

RESEARCH ARTICLE

Kinetics of the thermal disappearance of radicals formed during the radiolysis of L- α -anhydrous asparagine

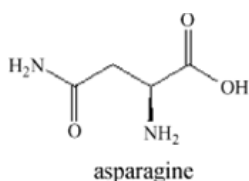
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Abstract: An EPR study of paramagnetic centers formed by irradiation of polycrystalline L- α -anhydrous asparagine (L-Asn) was performed. The EPR spectra of gamma irradiated samples at room temperature, shown the presence of three types of paramagnetic centers. A possible mechanisms of formation for the three radical species is suggested, based also on literature data. The kinetics of the disappearance of radicals during thermal annealing indicated a complex mechanism.

Keywords: L- α -anhydrous asparagine, ionizing radiation, paramagnetic centers, thermal disappearance, unpaired electron

1 Introduction

Amino acids which are the building blocks of proteins are among the simplest organic molecules of biological relevance and thus serve as convenient model systems in studies of radiation damage. Asparagine is an amino acid that plays an imperative role in the metabolic control of some cell functions in nerve and in brain tissue and is used by many plants as a nitrogen reserve substance.^[1] Asparagine is one of the 20 most common natural amino acids in living organisms. It has carboxamide as the side chain's functional group.



Asparagine has a high propensity to hydrogen bond, since the amide group can accept two and donate two hydrogen bonds. The well-known deamidation of L-Asn was documented because of its occurrence in all tissues.^[2]

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Oxidative damage enhances L-Asn instability^[3] which indicates a free radical route in asparagine degradation. L-Asn is a highly conserved amino acid in the family of cyclooxygenases, key enzymes in inflammation processes.^[4] Since these enzymes function in conditions of oxidative stress, the behavior of L-Asn residue toward free radical is important. Even if they do not belong to the active site, these residues are important in maintaining the three dimensional structure of proteins by their participation in hydrogen bond network.^[5]

Peptides containing asparagine residues undergo side chain cleavage during dissociative electron attachment and it was proposed that such losses could be used to ascertain the presence of this residue in unknown peptides.^[6] It was shown that sequences containing asparagine residues are prone understood despite their importance in many biological (radioprotection, radiotherapy) and industrial processes (radiosterilization of food and drugs).

The analysis of EPR spectra of L- α - anhydrous asparagine irradiated in solid state indicated the certain existence of three radicals, stable at room temperature, two radicals resulted from decarboxylation and deamination processes and the third radical from expulsion of a hydrogen atom from the amidic group.

2 Experimental

Polycrystalline samples of L- α - anhydrous asparagine have been irradiated with gamma rays at the room temperature by using a ¹³⁷Cs source (Gammator type). The irradiation dose was 1.05×10^2 Gy/h. The EPR spectra

were recorded at room temperature with an EPR ART 6 instrument (IFIN Magurele) which operates in the X band with high frequency modulation of 100 kHz. The g factors were determined using the Mn^{2+} ion in CaO matrix as a standard.

3 Results and discussions

Asparagine similarly to all amino acids, having an acidic and a basic group, neutralize intramolecularly resulting a zwitterion structure.^[7] Direct ionization of L- α - anhydrous asparagine molecules by gamma radiations it can be detected experimentally only at low temperatures (77 K) and should lead to the formation of a radical cation resulted from the expulsion of an electron followed by the formation of radical anion by the addition of the expelled electron. Regarding L- α - anhydrous asparagine, the location of the electron excess is considered to be on the carboxyl group.^[8] The theoretical and EPR study conducted by Strzelczak et al.^[9] upon irradiated anhydrous asparagines in polycrystalline solid state revealed the existence of three radicals species stable at room temperature. The nature of the radicals was established based on the simulated structures of the three entities, and the theoretical spectrum obtained from the overlapping of the simulated spectra was identical to the experimental one. The structures of the three radicals resulting from the decarboxylation, deamidation and dehydrogenation processes during L- α - anhydrous asparagine irradiation will be presented in the radiolysis mechanism.

In Figure 1 is shown the EPR spectrum of L- α - anhydrous asparagine sample irradiated with a 4.7×10^4 Gy dose.

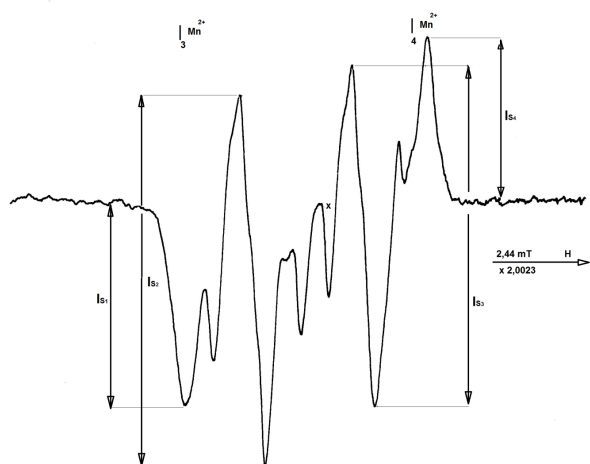


Figure 1. EPR spectrum of polycrystalline L- α - anhydrous asparagine sample irradiated with a 4.7×10^4 Gy dose

From Figure 1 it is found that the spectrum is complex consisting of 7 well solved components, two of the four central lines presenting high intensity. The chemical structure of the three radical entities can not be determined from the EPR spectra. There are two factors that make difficult the establishment of the chemical structure of the three radical species: first, the overlapping of the lines that form the spectrum of each radical and the second essential factor is the appropriate thermal resistance of the radicals. If the three paramagnetic centers had different thermal stabilities then, after heating the irradiated sample to a certain temperature, it would have to be noted the disappearance of some components accompanied by the significant modification of the spectrum structure. As will be seen in this study, this behavior is not found.

In Figure 2 is shown the spectrum of 4×10^4 Gy irradiated sample recorded before and after heating 140 minutes at 180°C .

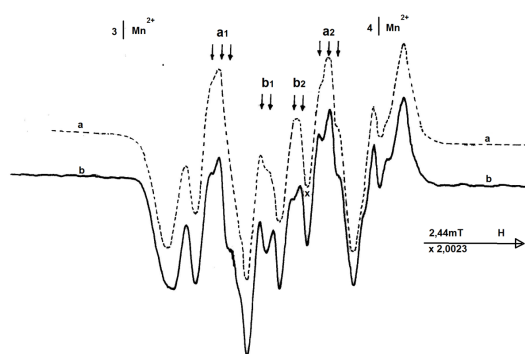


Figure 2. EPR spectrum of irradiated L- α -anhydrous asparagine sample; a) before heating b) after heating 140 minutes at 180°C

The results from Figure 2 is as the following:

- (1) Both the shape of the spectrum and the number of spectral components of the irradiated sample after heating are identical to the unheated sample.
- (2) It is noted that the four central components of the irradiated and heated sample have additional hyperfine splittings, marked with arrows.
- (3) After heating the irradiated sample there is an accentuation of both the hyperfine splittings in three intensive components marked with a_1 and a_2 as well as the two splitting of the central lines marked with b_1 and b_2 .

This behavior, correlated with the kinetic study to be presented, provides arguments for considering that three radical species whose spectra overlap are formed on L- α - anhydrous asparagine irradiation. Since each spectral line could belong to two radicals, in order to perform the kinetic study, the intensity decrease of the four main

components of the spectrum, noted Is_1 , Is_2 , Is_3 , Is_4 (Figure 1) was noted, study conducted with both irradiation dose and heating temperature.

In order to obtain information on the increase of the radicals concentration versus the absorbed dose, it is shown in Figure 3, for example, the intensity variation of the two components Is_1 and Is_2 versus irradiation dose.

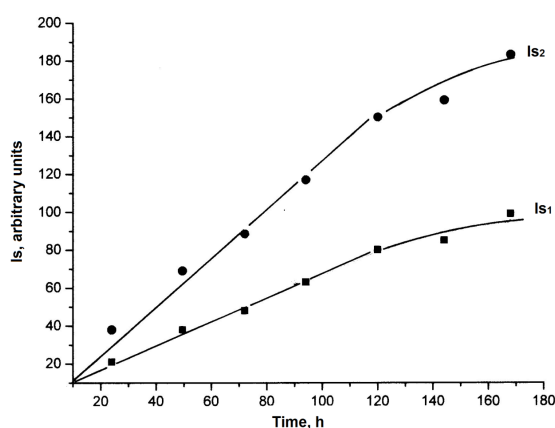


Figure 3. Variation of irradiation times (4.2×10^2 Gy/h dose rate) of Is_1 and Is_2 signals intensities of the EPR spectrum of irradiated L- α -anhydrous asparagine samples

From Figure 3 is noted a linear increase of the two signals intensities versus irradiation dose, up to 5×10^4 Gy, followed by a tendency to a constant value, for high doses. The signals are proportional to the amount of radicals in the irradiated samples. Since the radical concentration is a function of the absorbed dose, the substance may serve as a dosimeter material. The L- α -anhydrous asparagines radicals are unusually stable because of the crystalline form and the pure dry crystals, the signal loss is only about 3% during an year after irradiation. The substance has a linear dose response from 1 Gy up to 5×10^4 Gy. Using EPR spectroscopy as a detection method, the peak to peak amplitude Is_2 and Is_3 of the central spectral lines is proportional to the radical concentration which is proportional to the absorbed dose. The tendency to a constant value after a certain absorbed dose proves that as the concentration of radicals increases, the number of those that disappear under the action of the radiation increases too. When a constant concentration of radicals is reached, this means that the rates of the two processes become equal.

The study of the thermal behavior of the radicals formed during the radiolysis of the L- α -anhydrous asparagine, required firstly, plotting of the reaction isochronous. For this purpose, an irradiated sample with a dose of 4×10^4 Gy of L- α -anhydrous asparagine was gradually heated 5 minutes in stepwise (each step=

10°C), from room temperature up to the temperature of the complete disappearance of radicals.

After each isothermal heating temperature, the EPR spectrum of the sample was recorded under the same conditions at all temperatures. The results are shown in Figure 4.

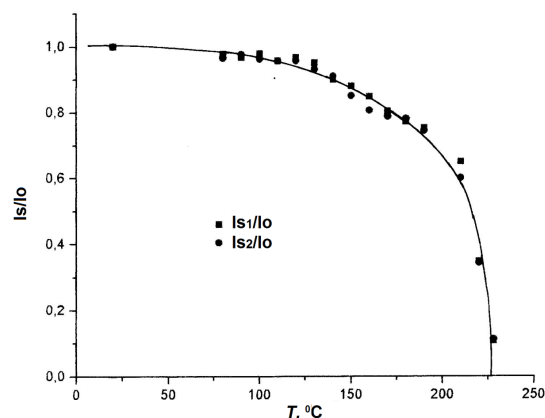


Figure 4. Isochronous variation of the EPR spectrum Is_1 and Is_2 signal intensities (arbitrary units) versus heating temperature of a 4×10^4 Gy irradiated L- α -anhydrous asparagine

Figure 4 shows that although the isochronous heating was performed on a wide temperature range, the radicals disappearance until 150°C does not occur. From this temperature it can be seen a slow decrease of the signals intensities Is_1 and Is_2 and then at 200°C the decrease occurs suddenly, the complete disappearance of the radicals under the mentioned work conditions taking place around 220°C , very close to the melting temperature of the sample.

The kinetic study of the paramagnetic centers thermal annealing required the plotting in isothermal conditions of the radicals concentrations variation, respectively the peak to peak signal intensity of the four components of the spectrum, Is_1 , Is_2 , Is_3 , Is_4 , versus isothermal heating time.

The temperatures for performing the thermal disappearance study of radicals complied with the conditions imposed by the chemical kinetics: for the low temperature the process of disappearance should be at least 50% and for the upper maximum temperature, the rate of radicals disappearance should not be too high in order to carry out a sufficient number of determinations required to establish the reaction order.

In these conditions the intensity of the Is_1 , Is_2 , Is_3 , Is_4 components of the EPR spectra was measured versus the isothermal heating time at 180°C , 190°C , 200°C and 207°C . To highlight the influence of the temperature increase on the thermal annealing rate of the radicals formed in the irradiated samples, it is shown in Figure 5

the decrease of the Is_2 EPR signal versus time, at the four temperatures 180°C, 190°C, 200°C and 207°C.

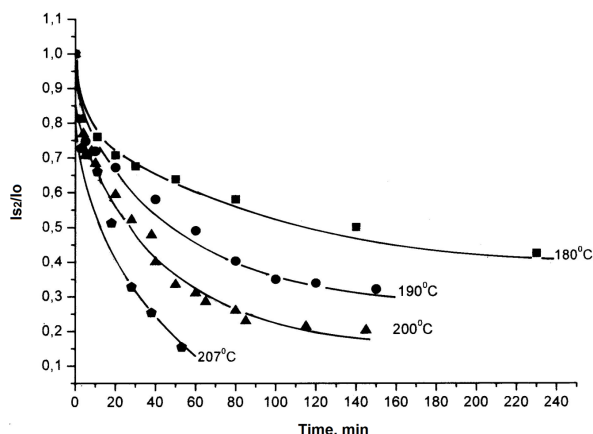


Figure 5. Isothermal variation of the intensity of the EPR signal Is_2 at the temperatures: 180°C, 190°C, 200°C, 207°C

The shape of the curves from Figure 5 shows that the higher the heating temperature of the irradiated samples of L- α -anhydrous asparagines was, the faster was the decrease of the radicals concentration. The four isotherms from Figure 5 present the same shape, firstly showing a high radicals concentration decrease then the tendency to a constant value and finally a decrease as the heating temperature increases. For long times of heating, the radical concentration tends to a constant value, which decreases as the heating temperature increases.

The explanation of the above-mentioned phenomenon is due to the increase of the mobility of the radical species with the temperature rise: when the thermal energy received from the outside exceeds that of the radicals with the surrounding molecules, they detach from the occupied position in the network and enter the reaction through different mechanisms depending on the temperature as will be shown from the kinetic study. The tendency to a constant value shows that at each temperature there are still radicals that do not disappear because they have higher thermal resistance being more strongly trapped in the crystalline network. Similar results were found by performing the same experiment on other irradiated organic substances^[10] and inorganic ones.^[11,12]

In order to study the kinetics of the thermal disappearance of radicals, we tried to fit our data in integral equations, used in chemical kinetics. For determining the reaction order and calculate the rate constants, the integral kinetic equations for all fractional and integers orders were plotted using the graphic method. The reaction order was established from the graphical representation which gave a straight line, the other representations were curves. For calculating the rate constants it was used the dimensionless parameter ($R=Is/I_0$) relative intensity in-

stead concentration, I_0 representing the initial intensity of the signal recorded at room temperature and Is the intensity of the same signal recorded after each isothermal heating time.

Regarding the kinetic study, it should be noted that the establishment of the global reaction order was made from the graphical representation of the kinetic equation which gave a straight line having the highest correlation coefficient. The same order was found at the same temperature for all four components. At 180 °C the highest correlation coefficient was obtained from the R^{-2} graphic representation versus the isothermal heating time. This behavior was found for all four Is_1 , Is_2 , Is_3 , Is_4 spectral components. For example, Figure 6 shows R^{-2} versus to isothermal heating time at 180°C for Is , proving that a third order kinetic was checked.

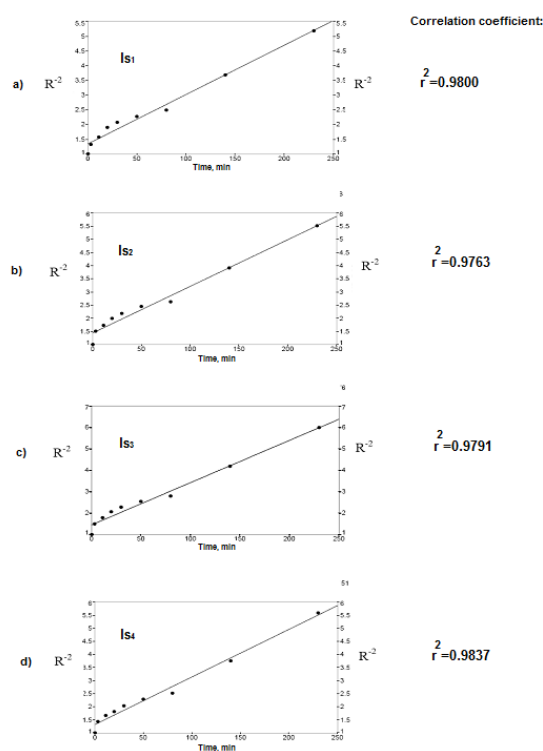


Figure 6. Variation of R^{-2} versus isothermal heating time at 180°C for all four components of the L- α -anhydrous asparagines spectrum

At 190°C, a high correlation coefficient was obtained from the $R^{-3/2}$ representation versus time, proving that the thermal annealing process of the radicals at this temperature verifies a fractional kinetic of 2.5 order (Figure 7).

At 200°C, the R^{-1} representation versus the heating time for all four components straight lines were obtained, proving that the thermal disappearance of the radicals follows a second order kinetics. Figure 8 exemplifies this

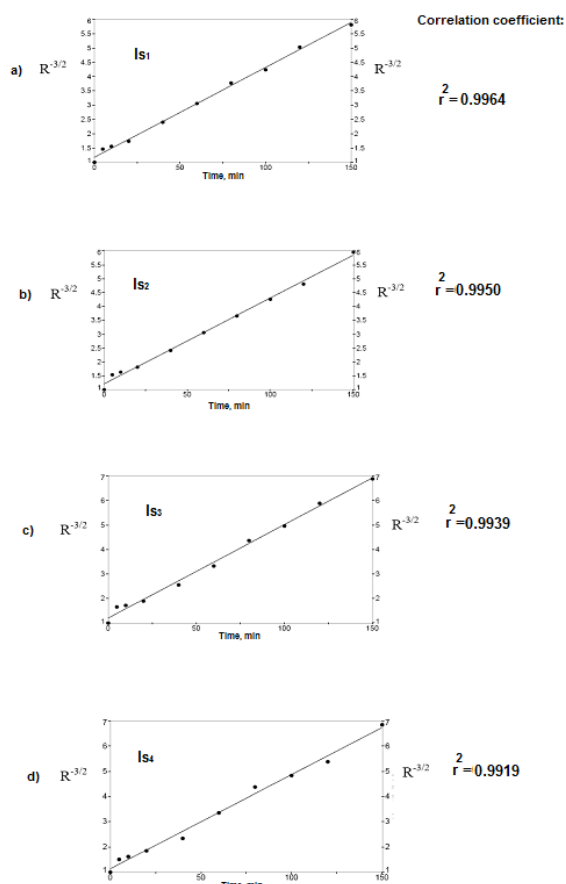


Figure 7. Variation of $R^{-3/2}$ versus isothermal heating at 190°C, for L- α -anhydrous asparagine sample, irradiated with a 7×10^3 Gy dose

representation.

At 207°C, the logarithm of the relative intensity ($\ln R$) plot versus time for the four components shown in a straight lines, confirming that the disappearance of the radical entities present a kinetic of the first order (Figure 9).

The values of the rate constants calculated from the slopes of the graphical representations corresponding to the global reaction orders: 3, 2.5, 2, 1 for all four spectral components are listed in Table 1.

The results of the kinetic study lead to the following conclusions:

(1) The most important finding is the variation of the reaction order. As the temperature rise from 180°C to 207°C, over a temperature range of about 30°C, the reaction order gradually decreased from 3 to 1.

(2) The high reaction orders of 3, 2.5, 2 from 180°C, 190°C, 200°C prove that the process of the radicals thermal disappearance is complex, this means that the three radicals disappear through several reactions involving

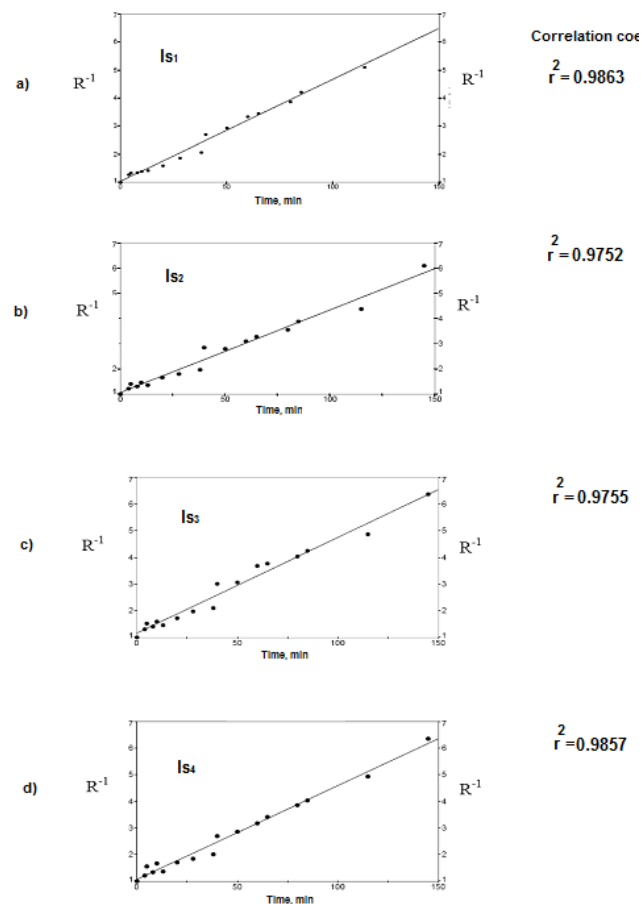


Figure 8. Variation of R^{-1} versus isothermal heating time at 200°C for L- α -anhydrous asparagine sample, irradiated with a 7×10^3 Gy dose

different mechanisms.

(3) The high values of the reaction orders (3, 2.5, 2) found, evidenced that three radical entities are formed on L- α -anhydrous asparagine irradiation.

The results of the thermal disappearance study of the trapped radicals in the crystalline lattice that occurs when heating the irradiated samples proves that the rate of this process, namely the reaction order, is influenced by the nature of the radicals having different thermal resistance. Indeed, increasing the temperature, the vibration of the crystalline network constituents increases and produces the weakening of the bonds that keep the radicals trapped.

Due to the large variation of the reaction order, the activation energy corresponding to a particular order could not be determined over a narrow temperature range.

At 170°C, none of the known kinetics equations were verified. This behavior is a particular case, proving that the thermal annealing process is strongly influenced by a physical one, which best respects the so-called “step-disappearance”.[13]

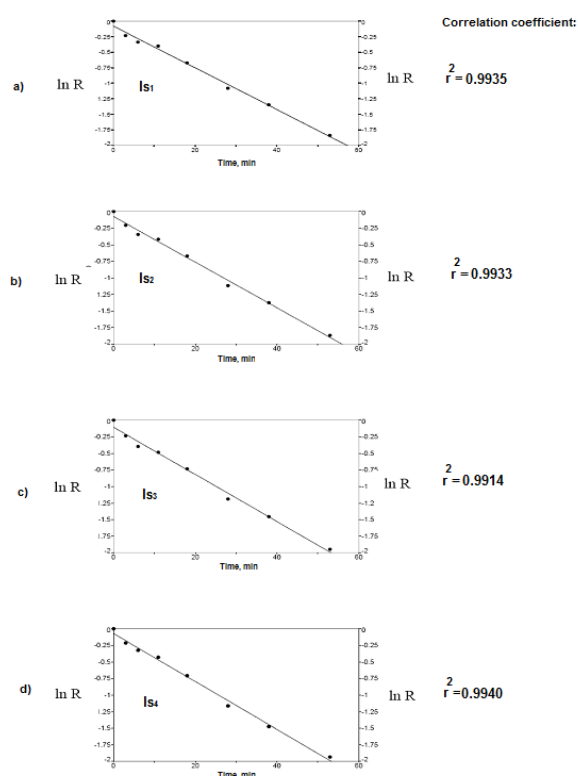


Figure 9. Variation of $\ln R$ versus isothermal heating time at 200°C for L - α -anhydrous asparagine sample, irradiated with a 7×10^3 Gy dose

The kinetic of the disappearance of the radicals formed in the irradiated samples of L - α -anhydrous asparagine and heated to 170°C could be explained using “the steps” model developed by Waite.^[14, 15] According to this model, the disappearance of radicals with a random space distribution involves two processes: a rapid process of recombination of nearby entities and a slow recombination process that occur due to vacancies formed after the diffusion of radicals within the network. Both processes lead to two approximations that are in the form

of the second order kinetic equations. For example, the variation of the R^{-1} ratio versus the isothermal heating time at 170°C for the four main components of the EPR spectrum is shown in Figure 10.

From the slopes of the straight lines plotted, the rate constants k_{A1} , k_{A2} , k_{A3} , k_{A4} for the rapid process and k_{B1} , k_{B2} , k_{B3} , k_{B4} for the slow process were calculated, the values are listed in Table 2.

Comparing the values of the rate constants determined according to the Waite model^[14] for the four intense components of the EPR spectrum, the following observations are noted:

- (1) The initial recombination process of the radicals is about 10 times faster than that of diffusion ($k_A - 10$ kB)
- (2) The values of the rate constants corresponding to the components $Is_1 - Is_4$, $Is_2 - Is_3$ respectively, are close to one another.
- (3) The rate constants of the Is_2 and Is_3 central components of the initial thermal annealing process have higher values than the initial ones of the Is_1 and Is_4 side components.

In conclusion, the high values of the reaction order presented above, highlighted at 170°C kinetic study, are reliable proof that the ESR spectra of the L - α -anhydrous asparagines irradiated samples result from the overlapping spectra of three stable radicals at room temperature, specified in the radiolysis mechanism.

4 Radiolysis mechanism

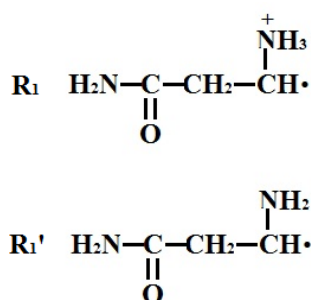
In the literature there is only one information regarding the radical species formed on L - α -anhydrous asparagines irradiation^[9] which contains the overlapping simulated three radicals spectra with the experimental EPR spectrum.

Direct ionization of the L - α -anhydrous asparagine molecule leads to the formation of the $\text{Asn}(\text{COO})^{\cdot+}$ radical cation having the spin density located on the deprotonated carboxyl group. This species being unstable, dis-

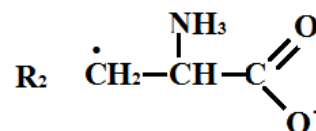
Table 1. The orders and rate constants of reactions of thermal disappearance of the paramagnetic centers formed on the irradiation of the L - α -anhydrous asparagine, calculated for the four main components of the ESR spectrum at 180°C , 190°C , 200°C and 207°C

Temp, $^\circ\text{C}$	Reaction order	k (s^{-1})				k , (s^{-1})
		Is_1	Is_2	Is_3	Is_4	
180°C	3	2.78×10^{-4}	2.95×10^{-4}	3.28×10^{-4}	3.01×10^{-4}	$3.00 \times 10^{-4} \pm 0.21$
190°C	2.5	5.23×10^{-4}	5.15×10^{-4}	6.35×10^{-4}	6.21×10^{-4}	$5.73 \times 10^{-4} \pm 0.63$
200°C	2	6.05×10^{-4}	5.45×10^{-4}	5.96×10^{-4}	5.85×10^{-4}	$5.82 \times 10^{-4} \pm 0.26$
207°C	1	5.58×10^{-4}	5.73×10^{-4}	5.91×10^{-4}	6.01×10^{-4}	$5.80 \times 10^{-4} \pm 0.19$

appears following two pathways that produce decarboxylation: first is CO₂ expulsion from the Asn(COO)^{·+} paramagnetic center accompanied by R₁ radical formation, a process that can occur simultaneously with ionization, the second pathway is the deprotonation of the amino group of Asn(COO)^{·+} radical followed by formation of the unstable radical Asn(COO)[·] which decarboxylates to form the radical R₁'. The decarboxylation process is experimentally proven highlighting the CO₂ formation in L- α -anhydrous asparagine irradiated samples using gas chromatography method.^[9]



The existence of the two intense components of the EPR spectrum marked with a₁ and a₂ (Figure 2) could be attributed to the R₁ and R₁' entities since the odd electron located on the carbon atom interacts with α proton. After heating the irradiated sample, it is noted the three hyperfine splitting accentuation of the two spectral lines proving the increase of the odd electron interaction with the nitrogen atom of the amino group linked to the same carbon atom. The mass spectrometry study of similar structure amino acids by Zubavichus^[16] led to the conclusion that the most unstable functional group of L- α -anhydrous asparagine is the amidic one. The direct ionization of L- α -anhydrous asparagine^[9] also leads to the radical cation Asn (CONH₂)^{·+} formation having the spin density located on the amide group. This entity is unstable and the analysis of the experimental data obtained by ESR method as well as the calculation of the bonds dissociation energies^[9] showed that the Asn (CONH₂)^{·+} radical cation performs the amide bond cleavage leading to R₂ radical formation.



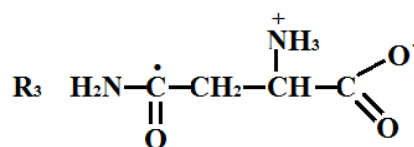
In the EPR spectrum of the irradiated L- α -anhydrous asparagine samples it was found the formation of four components resulted from the splitting of the two central lines marked with b₁ and b₂. This finding could be an argument to assign them to the R₂ radical due to the odd electron interaction with two non-equivalent α protons.

The radiolysis mechanism of L- α -anhydrous asparagine irradiated in the solid state at room temperature must include the formation of a radical anion.

In the primary interaction process, the expelled electron from the ionizing radiation after thermalization is captured by the carboxyl group of a parent molecule located at a certain distance from the ionizing site to form the Asn^{·-} anion.

In this radical, having an excess of negative charge, mutually neutralizes the positive charge of the amino group with the additional electron of the carboxyl group.

The entity resulting from this intramolecular electron transfer being unstable removes a hydrogen atom from the amino group. The hydrogen atom due to high reactivity extracts another hydrogen atom from the methylene group of the L- α -anhydrous asparagine molecule chain to form the deprotonated radical having the R₃ structure.^[17]



The formation of the R₃ radical is sustained by Strzelczak simulated spectrum.^[9] In the present study, the formation of radicals R₁, R₂, R₃ through L- α -anhydrous asparagine irradiation at room temperature is justified by the kinetic results of thermal disappearance characterized by superior and fractional order: the third order kinetics

Table 2. The rate constants for the radicals disappearance at 170°C second order kinetics, according Waite model

170 °C							
I _{s1}	I _{s4}	I _{s1}	I _{s4}	I _{s2}	I _{s3}	I _{s2}	I _{s3}
k _{A1} × 10 ⁴	k _{A4} × 10 ⁴	k _{B1} × 10 ⁵	k _{B4} × 10 ⁵	k _{A2} × 10 ⁴	k _{A3} × 10 ⁴	k _{B2} × 10 ⁵	k _{B3} × 10 ⁵
3.15	3.05	2.71	2.87	4.01	4.33	1.74	2.43

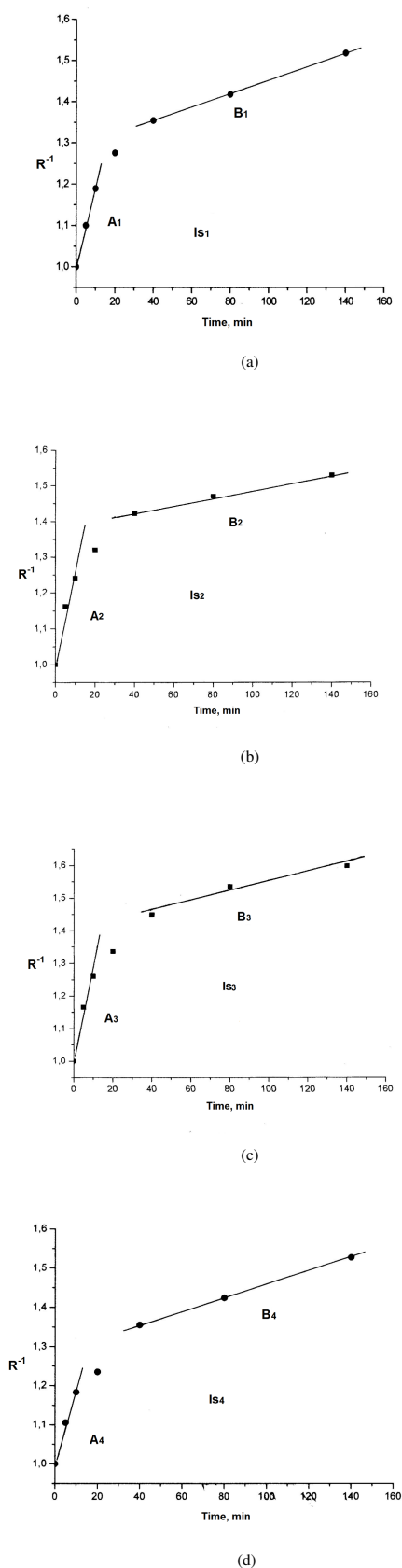


Figure 10. Variation of the R^{-1} ratio versus the isothermal heating time at 170°C for the disappearance of the radicals formed in L- α -anhydrous asparagines sample irradiated with a dose rate of 3×10^4 Gy, (a) Is₁, (b) Is₂, (c) Is₃, (d) Is₄

at 180°C proves the involvement of the three radicals. Decreasing the reaction order with temperature increasing is due to the disappearance of radicals with higher thermal resistance.

5 Conclusions

The primary process of L- α - anhydrous asparagine radiolysis consists in formation of two radical species: a radical cation and a radical anion. Theoretical and EPR study^[9] have shown that at room temperature the radical cation decarboxylates and deamides to form the radicals R_1 and R_2 , and the radical anion expels a hydrogen atom from the amide group and generates the radical R_3 . From the present study of the thermal annealing of radicals it was found the following kinetic behaviors: higher reaction order and their gradual decrease from 3 to 1 with increasing temperature. The results of the kinetic study are a confirmation of the three radical species formation reported in the literature.

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