Influence of Temperature and Oxygen Concentration on the Radiation Induced Oxidation of Phenylalanine

P. Krajnik [1], R. M. Quint [1], S. Solar [1], N. Getoff [1], and G. Sontag [2]

[1] Institut für Theoretische Chemie und Strahlenchemie, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria
[2] Institut für Analytische Chemie, University of Vienna, Währinger Str. 38, A-1090 Vienna, Austria

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The formation of tyrosine isomers by γ-radiolysis of neutral aqueous phenylalanine solutions was found to be strongly dependent on oxygen concentration and temperature. Changing the dose rate did not influence the degradation process. In the presence of 0.25×10^{-3} mol dm^{-3} oxygen at room temperature the yields of o-tyrosine as well as of m- and p-tyrosine drop from G(o-Tyr) = 0.5 and G(m-Tyr) = G(p-Tyr) = 0.4 at a dose of 0.3 kGy to 0.18 and 0.16 at 2.5 kGy, respectively. In solutions containing 1.25×10^{-3} mol dm^{-3} oxygen the initial yields remain unchanged but decrease at 2.5 kGy only to G(o-Tyr) = 0.3 and G(m-Tyr) = G(p-Tyr) = 0.20. Under the latter reaction conditions also 3,4-dihydroxyphenylalanine was found.

Samples irradiated in frozen state did not show remarkable radiolysis of phenylalanine and tyrosine formation. In the range between 5 and 20 °C no essential influence of temperature on the phenylalanine radiolysis and tyrosine yields was observable. The obtained results are important for methods using the tyrosine yields as markers for the detection of irradiated food. Storage conditions and irradiation temperature play an essential role on radiation induced changes of food.

Introduction

The radiolysis of aqueous solutions of phenylalanine (Phe) results in the formation of tyrosines (Tyr) [1]. This reaction is well known and was studied by several authors [2–5]. Since the water content of biological tissues is very high, approximately 78% [2], studies of radiation induced reactions in food can be carried out in aqueous solutions of amino acids.

The overall reaction mechanism was shown to base on the reaction of the primary radicals of water radiolysis (OH, H, and e_{aq}^-) with amino acids. Proteins are known to show a very high reactivity towards hydroxyl radicals [6]. The distribution of the individual radical attack on different amino acids, however, is not known with certainty. But in a crude approximation it can be related to the individual reactivities and abundances of amino acids. Phenylalanine, which is present in meats at a level of 2–4%, reacts very fast with OH (k(OH + Phe) = 6×10^9 dm^3 mol^{-1} s^{-1} [6, 7]). Due to this high affinity the fraction of phenylalanine scavenging these radicals should be substantial.

The OH radical itself is a very electrophilic species and attacks preferentially the electron rich benzene ring of phenylalanine [7, 8]. This addition occurs almost statistically, so a mixture of o-, m- and p-tyrosine is obtained [4]. In general, biological systems contain only p-tyrosine (present in all proteins at about 1–3%) and traces of o-tyrosine. The third isomer, m-tyrosine, has not been detected yet in such systems. Because of the significant distribution the use of o- and m-tyrosine as an indicator for γ-irradiated food appears reasonable [9–11]. In all previous publications o-tyrosine has been suggested as such marker rather than m-tyrosine because of the easier chromatographic separation procedure from p-tyrosine [9–13]. Recently, m-tyrosine has also been reported to be a detectable marker for the identification of irradiated chicken meat [5, 14].

Several discrepancies have been reported regarding the levels of o-tyrosine in unirradiated meat varying from 0.01 mg/kg [2, 10, 15] to 0.5 mg/kg [10]. The detected radiation yields of o-Tyr also show some inconsistencies depending on the applied method: GCMS, HPLC, different columns, and different detection methods influence the results. The amounts of o-Tyr formed by irradiation fluctuate from 0.05 mg/(kg.kGy) at 20 °C [3] to 1.1 mg/(kg.kGy) at 20 °C [10]. In all experiments the level of o-tyrosine produced by irradiation at commercially approved doses of 3–5 kGy exceeded that of unirradiated food.

The aim of the present work was to investigate more carefully all important parameters which can influence significantly the radical oxidation of phenyl-

Reprint requests to Prof. N. Getoff.

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alanine. In this study we show the effect of oxygen concentration present in the system during irradiation, the influence of temperature, radiation dose, and dose rate on the formation of the isomeric tyrosines.

Experimental

Phenylalanine (Aldrich 99%) was used as received. The solutions of phenylalanine were saturated with the respective gas (Ar, O_2, N_2O) for 30 min and irradiated in a ^{60}Co-γ-source (Gammacell 220, Nordion).

Low temperature experiments were carried out in cooling baths. The phenylalanine solutions were cooled for 30 min. For irradiation the samples were put into a Dewar flask, and cooling was maintained during irradiation in order to guarantee constant reaction temperatures.

Following cooling mixtures were employed: water (5 °C), ice/NaCl (−5 °C), dry ice/CCl_4 (−20 °C), and dry ice/acetone (−78 °C).

Analysis: The irradiated solutions were analyzed by HPLC (Spherisorb ODS2 250 mm column; eluent: H_2O:CH_3CN = 93:7). The chromatograph (Hewlett Packard 1050) was equipped with a multiple wavelength uv-detector with diode array and an electrochemical detector. The absorptions of the individual products were detected at 210 nm and at $E = +0.9$ V. The identity of the products was confirmed by comparison with the chromatograms of standards and the uv-spectra of the products.

Results and Discussion

The initiating interaction between ionizing radiation and aqueous solution is leading to water radiolysis, whereby several primary products are formed (yields (G value = number of species formed per 100 eV absorbed energy)[1] at pH = 7 are put in parentheses):

$$H_2O \longrightarrow \underset{(2.7)}{e^-_{aq}},\ \underset{(0.6)}{H},\ \underset{(2.8)}{OH},\ \underset{(0.45)}{H_2},\ \underset{(0.75)}{H_2O_2},\ \underset{(3.2)}{H^+_{aq}},\ \underset{(0.5)}{OH^-_{aq}}. \quad (1)$$

In the presence of oxygen, both H atoms and e^-_{aq} are transformed into peroxy radicals:

$$H + O_2 \xrightarrow[k_2 = 2.1 \times 10^{10}\ dm^3\ mol^{-1}\ s^{-1}\ [6]]{} HO_2^{\cdot}, \quad (2)$$

$$e^-_{aq} + O_2 \xrightarrow[k_3 = 1.9 \times 10^{10}\ dm^3\ mol^{-1}\ s^{-1}\ [6]]{} O_2^{\cdot -}, \quad (3)$$

$$HO_2^{\cdot} \underset{pK = 4.8\ [16]}{\rightleftharpoons} H^+ + O_2^{\cdot -}. \quad (4)$$

The reaction conditions in aqueous solutions, i.e. presence or absence of oxygen, can strongly influence the pathways of radiation induced processes. Therefore we observed the radiolysis of phenylalanine and the formation of o-, m-, and p-tyrosine in solutions saturated with argon, air, oxygen or N_2O in dependence of dose and dose rate. Since oxygen is present is tissues, samples containing oxygen are the most representative ones. The applied concentration of phenylalanine was between 2.5×10^{-4} mol dm^{-3} and 2.5×10^{-3} mol dm^{-3}. The radiolysis of phenylalanine under different conditions is shown in Figure 1.

In airfree media where oxydizing species (OH radicals) and reducing species (H and e^-_{aq}) are present, a G_i value of 3.0 was detected for the consumption of phenylalanine. In solutions containing oxygen $G_i(-\text{Phe}) = 2.8$, which corresponds to $G(\text{OH})$, indicating that $O_2^{\cdot -}$ transients do not contribute to the decomposition of Phe. Their rate constants with aromatic amino acids is very low, $k(O_2^{\cdot -} + \text{Phe/Tyr}) < 10$ dm^3 mol^{-1} s^{-1} [17]. The highest degradation ($G_i(-\text{Phe}) = 4.66$) is found in solutions saturated with N_2O, where $G(\text{OH}) = 5.5$. Obviously, high concentrations of OH radicals lead to most effective hydroxylation reactions.

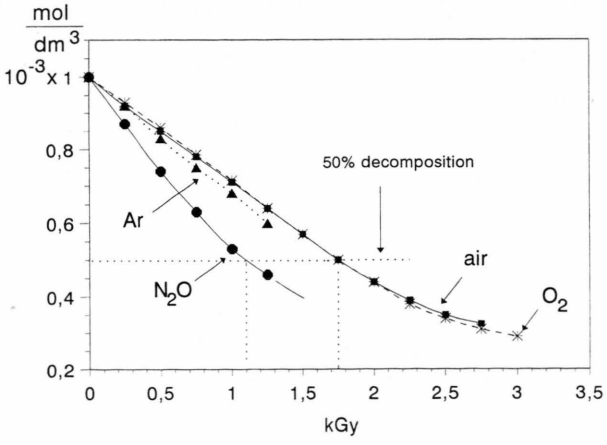


Fig. 1. Radiolysis of aqueous 1.0×10^{-3} mol dm^{-3} phenylalanine (pH = 6) as a function of dose in the presence of Ar, N_2O (2.8×10^{-2} mol dm^{-3}), air (0.25×10^{-3} mol dm^{-3}), and O_2 (1.25×10^{-3} mol dm^{-3}).

[1] For conversion into SI-units multiply the G value by 0.1036 to obtain $G(x)$ in μmol/J.

The principal OH radical attack occurs at the aromatic ring giving OH adducts in o-, m-, p-, and ipso positions, whereas H abstraction reactions were found to be negligible [4, 8]. Previous radiolysis studies reported that the primary distribution of hydroxycyclohexadienyl radical isomers is o:m:p = 50%:14%:30% [7], the remaining 6% were attributed to other pathways. These transients are the precursors of o-, m-, and p-tyrosine. Recently, Wang et al. [4] investigated the yields of these final products formed during γ-radiolysis in aqueous Phe solutions under various conditions. If strongly oxidizing reactants ($N_2O/Fe(CN)_6^{3-}$) are available, 80% of the OH radicals are leading to tyrosine isomers, whereby o:m:p = 37%:24%:19%. In the presence of N_2O/O_2 (4:1) the conversion factor reduces to 50% (o:m:p = 18:16:18) and drops drastically in N_2O solutions to 10% (o:m:p = 2.5%: 3.5%:4.5%). Besides the amount of OH species leading to the formation of tyrosines the ratio o:m:p is also influenced by the kind of oxidants. Decreasing the oxidation ability results in a more statistical distribution.

The results of our studies concerning the product formation in γ-irradiated aqueous solutions of Phe in dependence of dose under various reaction conditions are presented in Figure 2a–d.

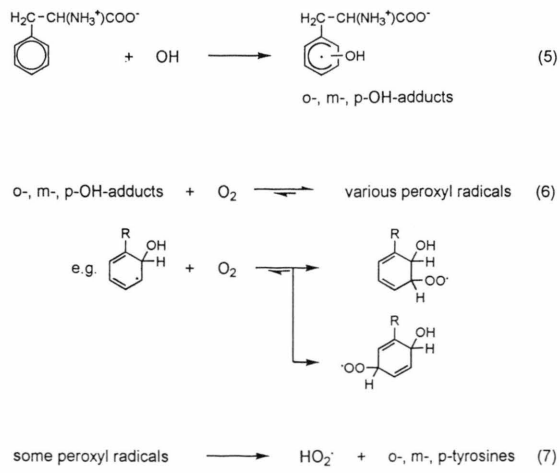
In airfree solutions (Ar and N_2O saturated, Fig. 2a, b) the yields of o-, m-, and p-tyrosines are rather low compared to the decomposed Phe, Figure 1, but are linearily increasing with dose up to 1.2 kGy. In N_2O saturated solutions equal amounts of isomers ($G_i = 0.22$) are formed, which differ slightly from the results of Wang et al. [4]. In the presence of oxygen the tyrosine yields increase essentially, but they exhibit a strong deviation from linearity with rising dose. The influence of oxygen concentration is clearly pronounced. In air ($[O_2] = 0.25 \times 10^{-3}$ mol dm^{-3}) with a dose of 1 kGy 32 µmol of o-Tyr and 27 µmol of m-Tyr and p-Tyr are formed, whereas in oxygenated solution ($[O_2] = 1.25 \times 10^{-3}$ mol dm^{-3}) 42 µmol o-Tyr and 34 µmol m- and p-Tyr are obtained (Figure 2c, d). In air, linear proportionality between tyrosine isomer formation and dose is observable in the range 0–0.3 kGy and in oxygenated solution up to 0.8 kGy. These facts indicate the involvement of the primary formed hydroxycyclohexadienyl radicals in more than one reaction pathway. Further, during γ-radiolysis OH radicals attack the primary products leading to polyhydroxy compounds. The formation of one of them is shown in Fig. 2d (3,4-dihydroxyphenylalanine, DOPA). The shape of the curve clearly demonstrates that it is a secondary product. Figure 3 shows a chromatogram of a typical reaction mixture. Irradiation at higher doses makes the analysis even more difficult because many di- and polyhydroxy compounds are appearing.

Table 1. Initial G values (G_i) of degradated phenylalanine, formation of o-, m-, p-tyrosine as well as % conversion from degradated phenylalanine into tyrosines, obtained after steady state irradiation of aerated neutral aqueous phenylalanine solutions. Dose rate: 73 Gy min^{-1}; * dose rate: 240 Gy min^{-1}.

Phe (mol dm^{-3})	G_i values					Conversion (Total Tyr)
	−Phe	o-Tyr	m-Tyr	p-Tyr	Total Tyr	%
2.5×10^{-4}	2.7	0.5	0.3	0.4	1.2	44
1.0×10^{-3}	2.9	0.5	0.4	0.4	1.3	45
1.0×10^{-3} *	2.8	0.5	0.4	0.4	1.3	46
2.5×10^{-3}	2.8	0.5	0.4	0.5	1.4	50

The initial G values (G_i), which are the same for aerated and oxygenated solutions, are summarized in Table 1 (concentration range $2.5–25 \times 10^{-3}$ mol dm^{-3} Phe). Changing the concentration of Phe only slightly effects the product formation and degradation of the substrate. On moving to higher concentrations the overall conversion raises from 44 to 50%. To some extent the domination of the o-isomers over the m- and p-isomers disappears.

The pronounced influence of oxygen concentration on the product formation (Fig. 2c, d) can be attributed to the reversible oxygen addition reaction on the various types of hydroxycyclohexadienyl radicals (6).



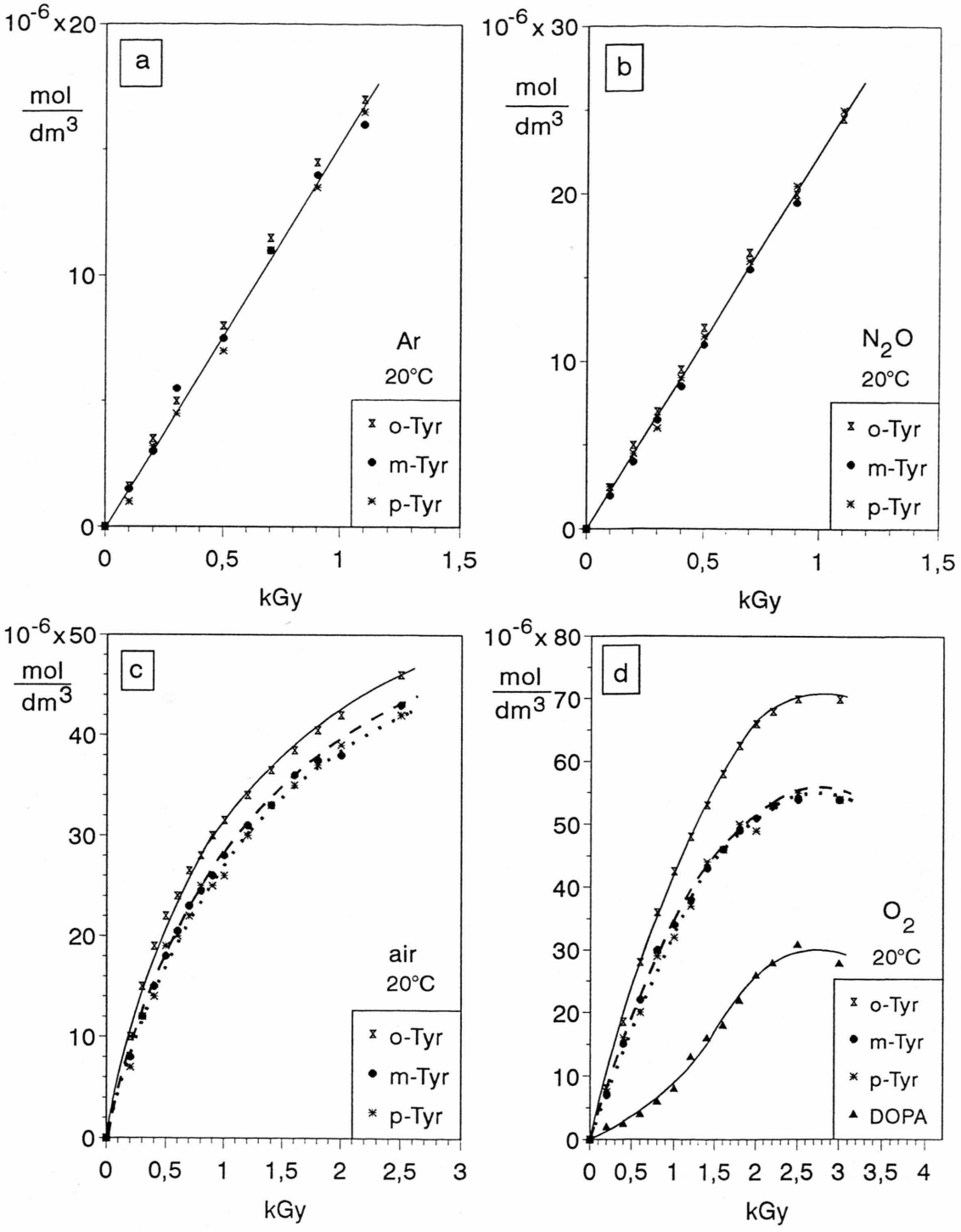


Fig. 2. Radiation induced formation of o-, m-, p-tyrosine, and 3,4-dihydroxyphenylalanine (DOPA; 2d) in the presence of Ar (a), N_2O (b), air (c), and O_2 (d).

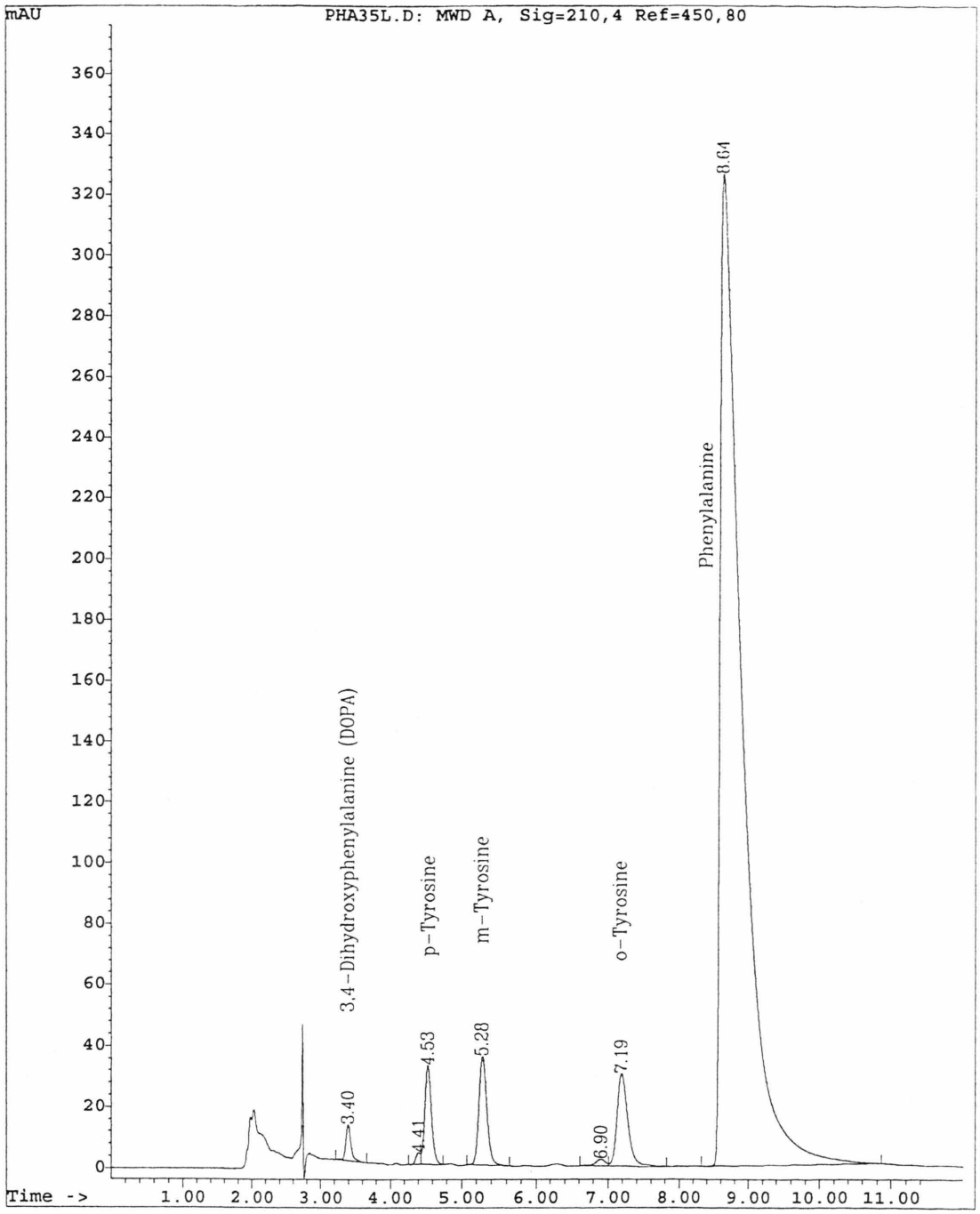


Fig. 3. HPLC chromatogram of irradiated 1.0×10^{-3} mol dm^{-3} phenylalanine. Dose 1 kGy, $T = 20\,^{\circ}C$, solution saturated with air. Detection wavelength 210 nm.

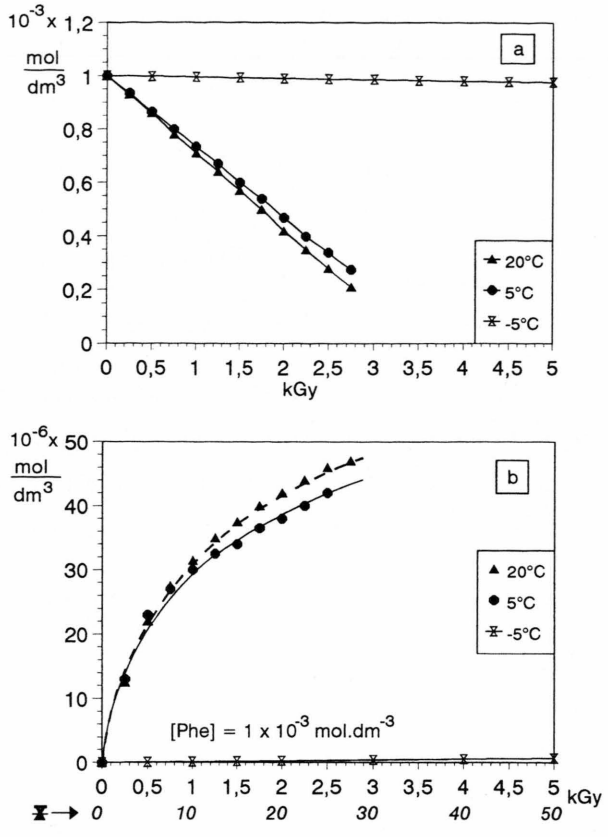


Fig. 4. a) Temperature dependence of the radiolysis of phenylalanine ($c = 1.0 \times 10^{-3}$ mol dm^{-3}). b) Temperature dependence of the formation of o-tyrosine (* = dose scale for the experiments at −5 °C).

The overall rate constant for the addition reaction is $k_{6f} = 1.8 \times 10^8$ dm^3 mol^{-1} s^{-1} and the back reaction $k_{6b} = 5.4 \times 10^4$ dm^3 mol^{-1} s^{-1} [4, 18]. Higher O_2 concentrations shift the equilibrium to the peroxyl radical site, enhancing the formation of o-, m, and p-Tyr (7).

The deviation of the product formation from linearity with increasing dose, which is more pronounced in aerated than in oxygenated solution (Fig. 2c, d) is a consequence of oxygen consumption during γ-radiolysis. The continuously produced H and e_{aq}^- during water radiolysis are scavenged by O_2 (2, 3).

Variation of dose rates did not influence the reaction. In several experiments dose rates between 60 Gy/min and 240 Gy/min were applied. Neither the product distribution nor the yields changed significantly on moving to higher dose rates (Figure 2c, d).

A parameter which strongly influences the reaction is the temperature during the irradiation. Three temperature ranges were observed: 25 °C, 5 °C, and −5 °C. The results shown in Fig. 4a, b indicate that in samples irradiated at temperatures below freezing, the amounts of radiation-induced tyrosins are significantly lower than those in samples irradiated at room temperature or temperatures above freezing. The formation of the tyrosines rapidly drops when the sample freezes. At a temperature of −20 °C the concentration of tyrosines is extremely low, at −78 °C no products could be detected at all.

The observed effect can be attributed to the fact that the mobility of the radiolytically generated free radicals is strongly reduced in ice as to be expected for diffusion controlled reactions. The yield of free radicals (H, OH, e_{aq}^-) resulting from radiolysis of water depends on the phase and the temperature. In ice the yield of OH radicals has a G value of approximately 1.0, whereas in water at 25 °C the G value is 2.8 [19]. Consequently, the availability of OH to react with phenylalanine to form tyrosines is decreased in samples irradiated in the frozen state, resulting in lower levels of radiation-induced tyrosines.

This phenomenon that the solid state is responsible for the low yields can clearly be shown by partially melting the sample or shorter cooling periodes which prevent complete crystallization. Then the tyrosine formation rapidly increases.

From the data shown in Fig. 4 it can also be concluded that when samples are irradiated above freezing, the levels of radiation induced tyrosines are nearly not effected by the temperature during irradiation.

Conclusion

The radiation induced formation of o-, m-, and p-tyrosine from phenylalanine strongly depends on the oxygen concentration in the solution as well as on the temperature during irradiation. Increasing the O_2 concentration leads to a pronounced enhancement of the formation of the three tyrosine isomers.

The phenlyalanine radiolysis and hence the formation of tyrosines at temperatures below 0 °C is found to be insignificant. The tyrosine yield obtained by irradiation at room temperature is slightly higher than the yield at 5 °C. Changing the dose rates, however, did not a affect the radiolysis products.

The presented results are of importance concerning the use of o- and/or m-tyrosine formation as a marker for irradiated food containing proteins. Storage conditions as well as irradiation temperature may strongly influence the chemical changes in irradiated food.

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