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Autoxidation of tryptophan in aqueous solutions

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Autoxidation of tryptophan was investigated in aqueous solutions by the gamma radiolytic technique. The oxygen uptake and formation of peroxide materials was followed as a function of pH, dose rate and concentration of tryptophan. The results obtained indicate that TrpH(OH)OO· radicals react with tryptophan by adduct formation thus propagating autoxidation. The chain propagation length (*CPL*) for a 2×10^{-2} mol dm^{-3} tryptophan solution at pH 9.5 and a dose rate 0.01 Gy s^{-1} was estimated to be ≈ 5.8, which shows that the autoxidation of tryptophan is a short chain reaction.

Keywords: autoxidation, tryptophan, peroxy radicals.

INTRODUCTION

The autoxidation of many food products, as a result of the direct or indirect action of molecular oxygen, usually proceed through free radical processes.[1] The consequence is deterioration of food which is generally associated with the autoxidation of the major food constituents - fatty and amino acids. Autoxidation of fatty acids is fairly well understood.[2] Less is known about the autoxidation of amino acids which leads to the development of an off flavor in food. Among the amino acids, tryptophan, TrpH, is quite sensitive to autoxidation. An oxygen uptake $G(-O_2) > 6.2$ was measured during the gamma radiolysis of oxygenated solutions of tryptophan.[3] Since $G(-O_2)$ depends on the concentration of tryptophan, it was concluded that short chain autoxidation reactions occur and that in the propagation step a TrpH-peroxy radical reacts with tryptophan. The following reactions were suggested as being probable:

$$\mathrm{TrpH(OH)OO\bullet + TrpH \rightarrow TrpH(OH)OO^- + TrpH^{\cdot +}} \quad (1)$$

$$\rightarrow \mathrm{TrpH(OH)OOH + Trp(-H)H\bullet} \quad (2)$$

$$\rightarrow \mathrm{TrpH(OH)OO - TrpH\bullet} \quad (3)$$

However, it is not clear which of these reactions is responsible for the autoxidation of tryptophan. In this work, using the radiolytic generation of free radicals, the autoxidation of tryptophan in aqueous solution was investigated in more detail.

It is well known that the hydrated electrons formed in the radiolysis of water (reaction 4) are efficiently converted into •OH radicals by reaction with N_2O (reaction 5).[4]

$$H_2O \rightsquigarrow \bullet OH\ (2.8),\ e_{aq}^{-}(2.7), \bullet H\ (0.55),\ H_2O_2\ (0.71),\ H_2\ (0.45) \qquad (4)$$

$$N_2O + e_{aq}^{-} + H_2O \rightarrow \bullet OH + OH^{-} + N_2 \qquad (5)$$

The numbers in parenthesis indicate the radiation chemical yields of the reactive species expressed as *G*-values (number of species per 100 eV energy absorbed).

Hydroxyl radicals react with tryptophan by adding to the double C=C bonds yielding TrpH–OH adducts.[5] These adducts react with oxygen producing the corresponding peroxy radicals. Hydrogen radicals react with TrpH yielding the corresponding TrpH–H adducts, while a small amount of the H-atoms reacts with oxygen yielding $HO_2\bullet$:

$$\bullet H + O_2 \rightarrow HO_2\bullet \qquad (6)$$

$$HO_2\bullet \rightleftharpoons O_2\bullet^{-} + H^{+} \quad pK_a = 4.88 \qquad (7)$$

Superoxide/perhydroxyl ($O_2\cdot^{-}$ / $HO_2\cdot$) radicals usually disproportionate to give hydrogen peroxide and oxygen.[6]

In gamma irradiated tryptophan solutions saturated with a mixture of N_2O and O_2, the reactions of TrpH – peroxy radicals were investigated by measuring the oxygen uptake and by following the formation of hydrogen peroxide and organic peroxides. To obtain some more information about the effect of the side alanine chain on the autoxidation of tryptophan, the oxygen uptake was also measured in solutions of tryptamine and indole-3-acetic acid. The mechanism of tryptophan autoxidation is discussed on the basis of the results obtained.

EXPERIMENTAL

Tryptophan (Sigma grade, Sigma) was used without additional purification. All other chemicals (Merck, Fluka) were of the highest purity available. Water was purified through a Millipore Milli Q system. The pH of the solutions was adjusted between 5 to 8 with phosphate buffer, while KOH was used to prepare alkaline solutions. Prior to irradiation, all solutions were saturated with high purity O_2 and N_2O (Tehnogas, Pančevo). Solutions containing a 4:1 (v/v) mixture of N_2O and O_2 were prepared by mixing saturated solutions using the syringe technique.

The irradiations were performed using a ^{60}Co- gamma source. The different dose rates were obtained by varying the distance of the samples from the source. The dose rates were determined by Fricke dosimetry.

The oxygen uptake was determined from measurements of the oxygen concentration before, during and after irradiation using a standard Clark electrode (Orion 97-08) fitted in an airtight irradiation vessel.

The total peroxides were determined by the iodide method.[7] Hydrogen peroxide was analyzed by the titanium sulfate reagent.[8] The yield of organic peroxides was determined as the difference between the results obtained by the iodide method and those of the titanium sulfate reagent. The product yields were obtained from the linear absorbance *vs*. dose plots with an experimental error better than ±10 %. A Perkin Elmer Lambda 5 spectrophotometer with 10 mm optical cells was used for the absorption measurements.

RESULTS

The oxygen uptake and formation of peroxide material were followed as a function of pH, dose rate and concentration of TrpH in solutions saturated with a 4:1 (v/v) mixture of N_2O and O_2. The results obtained by measuring $G(-O_2)$ and $G(R_2O_2 + H_2O_2)$ as a function of TrpH concentration are presented in Fig. 1.

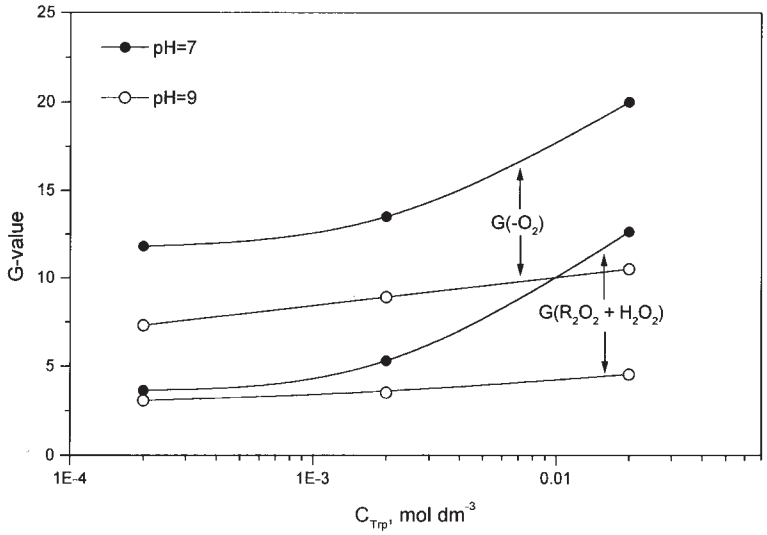


Fig. 1. Effect of tryptophan concentration on the oxygen uptake and formation of peroxide material (H_2O_2 and R_2O_2) in gamma irradiated aqueous solutions of tryptophan at pH 7 and 9. Dose rate 0.01 Gy s^{-1}.

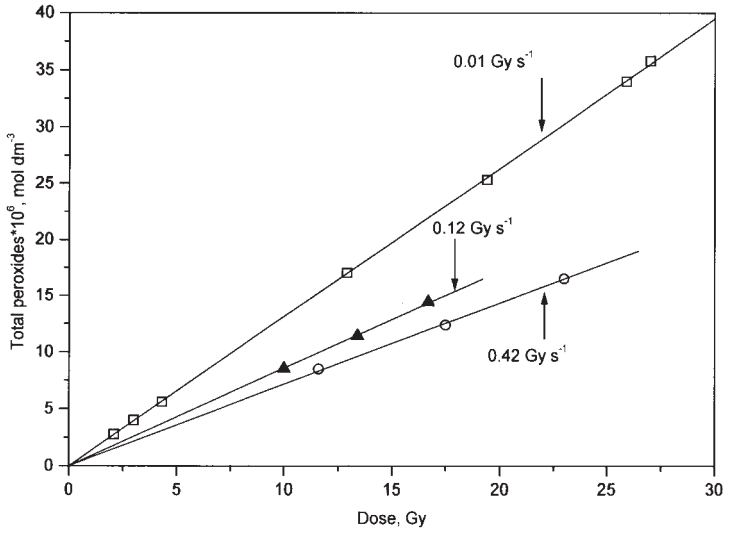


Fig. 2. Effect of the dose rate on the formation of total peroxides in gamma irradiated 2×10^{-2} mol dm^{-3} solutions of tryptophan at pH 9.

The formation of peroxides was followed as a function of dose rate in 2×10^{-2} mol dm^{-3} solutions of tryptophan at pH 9 and the results obtained are presented in Fig. 2.

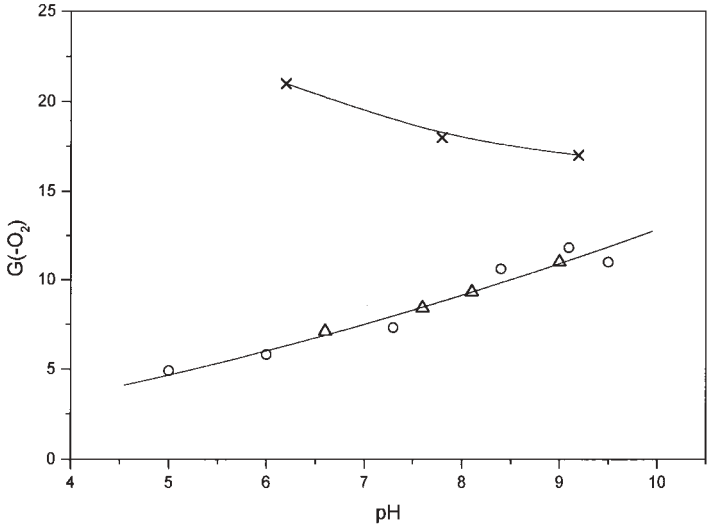


Fig. 3. Effect of pH on the oxygen uptake in gamma irradiated solutions of: 2×10^{-4} mol dm^{-3} tryptophan (o); 2×10^{-4}mol dm^{-3} tryptamine (Δ) and 1×10^{-2} mol dm^{-3} indole-3-acetic acid (x). Dose rate 0.01 Gy s^{-1}.

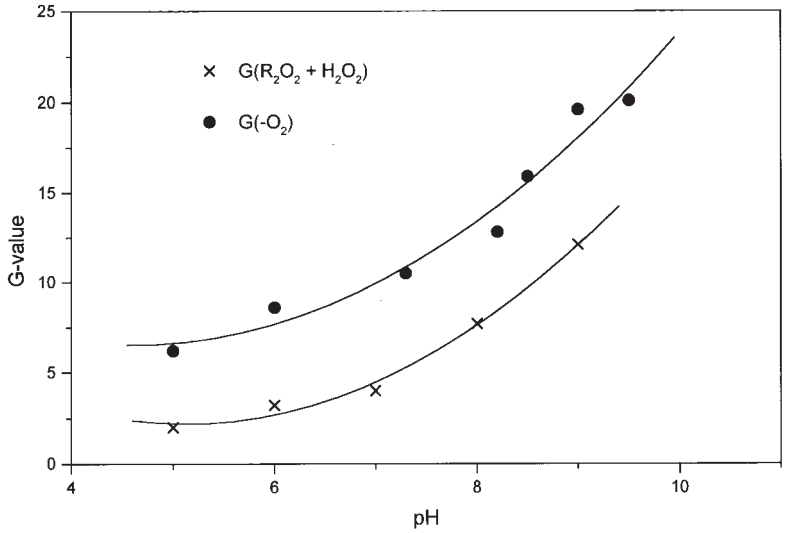


Fig. 4. Effect of pH on the oxygen uptake and formation of total peroxides in gamma irradiated 2×10^{-2} mol dm^{-3} solutions of tryptophan. Dose rate 0.01 Gy s^{-1}.

The oxygen uptake was measured in the pH interval from 5 to 9 in aqueous solutions of:

1. tryptophan (pK_a = 2.38 and 9.39) – 2×10^{-4} and 2×10^{-2} mol dm^{-3}

2. tryptamine (pK_a = 10.2) – 2×10^{-4} mol dm^{-3} and

3. indole-3-acetic acid (pK_a = 4.75) – 1×10^{-2} mol dm^{-3},

where the pK_a values were taken from Lange's Handbook of Chemistry, 1985. The results obtained are presented in Fig. 3.

The effect of pH on the formation of peroxides and oxygen uptake was followed in 2×10^{-2} mol dm^{-3} solutions of tryptophan at a dose rate 0.01 Gy s^{-1} (Fig. 4).

The yields of organic peroxides and hydrogen peroxide are measured for two initial concentrations of tryptophan and two dose rates in the pH interval from 7 to 9. The results obtained are presented in Table I.

TABLE I. The yields of organic peroxides and hydrogen peroxide in aqueous solutions of tryptophan, $N_2O : O_2 = 4 : 1$

DOSE RATE	pH	c(TrpH) = 2× 10^{-2} mol dm^{-3}		c(TrpH) = 2× 10^{-4} mol dm^{-3}	
Gy min^{-1}		$G(R_2O_2)$	$G(H_2O_2)$[a]	$G(R_2O_2)$	$G(H_2O_2)$[a]
0.5	7	3.15	0.82	1.21	1.15
	8	5.46	2.20	1.23	1.36
	9	9.79	2.29	1.31	1.63
25	7	2.90	0.51	1.01	0.75
	8	5.28	0.68	1.06	0.96
	9	5.56	0.80	1.10	1.05
> 50[b]	6	0.91	0	0.49	0

[a] $G(H_2O_2) = G(H_2O_2)_{meas.} - G(H_2O_2)_{calc.}$ [b] Calculated using data taken from Ref. 9

The yield of hydrogen peroxide in Table I represents the difference between the measured and the calculated $G(H_2O_2)$. The calculated, *i.e.,* the expected yield of hydrogen peroxide equals:

$$G(H_2O_2)_{calc.} = G(H_2O_2)_{mol.} + 0.5\ G(O_2^{\bullet-} + HO_2^{\bullet}) \qquad (8)$$

where $G(H_2O_2)_{mol.} = 0.7$ is the primary hydrogen peroxide.[4]

At higher concentrations of tryptophan, the competition of oxygen and tryptophan for •H and e_{aq}^- should be taken into account in the calculation of $G(H_2O_2)_{calc.}$. In addition, in 2×10^{-2} mol dm^{-3} tryptophan solution, due to track scavenging, G(TrpH-OH) is slightly increased thus decreasing $G(H_2O_2)_{mol.}$. The yield of TrpH-OH adducts is also higher in solutions containing N_2O which interferes with spur reactions. The procedure applied to calculate both G(TrpH-OH) and $G(H_2O_2)$ in tryptophan solutions saturated with 4:1 (v/v) mixture of N_2O and O_2 has been described elsewhere.[9] The yields of radicals and hydrogen peroxide for 2×10^{-2} mol dm^{-3} and 2×10^{-4} mol dm^{-3} solutions of tryptophan are given in Table II.

The increase in G(TrpH–H) due to track scavenging, which is not very important, was not taken into account. All data necessary to calculate the G-values given in Table II were taken from references 4 and 10.

TABLE II. Free radical yields and hydrogen peroxide yield in solutions of tryptophan, pH 5 – 9, $N_2O:O_2 = 4:1$

RADICAL	c(TrpH) = 2×10^{-2} mol dm^{-3}	c(TrpH) = 2×10^{-4} mol dm^{-3}
G(TrpH-OH)	6.06	5.83
G(TrpH-H)	0.53	0.11
G(Trp$^-$)	0.07	0
$G(R)_{total}$	6.66	5.94
$G(O_2^{\bullet-})$	0.07	0.07
$G(HO_2^{\bullet})$	0.02	0.44
$G(H_2O_2)_{mol}$•	0.56	0.71
$G(H_2O_2)_{calc.}$	0.61	0.97

The reaction of neutral tryptophan radicals (Trp•) with oxygen was examined by measuring the oxygen uptake in solutions containing KBr (TrpH + $Br_2^{\bullet-} \rightarrow$ TrpH$^{\bullet+}$ + 2Br$^-$).[11] In the system (1×10^{-4} mol dm^{-3} TrpH, 1×10^{-2} mol dm^{-3} KBr, 2.8×10^{-4} mol dm^{-3} O_2, pH ~ 7), where Trp• radicals are practically the only reactive species present during irradiation (G(Trp·) = 5.22), the oxygen uptake was estimated to be $G(-O_2) = 0.4$.

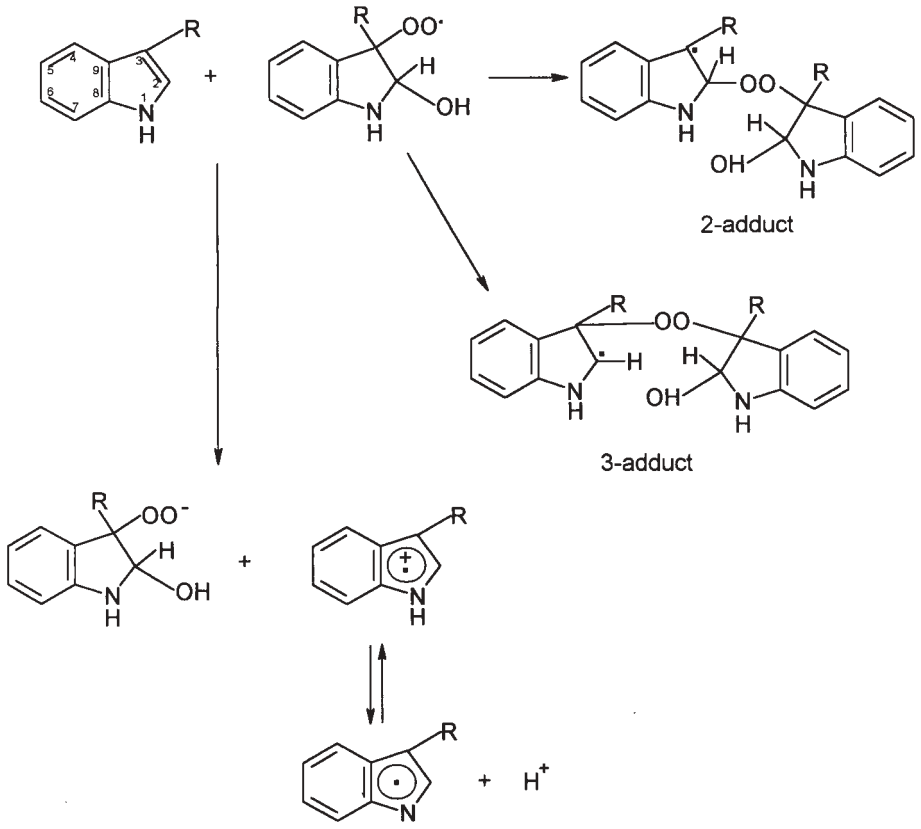
DISCUSSION

Since the autoxidation propagated by peroxy radicals is a chain reaction, its efficiency depends linearly on the solute concentration and inversely on the dose rate. As can be seen in Fig. 1, both the oxygen uptake and the yield of peroxide material increase with increasing concentration of tryptophan in the irradiated solutions. In addition, the yield of total peroxides varies inversely with the dose rate (Fig. 2). The results obtained show, as was previously suggested,[3] that tryptophan undergoes autoxidation reactions in oxygenated gamma irradiated solutions.

The autoxidation of tryptophan has not been observed directly probably because of its slowness. However, a reaction with Cl_3COO• has been reported.[12] The Cl_3COO• radical is a moderately strong oxidant and in most cases was found to react by one-electron oxidation.[13] An exception is its reaction with tryptophan and indoles where adduct formation was clearly established.[14] In both cases, the Cl_3COO• radical reacts by adding predominantly to the C(2) – C(3) double bond and only a minor fraction accounts for the one-electron oxidation. It is very likely that TrpH-peroxy radicals, as do Cl_3COO• radicals, react with tryptophan by adduct formation (reaction 3). It may be concluded that the adduct thus formed propagates autoxidation of tryptophan since Trp· radicals (reaction 1) are unreactive towards oxygen, from $G(-O_2) = 0.4$. H-abstraction from the allylic position in the side chain (reaction 2) cannot be excluded entirely. It is, however, generally slow and requires high activation energy.[2]

The increase of both the oxygen uptake and the yield of peroxides in alkaline media (Fig. 3) shows that the autoxidation of tryptophan occurs at pH > 6. In addition, by comparing $G(R_2O_2)$ and $G(H_2O_2)$ given in Table I with the yield of peroxides obtained

previously,[9] it is clear that autoxidation of tryptophan depends strongly on both the pH and the dose rate. As was expected, the incrases of $G(R_2O_2)$, $G(H_2O_2)$ and $G(-O_2)$ are more pronounced at higher concentrations of tryptophan.

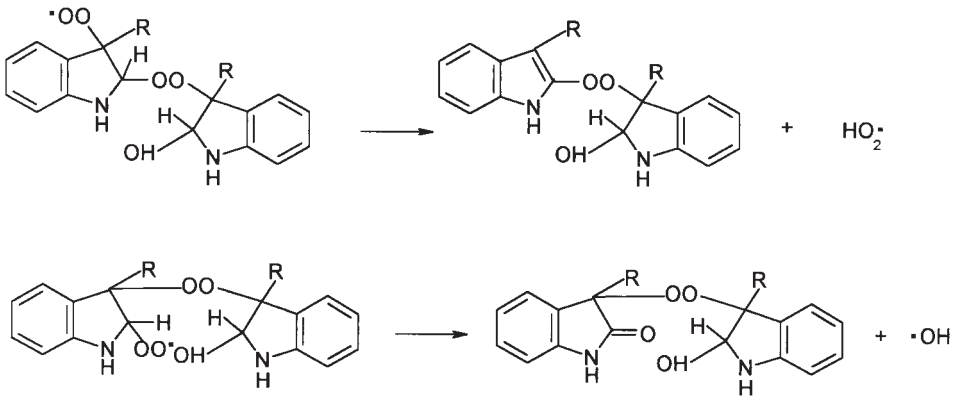


Scheme 1.

For tryptamine solutions, the same pH dependence of $G(-O_2)$ as for tryptophan was observed (Fig. 3), showing that the COO^- group does not affect the autoxidation of tryptophan. The different pH dependence of $G(-O_2)$ observed for indole-3-acetic acid, *i.e.,* the slight decrease of the oxygen uptake from $G(-O_2) = 21$ at pH 6 to $G(-O_2) = 17$ at pH 9, might be ascribed to the absence of the amino group in the side chain. It seems at first sight that the increase of $G(-O_2)$ in alkaline media observed in tryptophan and tryptamine solutions is due to the deprotonation of the amino group. However, the increase of the oxygen uptake was also observed at pH < 8, where the protonated amino group predominates. In addition, the same pH dependence of $G(-O_2)$ was observed in both tryptophan and tryptamine solutions, having different NH_3^+/NH_2 ratios according to their pK_a values.[15] These findings indicate that the propagation step of tryptophan autoxidation is a base-induced reaction. The base-induced changes of the transient absorption spectra of $Cl_3COO\cdot$-indole adducts were observed previously,[14] being influ-

enced by methyl substituents at various positions in the indole ring. While the spectrum of the CCl_3O_2–TrpH adduct resembles the spectra of trichloromethylperoxy adducts derived from 3-methylindole and 2,3-dimethylindole, the chain reaction propagated *via* CCl_3O_2–indole peroxy radicals was indicated only for 2,3-dimethylindole. The CCl_3O_2• radical is considered to be a moderately strong oxidant, and hence reacts with TrpH more selectively than •OH. The •OH radical initiates oxidation of tryptophan by adding to at least four positions in the indole ring.[5,9] Peroxy radicals are derived from C_2–, C_3– and C_5– (or C_7–) TrpH–OH adducts, while the C_8–adduct eliminates water yielding neutral tryptophan radicals, Trp·. The peroxy radicals formed at the benzene ring are not involved in the autoxidation of tryptophan since they decay by eliminating HO_2•/O_2•$^-$ and producing 5– or 7– hydroxytryptophan. This leaves the C_2– and C_3– peroxy radicals to react with tryptophan by adding to the C(2)–C(3) double bond. The formation of TrpH(OH)OO–TrpH• adducts is presented in Scheme 1.

By further oxygen uptake, these tryptophan adducts produce peroxy radicals. These peroxy radicals may decay by different modes of reactions. However, in a complex system such as tryptophan and without the possibility to follow directly the formation of the transient species, it is difficult to establish exactly the reactions in which they are involved. We suggest as most probable decomposition, although disproportionation can not be excluded entirelly. As can be seen (Scheme 2) the newly produced peroxy radicals may decompose by eliminating HO_2• and •OH radicals and producing peroxide material.



Scheme 2.

If they react by bimolecular decay, it is very likely that, similar to other organic peroxy radicals, a transient tetroxide is formed.[2] Fragmentation of the tetroxide leads eventually to the same products as obtained by decomposition of newly formed peroxy radicals. Therefore their disproportionation reactions are not discussed in more details.

The neutral tryptophan radical Trp· is at least formed by water elimination from the C_8 – TrpH–OH adduct and as a minor fraction in the reaction of peroxy radicals with tryptophan (Scheme 1). It may react either by disproportionation or with an O_2•$^-$ radical. According to the rate constants of these reactions,

$2Trp\bullet \rightarrow$ Products $2k=6.4\times10^8$ mol^{-1}dm^{3}s^{-1} [16]

$Trp\bullet + O_2\bullet^- + H^+ \rightarrow TrpH + O_2$ $k=4.5\times10^9$ mol^{-1}dm^{3}s^{-1} [9]

it is obvious that Trp• radical will preferentially react with $O_2\bullet^-$ since its bimolecular decay is very slow, $2k < 3.5$ mol^{-1}dm^{3}s^{-1}.[17] This explains the observed much lower increase of $G(H_2O_2)$ than $G(R_2O_2)$ (Table I).

In the view of the reactions given in Schemes 1 and 2, the obtained results indicate the following set of reactions for tryptophan autoxidation:

Initiation

$$TrpH + \bullet OH \rightarrow \bullet TrpH - OH \quad (9)$$

$$\bullet TrpH - OH + O_2 \rightarrow TrpH(OH)OO\bullet \quad (10)$$

Propagation

$$TrpH + TrpH\ (OH)OO\bullet \rightarrow TrpH(OH)OO - TrpH\bullet \quad (11)$$

$$TrpH(OH)OO - TrpH\bullet + O_2 \rightarrow TrpH(OH)OO - TrpHOO\bullet \quad (12)$$

$$TrpH(OH)OO - TrpHOO\bullet \rightarrow RR'O_2 + HO_2\bullet \quad (13)$$

2-adduct peroxy radical

$$TrpH(OH)OO - TrpHOO\bullet \rightarrow RR''O_2 + \bullet OH \quad (14)$$

3-adduct peroxy radical

Termination

$$2TrpH(OH)OO\bullet \rightarrow R_2O_2 + O_2 \quad (15)$$

The reactions 9–15 proposed for the autoxidation of tryptophan are consistent with the experimentally obtained results. The newly generated peroxy radicals (reaction 12) and the reactions in which they are involved (reactions 13, 14), as well as the disproportionation of tryptophan peroxy radicals (reaction 15) account for both the high $G(-O_2)$ and $G(R_2O_2)$ measured. The part of $HO_2\bullet/O_2\bullet-$ (reactions 13) which did not react with Trp• radicals will recombine yielding hydrogen peroxide.

The chain propagation length,[18]

$$CPL = [G(-O_2) - G(R_{in}) / 2] / G(R_{in}) \quad (16)$$

was estimated in 2×10^{-2} mol dm^{-3} solution of tryptophan at pH 9.5 and dose rate 0.01 Gy s^{-1} to be $CPL \approx 5.8$. To estimate the *CPL* in a tryptophan solution for the total yield of water radicals generated initially, $G(R_{in}) = 3.03$ was used since ≈50 % of the TrpH–OH adducts are not involved in autoxidation reactions.

In conclusion, in oxygenated solutions the TrpH– peroxy radicals produced during gamma radiolysis at pH > 6 at low dose rates initiate the autoxidation of tryptophan.

Compared to free-radical-radical reactions which are extremely efficient, the adduct formation (reaction 11) is very slow. As a consequence the autoxidation of tryptophan is a short chain reaction. Due to its slowness, the adduct formation is in competition not only with reactions 1 and 2 but also with other different modes of tryptophan peroxy radicals decay.[9] Therefore, the autoxidation of tryptophan is very complex and the reaction mechanism has not been fully resolved. However, measurements of $G(-O_2)$ and $G(R_2O_2)$ in radiation-induced autoxidation of tryptophan provide a useful model system for the study of air-induced oxidation of many organic systems.

И З В О Д

АУТООКСИДАЦИЈА ТРИПТОФАНА У ВОДЕНИМ РАСТВОРИМА

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Аутооксидација триптофана у воденим растворима испитивана је употребом гама радиолизе. Потрошња кисеоника и принос створених пероксида праћени су у зависности од pH, јачине дозе и почетне концентрације триптофана. Добијени резултати показују да адиција TrpH(OH)OO$^{\bullet}$ радикала на триптофан пропагира аутооксидацију. Дужина ланца пропагације која за 2×10^{-2} mol dm^{-3} триптофан на pH 9,5 и јачини дозе 0.01 Gy s^{-1} износи *CPL* ≈ 5.8 показује да је аутооксидација триптофана кратколанчана реакција.

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