December, 1984] © *1984 The Chemical Society of Japan* *Bull. Chem. Soc. Jpn.*, *57*, *3403—3407 (1984)* 3403

Pulse Radiolysis of Cycloserine in Aqueous Solutions. One-Electron Oxidation by OH Radicals

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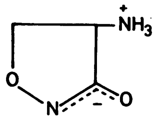
(Received May 23, 1984)

The reaction of (*R*)-4-amino-3-isoxazolidinone (cycloserine) with OH radicals was studied in aqueous solutions by optical pulse radiolysis at pHs 6.5—12. It was concluded, from a comparison of the transient spectra with those obtained in a reaction with N_3 radicals, that OH radicals attacked at the dissociated peptide group, $-\mathrm{N}^{-}\mathrm{CO}-$, and an oxidized radical, $-\dot{\mathrm{N}}\mathrm{CO}-$, was produced through one-electron oxidation. From a kinetic analysis, the reaction was considered to have proceeded by two steps: The first step was the formation of OH adducts, where the rate constant, $k(\mathrm{OH}+\text{cycloserine})$, was $8\times10^9\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}$ for the zwitterion form of cycloserine at pH 6.5 and $1.2\times10^{10}\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}$ for anion form at pHs 8—10. The second step was the formation of oxidized radicals at a rate of $3—4\times10^6\,\mathrm{s^{-1}}$. These radicals decayed with a second-order rate, suggesting a radical-radical recombination. D-Serine was detected as the major product.

The hydroxyl (OH) radical is one of the most reactive species generated in irradiated water. A number of investigations have been carried out on its reaction with peptides in aqueous solutions, and it has been established that the abstraction of hydrogen from the carbon adjacent to the peptiede nitrogen is the major reaction for linear peptides as well as cyclic dipeptides.[1–6] For the series of five-membered cyclic compounds containing one peptide linkage, such as pyrrolidinone derivatives, the reaction of OH radicals is known to be essentially the same as that on linear peptides.[7] Cycloserine, 4-amino-3-isoxazolidinone (I), which is one of the broad-spectrum antibiotics and is known as an effective inhibitor of transaminase enzyme systems, has a cyclic structure containing one peptide linkage. The sturcture of cycloserine in aqueous solution is a zwitterion as described in (I), the p*K* values of which are 4.4 and 7.4, respectively.[8] Therefore, it is expected that this compound has a high reactivity toward OH radicals and that the OH radicals will attack the dissociated pepetide groups at around biological pHs. In this work we investigated the site of attack of OH radicals and the reaction mechanism of intermediates by studying the transient spectra and kinetics with pH dependence of them.



Cycloserine (I)

Experimental

The 45 MeV linac of Hokkaido University, producing 10 ns pulses of 45 MeV electrons, was used for the optical pulse radiolysis experiments. The dose per pulse was 80—100 Gy, which was measured by the KCNS method. The optical detection system was basically the same as that designed by Hunt and Thomas.[9] The light from a high-pressure mercury lamp (HB-50101, Ushio) was chopped by a rotating chopper at 25 Hz and focused at an irradiation cell of which the optical path length was 2 cm. The absorption signals, after passing through a monochromator, were detected by a photomultiplier (R928, Hamamatsu TV), then followed on a transient digitizer (DM901, Iwatsu) and recorded on an X-Y recorder. The range of wavelengths examined was 270 to 350 nm.

The (*R*)-cycloserine was supplied by Aldrich. NaN_3 and other reagents were supplied by Wako Junyaku. All reagents were used without further purification. Solutions were prepared by using triply distilled water the day before the experiment and kept in a refrigirator. The pH values of the solutions were adjusted by adding sodium hydroxide and were determined with a pH meter (pH501, Yokogawa). Solutions were saturated with N_2O gas (1 atm, $(N_2O)=2.5\times10^{-2}$ mol dm^{-3}) just before irradiation in order to prevent the reaction by e_{aq}^-, in which N_2O converts them into OH radicals.

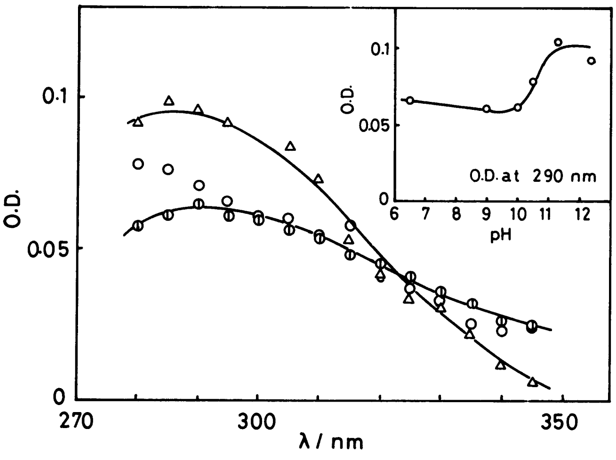


Fig. 1. The transient spectra produced on pulse radiolysis of 10^{-3} mol dm^{-3} cycloserine in presence of N_2O at pH 6.5 (○), 9.0 (⦶), and 12.4 (△). The insert shows the pH dependence of the absorbance at 290 nm.

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The radiolytic yield of serine, which was the major product, was determined as follows. A solution of 10^{-3} mol dm^{-3} cycloserine (saturated with N_2O) was irradiated by the γ rays of ^{60}Co for 10 min at a dose rate of 3×10^3Gy/h. The total absorption dose corresponded to a 10% conversion. Then, the solution was dried in a vacuum and the residue was dissolved in a buffer solution and passed through a conventional automatic aminoacid analyzer (JLC-6AH, JEOL). Upon elution with a buffer solution (pH 3.2), serine and 3-(aminooxy)alanine (which was produced by the hydrolysis of cycloserine in an acidic buffer solution) were separated. Cycloserine was separated by the buffer solution of pH 7.3. The evaluations of G(serine) and G(−cycloserine) were made after the correction for 3-(aminooxy)alanine. The optical isomerism of serine was examined by IR spectra using KBr discs of the residues. In this case, the N_2O saturated solutions of 2×10^{-2} mol dm^{-3} were γ-irradiated for 10 h at a dose rate of 3×10^3Gy/h and dried. The irradiation time was chosen so that most of the cyclsoerine could be decomposed.

Results

Transient Absorption Spectra. The absorption spectra were obtained 1.25 μs after a pulse for solutions containing 1.0×10^{-3} mol dm^{-3} cycloserine with various pH values from 6.5 to 12 and are presented in Fig. 1. The spectra, except the one at pH 6.5, have a broad maximum at 290 nm. The band maximum was not observed in the spectrum at pH 6.5. The insert in Fig. 1 shows the change of absorbance at 290 nm with pH. The increase in absorbance was observed above pH 10. The spectra shown in Fig. 1 were compared with those obtained in a reaction with N_3 radicals. The concentration of the N_3^- ion added was 10^{-2} mol dm^{-3}, and the spectra were obtained 10 μs after a pulse in order to avoid the contribution of the absorption due to the remaining N_3 radicals. Transient spectra are shown in Fig. 2. All the spectra at pH 6.5 to 12 showed broad absorption maxima at around 290 nm and were very similar to the spectra shown in Fig. 1. However, as shown in the insert of Fig. 2, the absorbance at 290 nm did not change within the whole pH range examined.

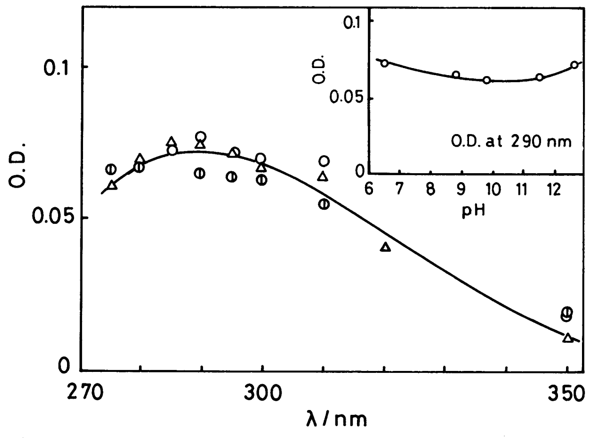


Fig. 2. The transient spectra produced on pulse radiolysis of 10^{-3} mol dm^{-3} cycloserine with 10^{-2} mol dm^{-3} NaN_3 in presence of N_2O at pH 6.5 (O), 9.0 (Φ), 12.4 (Δ). The insert shows the pH dependence of absorbance at 290 nm.

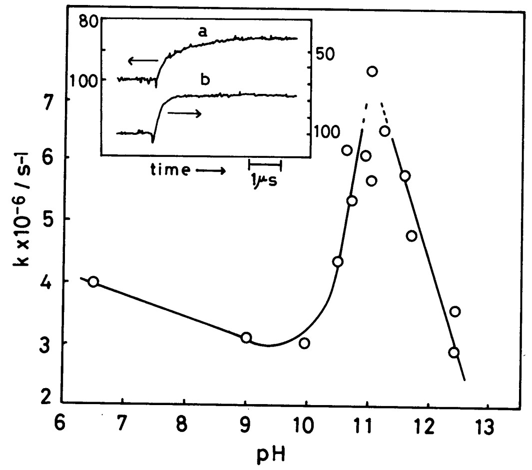


Fig. 3. The pH dependence of rate of formation of the intermediates measured at 290 nm on pulse radiolysis of 10^{-3} mol dm^{-3} cycloserine. The insert shows the example of signal traces at pH 9.9 (a) and 11.3 (b). The ordinates indicate the transmission, %.

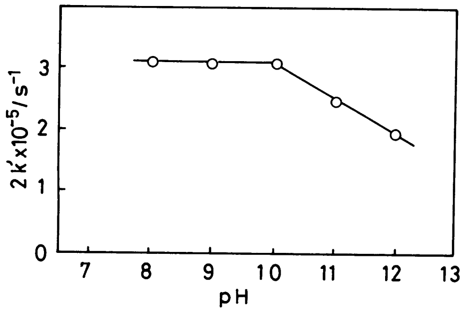


Fig. 4. The pH dependence of the decay rate measured in absorbance on pulse radiolysis of 10^{-3} mol dm^{-3} cycloserine. The true second-order decay rate, $2k$, is related with $2k'$ as follws: $2k=2k'\varepsilon_{290}l$, where ε_{290} is the molar extinction coeffident of intermediate at 290 nm and l is the optical path length.

Kinetics of the Formation and Decay of Intermediates. The rates of formation of transient species, shown in Fig. 1, were determined from the growth of the absorption signals at 290 nm for solutions of various pHs. The results are shown in Fig. 3. The insert presents a typical example of the signal traces of growth after pulse. The first-order rate constants are 3—4×10^6 s^{-1} between neutral and pH 10, increased between pH 10 and 11 and decreased above pH 11. It appeared that the base-catalyzed reaction, or deprotonation, occurred between pH 10 and 11.

The absorbance at 290 nm decayed by second-order kinetics. The pH dependence of the relative decay rates is shown in Fig. 4. The rates decreased above pH 10. The intermediates observed in the reaction of N_3 radicals also decayed by a similar second-order rate.

Determination of k*(OH+cycloserine).* The reaction rates of OH radicals with cycloserine were determined for solutions of various pHs by using the KCNS method.[10,11] The concentrations of cycloserine and KCNS used were 0.5 to 1.0×10^{-3} mol dm^{-3} and

1.0×10^{-3} mol dm^{-3}, respectively. The absorption due to the $(CNS)_2^{\cdot-}$ radical ions was measured at 475 nm. As the observed rates of growth of $(CNS)_2^{\cdot-}$ did not show simple first-order kinetics, the rate constants were evaluated by fitting the theoretical kinetic expressions to observed data. When the solution contained both cycloserine and CNS$^-$, the following competitive reactions occurred:

$$\mathrm{OH} + \mathrm{CNS}^- \xrightarrow{k_1} \mathrm{CNS} + \mathrm{OH}^-, \tag{1}$$

$$\mathrm{CNS} + \mathrm{CNS}^- \xrightarrow{k_2} (\mathrm{CNS})_2^{\cdot-}, \tag{2}$$

$$\mathrm{OH} + \text{cycloserine} \xrightarrow{k_c} \text{product}, \tag{3}$$

where k_1, k_2, and k_c are the rate constants in these reactions. The time dependence of the concentration of $(CNS)_2^{\cdot-}$ after pulse is given by the following equation, which is derived from the competitive reaction mechanism involving Reactions 1, 2, and 3,

$$\{(\mathrm{CNS})_2^{\cdot-}\}_t = \frac{k_1'k_2'(\mathrm{OH})_0}{k_2'-(k_c'+k_1')}\left\{-\frac{e^{-(k_c'+k_1')t}}{k_c'+k_1'}+\frac{e^{-k_2't}}{k_2'} + \frac{k_2'-(k_c'+k_1')}{k_2'(k_c'+k_1')}\right\}, \tag{4}$$

where $k_1'=k_1(\mathrm{CNS}^-)$, $k_2'=k_2(\mathrm{CNS}^-)$, and $k_c'=k_c$(cycloserine), and $(\mathrm{OH})_0$ is the initial concentration of OH radicals which was estimated from the maximum absorbance at 475 nm reached after pulse in the solution without cycloserine. The values of k_1 and k_2 were determined by fitting Eq. 4 with $k_c'=0$ to the observed traces of the build-up signal in the solution containing only CNS$^-$ ions. These values are k_1=1.0×10^{10} mol^{-1}dm^{3}s^{-1} and k_2=6×10^9 mol^{-1}dm^{3}s^{-1}, respectively, which agreed well with reported values.[12] The values of the k_c's are determined by fitting Eq. 4 to the observed traces of growth in solutions containing CNS$^-$ and cycloserine with various pHs. The results are listed in Table 1. The data of pH 12 was estimated by assuming the k_1=1.0×10^9 mol^{-1}dm^{3}s^{-1}.[10] The rates obtained at pHs 8 to 11 are 1.2—1.3×10^{10} mol^{-1}dm^{3}s^{-1}, and did not show the increase between 10 and 11 as observed in the formation rates in Fig. 3. The slightly slow rate obtained at pH 6.5 is reasonable because the net charge is zero at this pH.

Detection of Serine. The radiolytic yield of serine, G(serine), was determined to be 2.5 at pH 6.5—10, and 1.0 at pH 11. The yield of the decrease of cycloserine, G(−cycloserine), was also determined to be approximately 10.0 at pH<10. The IR spectra of the dried residues of the irradiated solution in which cycloserine mostly decomposed showed that the main product was D-serine.

Table 1. The rate constants of the reaction of OH radicals with cycloserine (×10^{10} mol^{-1} dm^3 s^{-1})

pH	k_1	k_2	k_c
6.5	1.0	0.65	0.8
9.0	1.0	0.65	1.2
10.0	1.2	0.65	1.3
11.3	1.2	0.65	1.2
12.5	0.1		0.13*

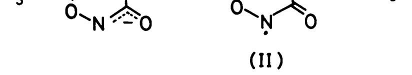
* This value was calculated by the comparison of absorbances at 475 nm in the experiment for only CNS$^-$ with that for CNS$^-$+cycloserine, assuming k_1=1.0×10^9 mol^{-1}dm^{3}s^{-1} as calculated by Simic and coworkers.[1—3]

Discussion

Upon the irradiation of solutions containing N$_3^-$ ions, N$_3$ radicals are known to be produced from a reaction of OH radicals with the N$_3^-$ ions,[13] as follows:

$$\mathrm{N_3^-} + \mathrm{OH} \rightleftharpoons \mathrm{N_3} + \mathrm{OH^-},$$
$$k(\mathrm{N_3^-}+\mathrm{OH}) = 1.2\times10^{10}\,\mathrm{mol^{-1}\,dm^3\,s^{-1}}. \tag{5}$$

It is also known that the reaction of N$_3$ radicals is exclusively one-electron oxidation and it is inert toward aliphatic H atoms, whereas the typical reaction of OH radicals is radical addition or H-atom abstraction. The intermediate, which is produced through one-electron oxidation of the isoxazolidinone ring by N$_3$ radicals, should be the isoxazolidinone radical (II) as described in Reaction 6.



(6)

(II)

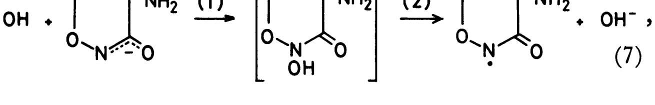
Under our experimental conditions, the production of N$_3$ radicals was completed within a pulse. The contribution of the absorption due to the N$_3$ radical (absorption maximum is 270 nm) to the transient spectra was negligible 10 μs after the pulse. Thus, the species responsible for the absorption band shown in Fig. 2 was the oxidized radical (II). Since the transient spectra at pH 8 to 10 shown in Fig. 1, where cycloserine exists as anion, are very similar to those in Fig. 2, we attributed the absorption band in Fig. 1 to the oxidized radical (II). Therefore, the site of attack of OH radicals was the dissociated peptide group, -N$^-$CO-, and the radicals were produced through one-electron oxidation by OH radicals. The spectrum at pH 6.5 was slightly different from others. Taking into account the slow k(OH+cycloserine), this would be induced by the lowered selectivity of OH radicals to the peptide group owing to the protonated amino groups, producing different species.

The absorbance at 290 nm in Fig. 1 and the rate of formation in Fig. 3 increased at the same pH range, pH 10—11, in the reaction of OH radicals, whereas these changes were not observed in the reaction with N$_3$ radicals. These results suggest that the change in the site of attack of OH radicals occur within this pH range. Since this pH range corresponds to the pK of the OH radical, $\mathrm{OH} \rightleftharpoons \mathrm{O^-}+\mathrm{H^+}$, p$K$=11.9,[14] H-atom abstraction by O$^-$ is expected to occur. As shown in Fig. 4, the rate of radical recombination was also found to decrease at this pH range, which suggested the formation of radical anions as intermediates in this pH region. There is one possibility that the amino groups were attacked by O^- or OH radicals, producing the anion radicals, since dehydrogenation of N–H bonds by these radicals in aliphatic amines on γ-radiolysis[15] and in simple aminoacids on pulse radiolysis[16] in alkaline solutions has been reported. Taking into account the increase in the rate-of-formation of the intermediates, it is also conceivable that the dehydrogenation by OH or O^- radicals occurred on the carbon adjacent to the amino group. In this case, the amino groups may be prompted to deprotonate and the intermediates of anion form could be produced. It is known that the peptide hydrogen adjacent to a radical carbon can dissociate upon pulse radiolysis of the derivatives of 2,5-piperazindione, in which the pK value of the radical is 9.6.[3] The result that pH dependence was observed in the rates-of-formation of the oxidized radical, as shown in Fig. 3, whereas the values of k(OH+cycloserine) were independent of pH, as shown in Table 1, implies that the oxidized radicals observed in Fig. 1 were not formed immediately as the result of the attack of OH radicals, but were formed from a precursor, OH adducts of cycloserine, and therefore k(OH+cycloserine) represents the rate of the formation of OH adduct. This implication is supported by the fact that the rate-of-formation of the oxidized radicals did not change significantly in 10^{-2} mol dm^{-3} solutions.

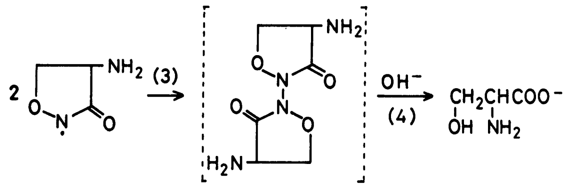
As the oxidized radicals decayed by second-order kinetics, the reaction mechanism of these species was considered to be predominantly through radical-radical recombination. Probably, two oxazolidinone rings become linked through N atoms to lead to the formation of a hydrazine-type bond. This was assumed from the analogy with the previous report that the oxidation of *N*-acyl-*O*-alkylhydroxylamine, $RCONHOR^-$, resulted in the formation of a hydrazine-type compound through N–N coupling.[17]

In this experiment, the value of G(OH) was the sum of $G(e_{aq}^-)$ and the original G(OH), that is, 5.6. If the OH radicals react with cycloserine quantitatively and the decomposition of one hydrazine-type intermediate produce two serine molecules, G(serine) and G(−cycloserine) will be equal to G(OH). However, the radiolytic yield of serine was roughly a half of G(OH) and G(−cycloserine) was higher than G(OH). Thus, the one molecule of serine would be formed through the deomposition of one dimerized intermediate, and some additional cycloserine molecules would react secondarily with the entity which remained after the elimination of serine from the dimerized species.

On the basis of the above considereation, the whole reaction scheme of the radiolysis of the cycloserine anion by OH radicals can be described according to reactions (7) and (8) at a pH range from 8 to around 10:



(7)



(8)

The rate constants of step (1) and (2) were 1.2×10^{10} mol^{-1}dm^{3}s^{-1} and 3—4×10^6s^{-1}, respectively. The second-order rate constant of step (3), $2k$, is equal to the products of the apparent rate constant, $2k'$, the molar extinction coefficinent of the oxidized radical (II) at 290 nm, ε_{290}, and the optical path length of the cell. The value of $2k'$ was 3×10^5s^{-1}, as shown in Fig. 4. The value of ε_{290} was roughly estimated to be 630 mol^{-1}dm^{3}cm^{-1} by using the absorbance of the oxidized radical in the reaction of N_3 radicals observed in Fig. 2 and the initial concentration of OH radicals which was estimated to be 5.6×10^{-5} mol dm^{-3} in the pulse of 100 Gy. The resulting rate constant of step (3) was $2k=3.8\times10^8$ mol^{-1}dm^{3}s^{-1} at pH range of 8 to 10.

It is considered that complex processes, such as the attack of OH or O^- radicals on multiple sites, may be occurring at a pH above or around 11.

The authors wish to thank Dr. Takeaki Enoto and Messers. Hiroaki Tanida and Koichi Sato for the help in operating the linac, and Miss Yumiko Misu for the analysis of aminoacids.

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