The Superoxide Radical Reacts with Tyrosine-derived Phenoxyl Radicals by Addition rather than by Electron Transfer

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Radiolytically generated azide radicals have been used for the formation of tyrosyl radical, TyrO$^{\bullet}$, from tyrosine. The TyrO$^{\bullet}$ radicals combine ($2k = 4.5 \times 10^8$ dm^3 mol^{-1} s^{-1}, determined by pulse radiolysis) yielding bityrosine in a > 90% yield. Bityrosine formation is not suppressed in the presence of oxygen [k(TyrO$^{\bullet}$ + O_2) < 1×10^3 dm^3 mol^{-1} s^{-1}].

When TyrO$^{\bullet}$ and $O_2^{\bullet -}$ radicals are generated side by side in a 1:1.2 ratio, bityrosine formation is strongly suppressed and (2*S*,3a*R*,7a*S*)- and (2*S*,3a*S*,7a*R*)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indole-2-carboxylic acids **10** become the major final products. Their hydroperoxidic precursor is only short-lived ($t_{\frac{1}{2}}$ = 4.2 h at room temperature and pH 8). Upon its decay H_2O_2 is released. Product **10** is believed to be formed by the addition of $O_2^{\bullet -}$ to the *ortho*- and *para*-position of the phenoxyl radical, followed by protonation, ring closure and hydrolysis.

Based on material balance considerations an electron transfer from $O_2^{\bullet -}$ to TyrO$^{\bullet}$, although thermodynamically feasible, must play a minor role ($\leqslant$10%). The rate constant k($O_2^{\bullet -}$ + TyrO$^{\bullet}$) has been determined by pulse radiolysis to be 1.5×10^9 dm^3 mol^{-1} s^{-1}.

In many free-radical reactions addition reactions dominate over electron transfer reactions despite the fact that the redox potential is high enough to allow an electron transfer to occur. A typical example is the OH-radical. Its redox-potential[1] at pH 7 is 1.8 V yet in its reactions with compounds whose redox potentials are much lower than this value it adds to C=C double bonds or abstracts an H-atom rather than undergoing electron transfer.

At pH 7 the redox potentials of the superoxide radical anion, $O_2^{\bullet -}$, and of the tyrosine-derived phenoxyl radical, TyrO$^{\bullet}$ **2**, are −0.33 V and +0.93 V, respectively.[1] Thus the electron transfer from $O_2^{\bullet -}$ to TyrO$^{\bullet}$ [*cf.* reaction (10)] is thermodynamically favoured and, previously, has always been considered to be the major reaction in their interaction.[2–4] However, it will be shown in this paper that these two radicals mainly add to one another, and that electron transfer must be a very minor process if it occurs at all.

Experimental

L-Tyrosine (Janssen), sodium azide (Merck) and *N,O*-Bis-(trimethylsilyl)trifluoracetamide (BSTFA; Macherey-Nagel) were used as received. Nitrous oxide (Messer-Griesheim) was freed from remaining traces of oxygen by passage through an Oxisorb column (Messer-Griesheim). A mixture of N_2O–O_2 (4:1) and pure O_2 was obtained from the same supplier.

Aqueous solutions containing tyrosine (5×10^{-4} mol dm^{-3}) and sodium azide (2×10^{-2} mol dm^{-3}) were made up in Milli-Q-filtered (Millipore) water and saturated with either N_2O, N_2O–O_2 or O_2 and γ-irradiated in a panorama ^{60}Co-γ-source (Nuclear Engineering) at a dose rate of 0.24 Gy s^{-1}.

Determination of the products was by HPLC (pump: Merck-Hitachi Model L-6200; detector: Merck-Hitachi Photodiode Array Detector Model L-3000) on a 250 × 4.6 mm Nucleosil-5-C_{18} column (Merck) using water as the eluent. For NMR spectroscopy, material (20–30 mg of each product) was isolated by repetitive HPLC (pump: Gilson Model 303; detector: Spectra-Physics Model 770, fraction collector: Gilson Model 201) on a 250 × 20 mm Nucleosil-7-C_{18} column (Macherey-Nagel) using water–methanol 97:3 (v/v) as the eluent. Detection was at 230 nm. For the determination of peroxidic material Allens reagent[5] was mixed with the column effluent with the help of a Merck-Hitachi reaction pump (Model 655A-13). The retention times as shown in Figs. 2 and 3 (see below) are prolonged, since a reaction coil was added. In the absence of such a reaction coil retention times of **10**, L-tyrosine and bityrosine **5** were 4.2 min, 9 min and 13 min, respectively.

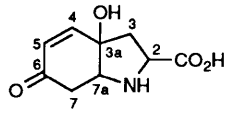
The NMR spectra were taken on a 400 MHz instrument (Bruker AM 400) using tetramethylsilane (TMS) as the standard. The NMR data for (2*S*,3a*R*,7a*S*)- and (2*S*,3a*S*,7a*R*)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indole-2-carboxylic acids **10** were obtained from a mixture of the two stereoisomers and are presented in Table 1.

The data, backed up by ^{13}C-DEPT and ^{13}C/^{1}H-COSY experiments, are consistent with constitution of **10** for both isomers. After several days at room temperature, both 7-H were

Table 1 NMR data of (2*S*,3a*S*,7a*R*)- and (2*S*,3a*R*,7a*S*)-3-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indole-2-carboxylic acids **10** (43:57 mixture) obtained by ^{1}H and ^{13}C NMR spectroscopy (^{13}C/^{1}H-COSY and DEPT, in D_2O) (minor isomer/major isomer)

Position	Signal	δ	J_{H-H}	
2	dd	4.25/4.38	$J^3_{2,3\alpha}$ = 5.0/9.0	$J^3_{2,3\beta}$ = 10.0/8.0
3α	dd	2.45/2.38	$J^3_{3\alpha,2}$ = 5.0/9.0	$J^2_{3\alpha,3\beta}$ = 14.0/14.0
3β	dd	2.66/2.64	$J^3_{3\beta,2}$ = 10.0/8.0	$J^2_{3\beta,3\alpha}$ = 14.0/14.0
4	d	6.89/6.91	$J^3_{4,5}$ = 10.3/10.3	
5	d	5.99/6.03	$J^3_{5,4}$ = 10.3/10.3	
7α	dd	2.92/2.90	$J^3_{7\alpha,7a}$ = 6.0/6.0	$J^2_{7\alpha,7\beta}$ = 17.0/17.2
7β	dd	2.72/2.70	$J^3_{7\beta,7a}$ = 10.5/10.0	$J^2_{7\beta,7\alpha}$ = 17.0/17.2
7a	dd	4.11/4.20	$J^3_{7a,7\alpha}$ = 6.0/6.0	$J^3_{7a,7\beta}$ = 10.5/10.0

Position	DEPT	δ
2	CH	66.6/67.1
3	CH_2	42.6/42.8
3a	C	78.2/79.5
4	CH	152.3/152.4
5	CH	131.4/131.6
6	C	199.8/199.9
7	CH_2	39.8/40.7
7a	CH	62.3/63.4
8	C	176.5/176.5



replaced by deuterium. The stereochemical assignments were derived as follows. The three proton–proton coupling constants exhibited by 7α-H, 7β-H and 7a-H are virtually identical for both stereoisomers. Given the known strong dependence of these parameters on slight changes of ring conformation, this indicates identical relative configurations at 3a/7a (either *cis* or *trans* arrangement of OH and H) for both isomers. Given the known 2*S* configuration for both isomers, this means that the ring systems, disregarding the carboxy group, are identical for both isomers but of opposite chirality. The high $J^3_{7a,7\beta}$ value of 10.5/10.0 Hz indicates a purely diaxial *trans* arrangement of these two protons. For the *trans* relative configuration of 3a-OH and 7a-H, there is only one possible conformation for the cyclohexenone ring and this conformation features the required diaxial arrangement. For the *cis* relative configuration, two similarly probable low-energy conformations are possible, one of them featuring the required diaxial arrangement but the other one featuring only *gauche* arrangements for which J^3 values are known to be ≤6.5 Hz. Since an equilibrium between the two conformations leading to averaged *J* values would exist which, moreover, would have somewhat different positions for the two isomers, the *cis* relative configuration can be dismissed. The same conclusion is reached from the high absolute value of 17.0/17.2 Hz for $J^3_{7\alpha,7\beta}$ which indicates the carbonyl plane bisecting the 7α-H/C-7/7β-H angle,[6,7] a feature realized only for the *trans* but for neither conformer of the *cis* relative configuration. An assignment of the (2*S*,3a*R*,7a*S*) and (2*S*,3a*S*,7a*R*) configurations thus derived to the individual isomers has not been carried out.

For analysis by gas chromatography–mass spectroscopy (Hewlett-Packard Model HP5890 II; column: 15 m PS-343.5, injection temperature 200 °C, temperature-programmed 50–250 °C, 6° min^{-1}; Hewlett-Packard MSD 5971A) samples were brought to dryness by rotary evaporation and trimethylsilylated with BSTFA in pyridine prior to injection. The tri-TMS ethers of the two isomers of **10** (*M* = 413 dalton) eluted at 33.8 and 34.1 min. Their mass spectra are characterized by *m/z* (%): 413 (1), 398 (2), 370 (2), 323 (2), 296 (100), 280 (1), 269 (2), 206 (13), 147 (7), 73 (58). The hexa-TMS ether of bityrosine (*M* = 792 dalton) eluted at 37.3 min. Its mass spectrum is characterized by *m/z*(%) 777 (1), 749 (4), 676 (7), 575 (71), 218 (100) and 73 (61).

For pulse radiolysis a 2.8 MeV van de Graaff electron accelerator (High Voltage Engineering) delivering pulses of 0.4–4 μs duration was used.[8] Dosimetry was done as reported earlier.[9]

Results and Discussion

TyrO$^\bullet$ and $O_2^{\bullet -}$ have been generated radiolytically (*cf.* Ref. 10). Using (*a*) N_2O-, (*b*) N_2O–O_2 (4:1)- or (*c*) O_2-saturated aqueous solutions of tyrosine, Tyr **1**, (5×10^{-4} mol dm^{-3}) in the presence of azide ions (2×10^{-2} mol dm^{-3}) conditions prevail[11] where essentially only TyrO$^\bullet$ radicals **2** (*a*,*b*) are generated,[12] either in the absence (*a*) or in the presence of oxygen (*b*), or TyrO$^\bullet$ and $O_2^{\bullet -}$ in a molar ratio of about 1:1.2 (*c*). The main reactions are represented by reactions (1)–(6).

$$H_2O \xrightarrow[\text{radiation}]{\text{ionizing}} e^-_{aq}, {}^\bullet OH, H^\bullet, H^+, H_2O_2, H_2 \quad (1)$$

$$e^-_{aq} + N_2O \longrightarrow {}^\bullet OH + N_2 + OH^- \quad (2)$$

$$e^-_{aq} + O_2 \longrightarrow O_2^{\bullet -} \quad (3)$$

$$H^\bullet + O_2 \longrightarrow HO_2{}^\bullet (O_2^{\bullet -} + H^+) \quad (4)$$

$${}^\bullet OH + N_3^- \longrightarrow OH^- + N_3^\bullet \quad (5)$$

The radiation chemical yields (*G* values) for TyrO$^\bullet$ are $G(TyrO^\bullet) \approx 5.6 \times 10^{-7}$ mol J^{-1} under conditions (*a*) and (*b*) and $G(TyrO^\bullet) = 2.8 \times 10^{-7}$ mol J^{-1}, $G(O_2^{\bullet -}) = 3.3 \times 10^{-7}$ mol J^{-1} under condition (*c*).

Under conditions (*a*) and (*b*) bityrosine **5** is the only product detected. Since $G(\mathbf{5}) = 2.6 \times 10^{-7}$ mol J^{-1} (*cf.* Fig. 1) is close to 1/2 $G(\mathbf{2})$, the other potential products listed in ref. 2 must be of minor importance. The intermediates **3** and **4** are very short-lived [reactions (8) and (9)] since such cyclohexadienones undergo rapid enolization.[2,13,14]

It has been reported before that TyrO$^\bullet$ radicals **2** do not react with oxygen.[4] This is in good agreement with our data. From our value of $2k_7 = 4.5 \times 10^8$ dm^3 mol^{-1} s^{-1} (see below) and the dose rate of our experiments (0.24 Gy s^{-1}) it is calculated that an upper limit is set at $k(\mathbf{2} + O_2) < 1 \times 10^3$ dm^3 mol^{-1} s^{-1}, in good agreement with an earlier report.[4]

In O_2-saturated solutions, conditions (*c*), the yield of bityrosine **5** is greatly reduced, $G(\mathbf{5}) = 0.26 \times 10^{-7}$ mol J^{-1}. The major final products are now (2*S*,3a*R*,7a*S*)- and (2*S*,3a*S*,7a*R*)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indole-2-carboxylic acid **10** (for their identification by NMR and mass spectrometry see the Experimental section) which are formed with a *G* value of $G(\mathbf{10}) = 2.1 \times 10^{-7}$ mol J^{-1} (Fig. 1), *i.e.*, there is again a very good material balance. This leaves very little room (in the order of 10%) for the electron-transfer reaction (10).

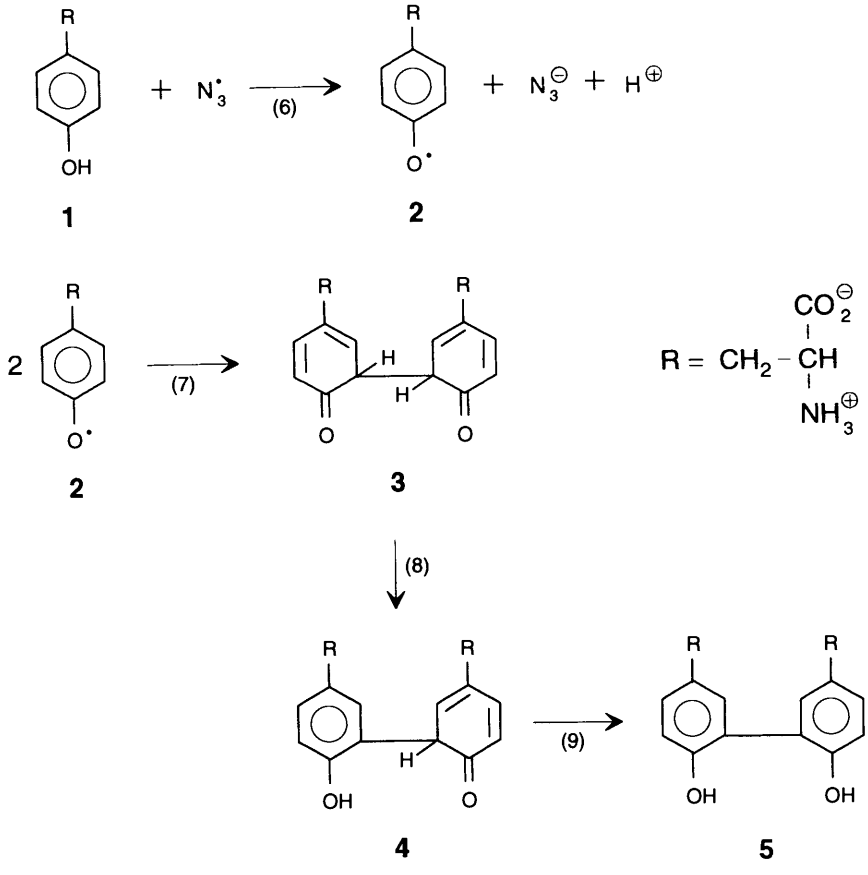
Right after the irradiation, besides bityrosine **5** (eluting after tyrosine, not shown in Fig. 2), further material with a retention time of 10.5 min has been identified by HPLC (Fig. 2). When this HPLC analysis was carried out in the post-column derivatization mode using Allen's reagent[5] (essentially KI) for the detection of H_2O_2 and other hydroperoxides (Fig. 3) two hydroperoxides were detected: hydrogen peroxide (retention time 8.3 min) which elutes together with the azide ion and an organic hydroperoxide eluting at 10.5 min, *i.e.*, at the same position as the new product shown in Fig. 2.

The peak with a retention time of 10.5 min is attributed to the two (non-resolved) hydroperoxides **8** and/or **9**. This hydroperoxidic material is unstable and decays into **10** which elutes at 9.5 min (*cf.* inset in Fig. 2). The NMR data shown in Table 1 clearly indicate that the peak at 9.5 min is in fact due to two (non-resolved) products, the two isomers of **10**. As TMS-derivatives these two isomers can, however, be separated well by analytical gas chromatography (see the Experimental section).

At room temperature the peroxidic material decays by first-order kinetics with a half-life of 4.2 h (Fig. 4). While the organic hydroperoxide(s) decay(s) the yield of hydrogen peroxide increases (*cf.* inset in Fig. 3).

The proposed mechanism for the formation of **8**, **9** and **10** is shown by the reactions (12)–(17). The superoxide radicals add to the *para*- and/or *ortho*-positions of the TyrO$^\bullet$ radical. The initially formed anions of 4-alanyl-4-hydroperoxycyclohexa-2,5-dienone and 4-alanyl-2-hydroperoxycyclohexa-3,5-dienone will be rapidly protonated by water [pK_a values ≥ 11, *cf.* p$K_a(H_2O_2)$ = 11.7] resulting in the hydroperoxides **6** and **7** [reactions (12) and (13)]. Enones are known to undergo with amines the Michael-addition-type reactions (14) and (15). Such a reaction can be very rapid indeed (*cf.* ref. 15). The hydrolysis of **8** and **9** leads to **10** and H_2O_2 [reactions (16) and (17)]. The mechanism of this hydrolysis has not been studied, but if it proceeds *via* a carbocation (protonation of the hydroperoxide by water) a common intermediate would be reached from **8** as well as from **9**. Its reaction with water (OH^-) would lead to **10** which is thermodynamically favoured over its non-conjugated isomer with the OH group in α-position to the carbonyl function.

A possible mechanism of this hydrolysis is shown in reactions (18)–(23). Hydration of the carbonyl function [reactions (18) and (20)] would facilitate the elimination of HO_2^- [reactions



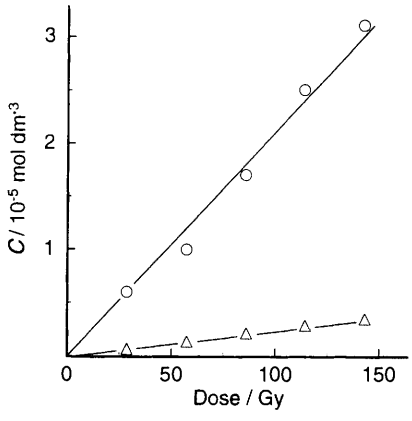


Fig. 1 Formation of (2*S*,3a,*R*,7a*S*)- and (2*S*,3a*S*,7a*R*)-3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1*H*-indole-2-carboxylic acid **10** (○) and bityrosine **5** (△) in the γ-radiolysis of L-tyrosine in O_2-saturated aqueous solution in the presence of 2 × 10^{-2} mol dm^{-3} azide at pH 8 and a dose rate of 0.24 Gy s^{-1} as a function of dose

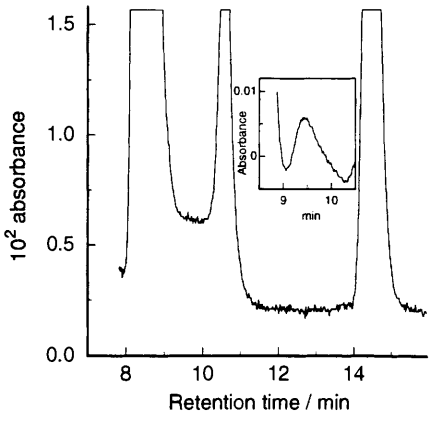
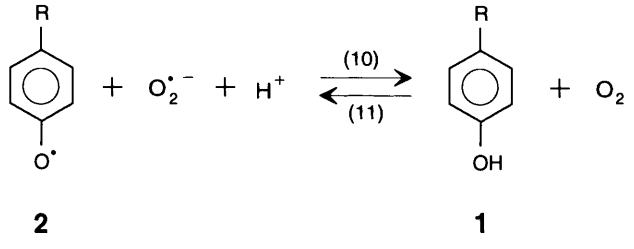


Fig. 2 HPLC chromatogram of a γ-irradiated O_2-saturated aqueous solution of L-tyrosine (conditions as in Fig. 1) on a 250 × 4.6 mm Nucleosil-5-C_{18} column with water as the eluent. Retention times prolonged due to the presence of a reaction coil (*cf.* Fig. 3): peak at 8.8 min, azide ion; 10.5 min, attributed to **8**/**9**; 14.5 min, L-tyrosine. Inset: elution profile of **10**, t_R 9.5 min, formed after the decay of the peroxidic material at t_R 10.5 min.



(19) and (21)], a group not readily replaced in bimolecular nucleophilic substitution reactions. The regio- and stereospecific introduction of OH into the molecule occurs most likely *via* an intermediate 1,4-epoxide (for a similar 1,2-epoxidation reaction see, *e.g.*, ref. 16). It is noted that the stereoisomeric 1,4-epoxide with 7a-H *cis* rather than *trans* to 3a-O would be prohibitively strained and thus is not formed.

It has been shown above that hydrogen peroxide is released during the decay of the organic hydroperoxide(s). Organic hydroperoxides often react slowly with Allen's reagent. This prevents their quantitative determination by post-column reaction unless reference material is available. Because of the short lifetime of the peroxidic material, isolation of enough material for this purpose was not possible in the present case. However, hydrogen peroxide reacts sufficiently rapid with Allen's reagent and its yield can be quantified by this technique. Prior to the decay of the organic hydroperoxide(s) hydrogen peroxide has been formed in reaction (1) and in the dismutation

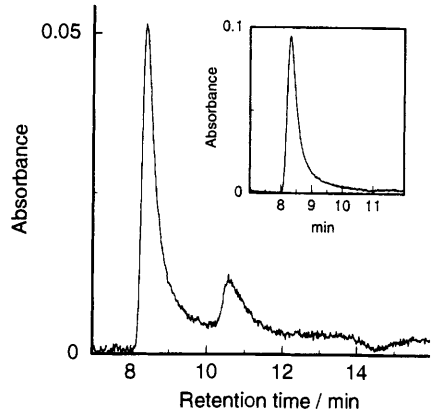


Fig. 3 HPLC chromatogram of a γ-irradiated O_2-saturated aqueous solution of L-tyrosine (conditions as in Fig. 2). Post-column detection of hydroperoxides using Allen's reagent: peak at 8.5 min, hydrogen peroxide; 10.5 min; attributed to **8**/**9**. Inset: chromatogram after the decay of the organic hydroperoxide(s) at t_R 10.5 min.

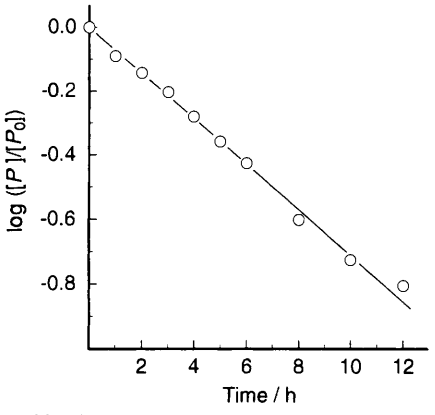


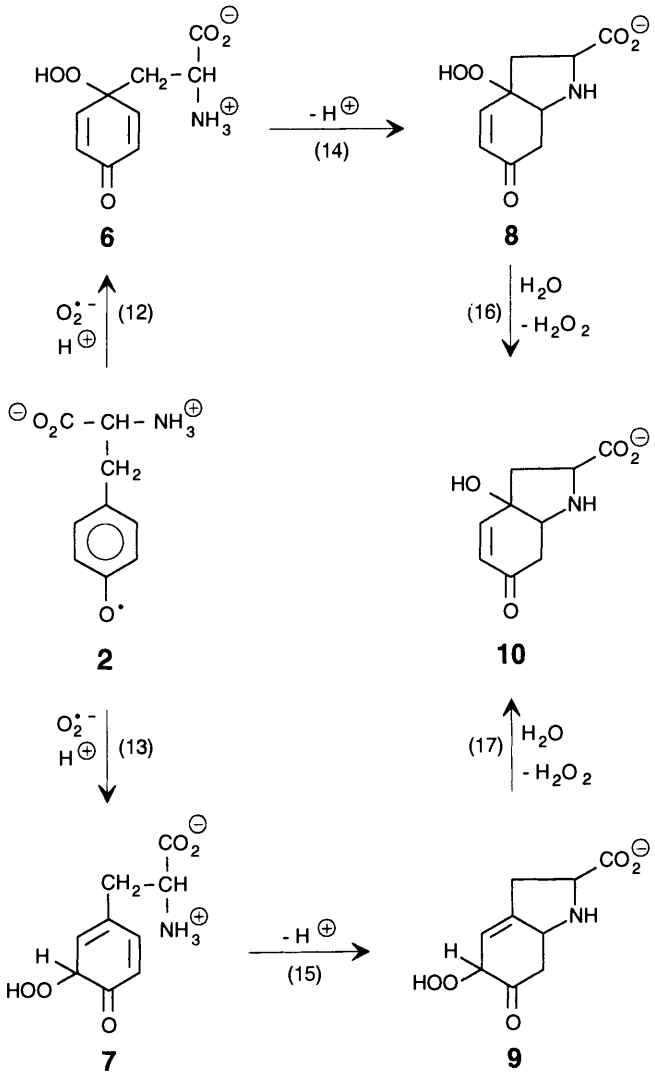
Fig. 4 Decay kinetics of the organic hydroperoxide(s) attributed to **8**/**9** at room temperature (*ca.* 20 °C, 2 × 10^{-2} mol dm^{-3} azide, pH 8)

Table 2 Rate constants for the self-termination of TyrO• radicals and their reaction with $O_2^{\bullet -}$ as determined by pulse radiolysis

Reaction	$2k$/dm^3 mol s^{-1}	Reference
TyrO• + TyrO•	1.2 × 10^9	18
	4.0 × 10^8	4
	6.5 × 10^8	3
	4.5 × 10^8	This work
TyrO• + O_2	< 1 × 10^3	4
	< 1 × 10^3	This work
TyrO• + $O_2^{\bullet -}$	1.7 × 10^9	3
	1.5 × 10^9	This work
Gly-TyrO• + $O_2^{\bullet -}$	~ 10^9	2

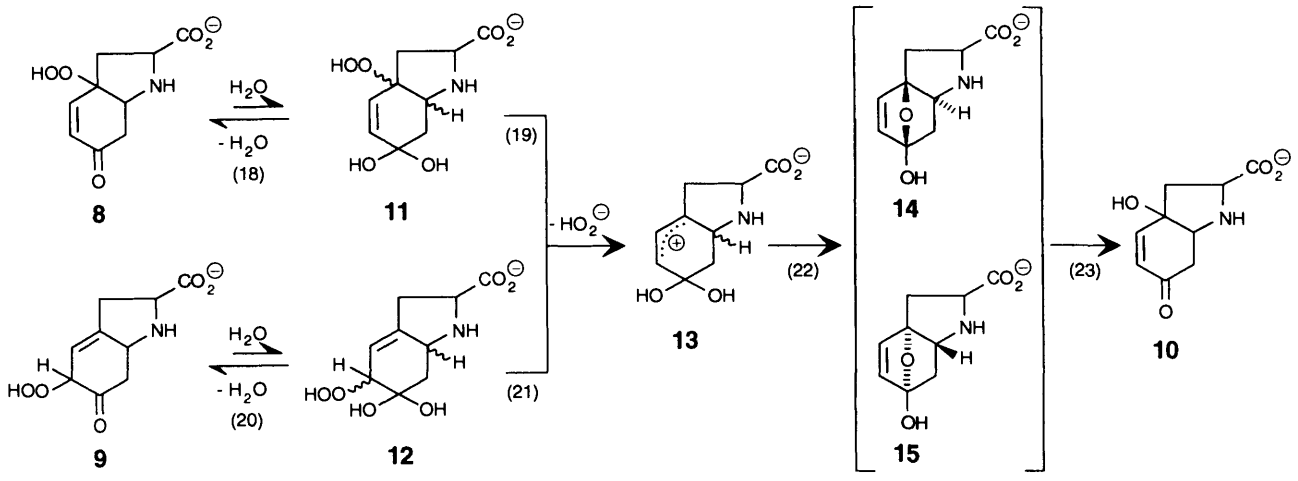
of $HO_2^{\bullet}/O_2^{\bullet -}$ which has not been scavenged by the TyrO• radicals. Upon hydrolysis of **8**/**9** the yield of hydrogen peroxide should increase by a factor of approximately 2.5 [primary yields result from reaction (1), G = 0.8 × 10^{-7} mol J^{-1}, and from the disproportionation of the $HO_2^{\bullet}/O_2^{\bullet -}$ radicals which have not reacted with the TyrO• radicals]. An approximate twofold increase can be seen from the data shown in Fig. 3. Considering that the organic hydroperoxide(s) must have already, to some extent, decayed and hence have produced hydrogen peroxide during irradiation and the time that elapsed between irradiation and analysis, this is a reasonable value, since only some 15% decay prior to analysis is required to bring this ratio close to the observed value.

In a previous study the reaction of the TyrO• radical with



$O_2^{\bullet -}$ has already been studied by pulse radiolysis and a rate constant k(TyrO• + $O_2^{\bullet -}$) = 1.7 × 10^9 dm^3 mol^{-1} s^{-1} has been obtained.[3] This value is much higher than one would calculate on the basis of the known rate constant of $O_2^{\bullet -}$ decay at pH 8.0,[17] the bimolecular rate constant of TyrO• reaction and the yields of bityrosine **5** and **10** at the dose rate of our experiments. The determination of rate constants of $O_2^{\bullet -}$ on the basis of steady-state assumptions in low-dose-rate experiments are always fraught with potential errors since adventitious transition metal ions such as copper ions may speed up $O_2^{\bullet -}$ dismutation and thus reduce the steady-state concentration of $O_2^{\bullet -}$. We have hence repeated the pulse radiolysis experiments using a ten-fold excess of $O_2^{\bullet -}$ over the TyrO• concentration. This was achieved by pulse-irradiating an O_2-saturated aqueous solution (pH 9) containing tyrosine (5 × 10^{-4} mol dm^{-3}), azide ions (3 × 10^{-3} mol dm^{-3}) and formate ions (5 × 10^{-2} mol dm^{-3}). Under these conditions the TyrO• radicals (monitored by their absorption at 405 nm) decayed by first-order kinetics. The observed first order rate constant was plotted as a function of the $O_2^{\bullet -}$-concentration generated in the pulse (Fig. 5). From the slope of the straight line in this figure a rate constant k(TyrO• + $O_2^{\bullet -}$) = 1.5 × 10^9 dm^3 mol^{-1} s^{-1} is calculated, in good agreement with the earlier value (*cf.* Table 2).[3]

In the course of these studies we have also determined the value for the self-termination of two TyrO• radicals. In these experiments N_2O-saturated solutions (pH 9) containing tyrosine (10^{-3} mol dm^{-3}) and azide ions (5 × 10^{-2} mol dm^{-3}) were pulse-irradiated. The decay of the TyrO• radicals was



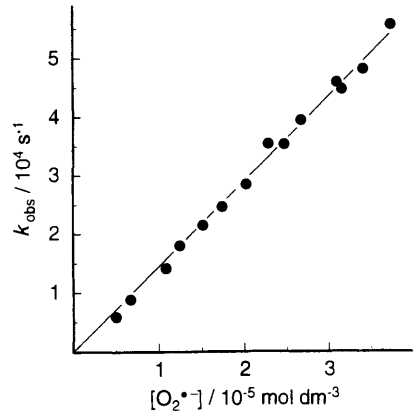


Fig. 5 Pulse radiolysis of an aqueous O_2-saturated solution (pH 9) of tyrosine (5×10^{-4} mol dm^{-3}), azide ions (3×10^{-3} mol dm^{-3}) and formate ions (5×10^{-2} mol dm^{-3}) yielding $[O_2^{\bullet -}] \geqslant 10[TyrO^{\bullet}]$. The rate of decay of the TyrO$^{\bullet}$ absorption at 405 nm is plotted against the initial $O_2^{\bullet -}$ concentration. The $O_2^{\bullet -}$ concentration was varied by varying the dose per pulse.

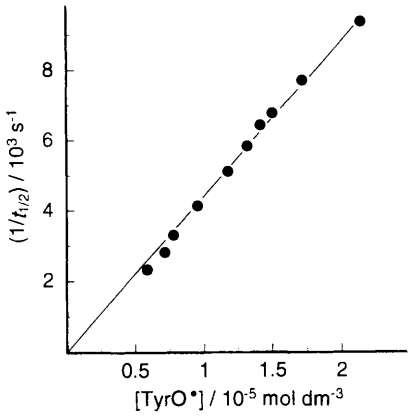


Fig. 6 Pulse radiolysis of an aqueous N_2O-saturated solution (pH 9) of tyrosine (10^{-3} mol dm^{-3}) and azide ions (5×10^{-2} mol dm^3). The inverse of the first half-life of the TyrO$^{\bullet}$ radical decay monitored at 405 nm is plotted against the total radical concentration (90% TyrO$^{\bullet}$ + 10% tyrosine H-adduct radicals).

always of second-order kinetics. A plot of the inverse of the first half-life against the initial radical concentration [90% TyrO$^{\bullet}$ + 10% tyrosine-H-adduct(s)] yielded a straight line (Fig. 6).

From its slope a bimolecular termination of the TyrO$^{\bullet}$ radicals of $2k$(TyrO$^{\bullet}$ + TyrO$^{\bullet}$) = 4.5×10^8 dm^3 mol^{-1} s^{-1} has been calculated. In this calculation the assumption has been made that the decay of the TyrO$^{\bullet}$ radicals is mainly determined by their self-termination and that the H-adduct radicals interact with the TyrO$^{\bullet}$ radicals with approximately the same rate constant. In Table 2 the rate constants for the self-termination of the TyrO$^{\bullet}$ radicals as determined earlier are compared with our value. It can be seen that there is a good agreement between our value and the more recently determined ones.

It is worth mentioning that, among other products, bityrosine and **10** are formed in the reaction of $^1O_2$ with Tyr and also in the direct photolysis of Tyr in the presence of O_2.[19] The above reactions certainly contribute in the reactions on the route to these products.

Note added in proof: M. Jonsson, J. Lind, T. Reitberger, T. E. Eriksen and G. Merényi (*J. Phys. Chem.*, in the press) have measured recently by pulse radiolysis the redox-potentials of 15 different phenoxyl radicals and their rate constants with the superoxide radical. They also come to the conclusion that addition must compete with electron transfer, but from their data (no detailed product study) one would estimate that electron transfer from the superoxide radical to the TyrO$^{\bullet}$ radical should be more prominent than found in the present study.

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