radicals [24]. Because of this, *E* values of the $^{15}$N-NMR signal of $^{15}NO_2^-$ were neither determined nor calculated.

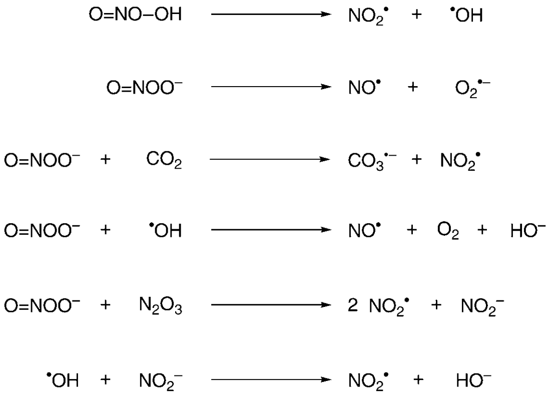
**5. Conclusions.** – During the decay of peroxynitrite in aqueous solution, various $^{15}$N-CIDNP effects are observed depending on reaction conditions like temperature, pH value, peroxynitrite concentration, and the immediate proximity of additional reactants like $CO_2$ or $NO_2^-$. The nuclear polarizations indicate the presence of radicals formed by direct or induced decomposition of peroxynitrite (*Scheme 9*). The $^{15}$N-CIDNP effects

Table 7. *$^{15}$N-CIDNP Enhancement Factors* E *and* E$_{calc}$ *during Peroxynitrite Decay and Nitration in the Presence of* $CO_2$

| System | pH or solvent | Compound | $T_1$/s | $E$ or $E_{calc}$ | Ref. |
|---|---|---|---|---|---|
| $O{=}^{15}NOO^-$ | 7.6a) | $^{15}NO_3^-$ | | *ca.* 0 | [15] |
| $O{=}^{15}NOO^-$/Tyrac | 7.6b) | $^{15}NO_3^-$ | 90 | 60 | |
| $O{=}^{15}NOO^-$/Tyrac | 12.5 | $^{15}NO_3^-$ | 90 | 110 | [15] |
| $O{=}NOO^-/Na^{15}NO_2$/Tyrac | 7.6 | $^{15}NO_3^-$ | | > – 10 | |
| | | 3-$^{15}NO_2$-Tyrac | | > – 400 | |
| $H_2O_2$/($^{15}$N)NANT | 7.5 | $^{15}NO_3^-$ | 90 | 110 | |
| $H_2O_2$/($^{15}$N)NANT/Tyrac | 4.0 | $^{15}NO_3^-$ | 90 | 310 | |
| | | 3-$^{15}NO_2$-Tyrac | 24 | – 1430 | |
| $H_2O_2/Na^{15}NO_2$ | 5.25c) | $^{15}NO_3^-$ | 90 | 290 | [17] |
| $H_2O_2/Na^{15}NO_2$ | 5.25d) | $^{15}NO_3^-$ | 90 | 320 | [17] |
| $H_2O_2/Na^{15}NO_2$/Tyrac | 5.25 | $^{15}NO_3^-$ | 90 | 430 | |
| | | 3-$^{15}NO_2$-Tyrac | 24 | – 1520 | [18] |
| $H_2O_2/Na^{15}NO_2$ | 4.7e) | $^{15}NO_3^-$ | | ~0 | |
| $H_2O_2/Na^{15}NO_2$ | 4.7 | $^{15}NO_3^-$ | 90 | 240 | |
| $H_2O_2/Na^{15}NO_2$/Tyrac | 4.7 | $^{15}NO_3^-$ | 90 | 260 | |
| | | 3-$^{15}NO_2$-Tyrac | 24 | – 1740 | |
| $H^{15}NO_3$/phenol | AcOH | 2-($^{15}$N)nitrophenol | 90 | – 1180 | [23] |
| $H^{15}NO_3$/Tyrac | AcOH | 3-$^{15}NO_2$-Tyrac | 16 | – 1440 | [18] |
| $[PhO^\bullet, {}^{15}NO_2^\bullet]^F$ | AcOH | '*c*' product | | – 1260f) | [23] |
| $[Tyrac^\bullet, {}^{15}NO_2^\bullet]^F$ | AcOH | '*c*' product | | – 1260f) | [23] |
| | $H_2O$ | '*c*' product | | – 1280f) | |
| $[CO_3^{\bullet-}, {}^{15}NO_2^\bullet]^S$ | $H_2O$ | '*c*' product | | 370f) | |

a) *T ca.* 273 K. b) *T* 268 K. c) Without addition of $NaHCO_3$. d) In the presence of $NaHCO_3$. e) In the absence of $CO_2$. f) Calculated by using *Pedersen*'s treatment [25].

Scheme 9. *Formation of Radicals during Decay of Peroxynitrite*



are partially generated during decay and formation of peroxynitric acid, a reactive intermediate during the peroxynitrite decay (*Scheme 8*).

In the presence of *N*-acetyl-L-tyrosine, the life-time of peroxynitrite is prolonged because of the decrease of nitrogen dioxide and subsequently of $N_2O_3$. As a consequence of this trapping, the nitration product 3-nitro-*N*-acetyl-L-tyrosine is formed showing $^{15}$N-CIDNP in the $^{15}$N-NMR signal of the nitro group which indicates the formation by recombination of the radicals Tyrac$^\bullet$ and $NO_2^\bullet$ (*Scheme 10*). Quantitative $^{15}$N-CIDNP investigations in the presence of $CO_2$ and Tyrac show that radical reactions are the most important pathways leading to $NO_3^-$ and 3-$NO_2$-Tyrac. Thus, there is no need to assume reaction mechanisms other than the radical-derived ones to account for the peroxynitrite-mediated chemistry in the presence of physiological $CO_2$ concentrations.

Scheme 10. *Formation of $NO_3^-$ and 3-$NO_2$-Tyrac* via *Radicals*

$$CO_3^{\bullet -} + NO_2^\bullet \longrightarrow CO_2 + NO_3^-$$

$$^\bullet OH_2 + \text{Tyrac} \longrightarrow H_2O + \text{Tyrac}^\bullet$$

$$NO_2^\bullet + \text{Tyrac} \longrightarrow HNO_2 + \text{Tyrac}^\bullet$$

$$CO_3^{\bullet -} + \text{Tyrac} \longrightarrow HCO_3^- + \text{Tyrac}^\bullet$$

$$NO_2^\bullet + \text{Tyrac}^\bullet \longrightarrow \text{3-}NO_2\text{-Tyrac}$$

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