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Author(s): M. D. Shattuck, Y. Ma, M. Itoh, H. Shields

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An ESR Study of Radical Kinetics in L- α -Amino-*n*-butyric Acid Hydrochloride Containing L-Cysteine Hydrochloride

M. D. SHATTUCK, Y. MA, M. ITOH, AND H. SHIELDS

Wake Forest University, Department of Physics, Winston-Salem, North Carolina 27109

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On annealing at temperatures near 100°C, carbon-centered radicals migrate to sulfur-centered radicals in X-irradiated crystals of L- α -amino-*n*-butyric acid hydrochloride, CH₃CH₂CH(NH₃-Cl)COOH, containing L-cysteine hydrochloride, SHCH₂CH(NH₃Cl)COOH. Samples containing 0, 0.5, 1.0, and 1.5% L-cysteine hydrochloride were studied. When no cysteine is present, the carbon-centered radical formed by X irradiation, CH₃CH₂ĊOOH, decays according to a second-order diffusion-controlled rate equation. In samples containing cysteine, the same carbon-centered radicals are formed, but on annealing, they migrate to cysteine, where a perthiyl radical, RSS, is formed. The transfer of carbon-centered radicals to perthiyl radicals follows a pseudo first-order rate equation with an activation energy of 1.15 eV. A decrease in the initial concentration of the carbon-centered radicals or an increase in the initial concentration of cysteine results in an increase in the transfer efficiency. The rate of growth of the perthiyl radical depends on both the initial concentration of cysteine and the initial concentration of carbon-centered radicals. The pseudo first-order rate constant increases when either the initial carbon-centered radical concentration increases or the initial cysteine concentration increases. The mechanism by which radicals move from one lattice site to another in the crystalline material is most likely hydrogen abstraction from a neighboring molecule. © 1989 Academic Press, Inc.

INTRODUCTION

When proteins in the solid state are exposed to ionizing radiation, free radicals resulting from broken bonds are stabilized. An unusually high percentage of sulfur-centered radicals form in proteins containing a small percentage of cysteine or cystine (1, 2). The understanding of the pathways for the formation of the sulfur-centered radical in irradiated proteins is incomplete (3).

In this study we have chosen a simple crystalline amino acid system (L- α -amino-*n*-butyric acid hydrochloride, CH₃CH₂CH(NH₃Cl)COOH, containing cysteine hydrochloride, SHCH₂CH(NH₃Cl)COOH, as an impurity) in which we can study the migration of radiation damage from a host molecule to a sulfur-containing impurity. Cysteine and α -amino-*n*-butyric acid molecules are identical except for the SH in cysteine and the CH₃ group in amino-*n*-butyric acid at the termination of the hydrocarbon chain. In this mixed material, the concentration of the sulfur impurity can be controlled. Although the crystalline structure of the mixed amino acid system is not the same as that of a protein, an understanding of radical transfer from one amino acid component to another should aid in understanding electron or radical transfer

in proteins. We find that the percentage transfer from an initial carbon-centered radical to a perthiyl radical depends strongly on both irradiation dose and the concentration of cysteine (the sulfur component). Previous studies of the kinetics of radicals in irradiated amino acids have been on single-component systems (4-8).

EXPERIMENTAL PROCEDURE

L- α -Amino-*n*-butyric acid and L-cysteine were obtained from Sigma Chemical Co. and used without further purification. Amino-butyrlic acid hydrochloride samples containing small percentages of cysteine hydrochloride were prepared by dissolving amino butyric acid and cysteine in 5 *N* HCl and then evaporating the solution in a nitrogen atmosphere to prevent cysteine from oxidizing to cystine. The solution contained three HCl molecules for each molecule of amino-butyrlic acid and cysteine. The concentration of the L-cysteine hydrochloride in the L-amino-butyrlic acid hydrochloride matrix was determined by Galbraith Laboratories (Knoxville, TN).

The polycrystalline samples were exposed to 50 kV X rays and then evacuated to a pressure of about 4×10^{-5} torr before annealing in an oven. After annealing, the samples were transferred to a 9 GHz Varian E-line spectrometer interfaced to a 9845 HP computer. The spectra were observed at room temperature and the relative number of radicals was obtained by double integration of the observed first derivative spectra. The number of radicals in some of the samples was determined using the Varian strong pitch sample as a standard.

An experiment was done to eliminate the possibility that the cysteine component was on the surface of the polycrystalline material rather than included as a substitutional impurity within the crystallites. A single crystal containing L-cysteine hydrochloride was first irradiated, and then the ESR spectrum was observed. Next, the outer surface was dissolved away, and then the ESR spectrum of the crystal with the new surface was observed. The ESR spectra before and after the removal of the surface were the same. These experiments indicate that cysteine is found in the interior of the crystallites, most likely at the lattice sites of the butyric acid.

The single crystal ESR spectra of the cysteine component had the expected orientation dependence of the crystal with respect to the external magnetic field (9). The specific orientation dependence of the spectra provides further support for the substitution of cysteine for butyric acid in the host lattice.

CARBON-CENTERED RADICAL RECOMBINATION

The semi-stable radical at room temperature in L- α -amino-*n*-butyric acid hydrochloride after X irradiation is $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ (9-11). The spectrum of the polycrystalline material used in this study is essentially a six-component spectrum attributed to the interaction of the unpaired electron on the α -carbon with the α -hydrogen and two β -hydrogens. The unequal coupling with the β -hydrogens average 10 and 27 G and the average of the α -hydrogen coupling is close to that of the larger β -hydrogen coupling, resulting in an approximate six-component spectrum.

Trace A in Fig. 1 shows the powder spectrum immediately after X irradiation. During the first 2 min of heating, the outside shoulders disappear, and there is a shift in the intensities of the outside peaks of the principal components of the spectrum. These early changes in the spectrum are attributed to remnants of transient precursor radical species which are slowly converting to the more stable room temperature radical. There may also be changes in the initial conformation of the room temperature species. After the first 2 min of heating at temperatures near 100°C, no further change in the positions or shapes of the peaks in the spectrum indicates the presence of a single radical species. Evidence for a single species is also obtained from single crystal studies on samples which annealed at room temperature for several days or were

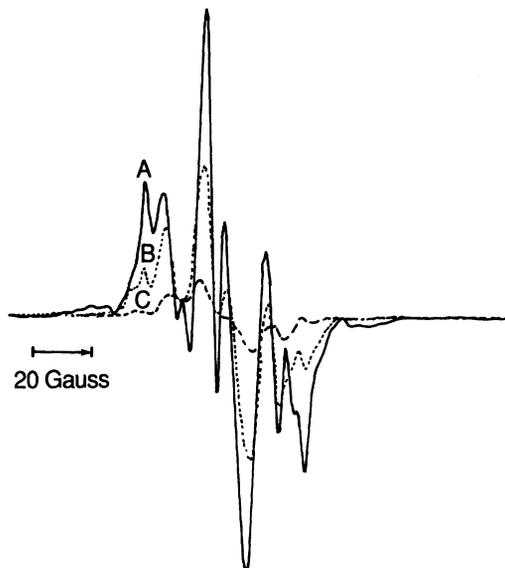


FIG. 1. First derivative, 9 GHz ESR spectra showing the decrease in the number of radicals in X-irradiated L- α -amino-*n*-butyric acid hydrochloride as a function of time annealed at 100°C. Annealing times were: plot A, 0 min; plot B, 10 min; plot C, 100 min.

heated for 1 or 2 min at higher temperatures (9). Data taken during the first 2 min of decay were not used in the analysis of the data.

When samples are annealed at temperatures above room temperature (see Fig. 1), the decay of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radical does not strictly follow second-order kinetics. The decay has a faster initial rate and then a slower rate after annealing for several minutes. The rates for the initial and final stages of annealing, taken from a second-order plot, are 9.6 and 5 molecules/radical \cdot s, respectively. A diffusion-controlled second-order equation results in a better fit of data to theory over the entire range of these data. The diffusion-controlled second-order rate equation derived by Waite (12) and put in a linear form by Wen *et al.* (13) is

$$\left(\frac{1}{[C]} - \frac{1}{[C_0]} \right) \frac{1}{t^{1/2}} = kt^{1/2} + \frac{2r_0k}{(\pi D)^{1/2}}. \quad (1)$$

Here $[C_0]$ and $[C]$ are the free radical concentrations at zero time and time t , respectively, k is $4\pi r_0 D$, r_0 is a separation distance within which free radicals react, and D is the sum of the diffusion coefficients of the reacting species. The good fit of the data in Fig. 2 to a straight line and the nonzero intercept (a zero intercept is expected for a second-order plot) both support the role of radical diffusion in the decay of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radicals. An even better fit of the data (Fig. 3) is found in a second-order type plot where the exponent of the concentration is 1.5 rather than 1. However, the diffusion-controlled second-order mechanism and plot is the most reasonable explanation of the decay. But the good fit of the data to a $1/C^{1.5}$ plot is an indication of a more complex decay.

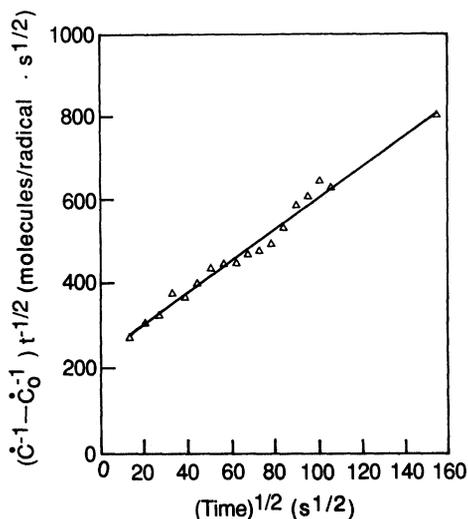


FIG. 2. Radical decay data for L-amino-*n*-butyric acid hydrochloride plotted according to a diffusion-limited second-order decay equation (Eq. 1).

An activation energy of 1.1 eV for the decay of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radical was determined from the Arrhenius equation by plotting the logarithm of the rate constant as a function of reciprocal temperature. The rate constants for the decay were determined from the diffusion-controlled second-order equation.

CARBON-CENTERED RADICAL TRANSFER TO CYSTEINE

In X-irradiated samples of L- α -amino-*n*-butyric acid hydrochloride which contain about 1% L-cysteine hydrochloride, the predominate radical at room temperature is

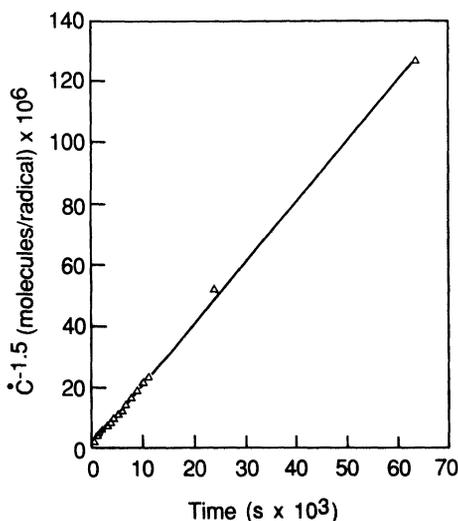


FIG. 3. A plot of L- α -amino-*n*-butyric acid hydrochloride radical concentration to the -1.5 power as a function of time for the data plotted in Fig. 2.

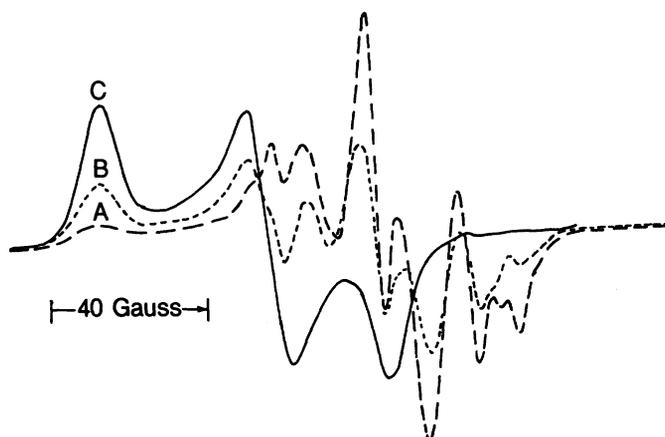


FIG. 4. First derivative, 9 GHz ESR spectra showing the transformation from an initial carbon-centered radical to a perthiyl radical as a function of annealing time at 100°C for a sample of L- α -amino-*n*-butyric acid hydrochloride containing 1.5% L-cysteine hydrochloride after exposure to 0.3 kGy. Annealing times were: plot A, 0 min; plot B, 4 min; plot C, 4 h.

the same as that in a sample with no cysteine. When a sample containing cysteine is annealed at temperatures above room temperature, the carbon-centered radicals are transformed into sulfur-centered radicals. The sulfur-centered radical has been identified as a perthiyl radical, RSS^\bullet (9). The identity of the perthiyl radicals, to which the carbon-centered radicals transfer, is based on the analysis of ^{33}S hyperfine components which are clearly from two sulfur nuclei on which unequal spin density is localized. The growth of the number of perthiyl radicals and the decrease of the carbon-centered radicals for a sample containing (1.5%) cysteine and annealed at 100°C is shown in Fig. 4. Resonances from the perthiyl radical grow in on the low-field side of the spectrum while the resonances from the carbon-centered radical become smaller on the high-field side of the spectrum. The spectrum shown after 240 min of annealing has the characteristic shape of a spectrum from a perthiyl radical (14). An analysis of the kinetics of the growth of the perthiyl radicals and the decay of the carbon-centered radicals was based on the growth of the perthiyl peak on the low-field side of the spectrum and the decrease of the carbon-centered radical hyperfine peaks on the high-field side of the spectrum. This type of analysis is possible since no carbon-centered radical hyperfine structure is under the low-field perthiyl peak and no perthiyl radical component is under the carbon-centered radical hyperfine structure on the high-field side of the spectra. The data were taken from polycrystalline samples rather than from single crystals to eliminate variations in intensity measurements. These variations occurred because of slight differences in orientation and placement in the ESR cavity which occur when a sample is heated in an oven and returned to the cavity for periodic measurement of the ESR spectrum.

The percentage of carbon-centered radicals transferring to perthiyl radicals on annealing depends on both the radiation dose and the concentration of the cysteine impurity. The data plotted in Fig. 5 show that the total number of radicals remains essentially constant for a 1.5% sample exposed to 0.3 kGy and annealed at 100°C. The carbon-centered radicals transfer almost completely to the perthiyl radicals.

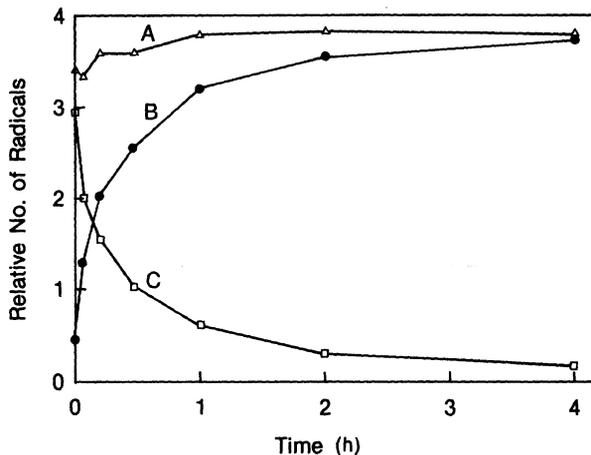


FIG. 5. Plots of the relative number of radicals as a function of annealing time for the sample described in Fig. 4. Plot A is for the total number of radicals, plot B the perthiyl radicals, and plot C the carbon-centered radicals.

When the radiation exposure is increased, as shown in Fig. 6, the percentage of carbon-centered radicals transferring and the total number of radicals present decreases on annealing. Since the perthiyl radical decays slowly at the annealing temperatures, it appears that there is considerable carbon-centered radical recombination as well as transfer to the perthiyl radical. The data for the growth of the perthiyl radical follow pseudo first-order kinetics rather than second-order or diffusion-controlled second-order kinetics as in the case of the pure butyric acid hydrochloride, because the con-

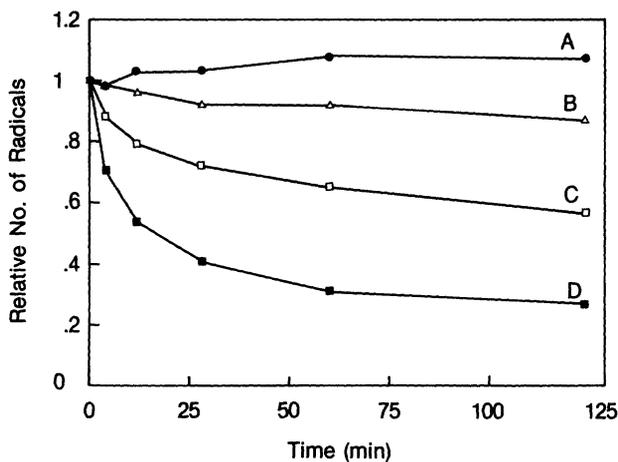


FIG. 6. Plots showing the relative number of radicals as a function of annealing time at 100°C for samples containing 1.5% L-cysteine hydrochloride, and receiving different amounts of irradiation. Plot A was exposed to 0.3 kGy, plot B to 0.9 kGy, plot C to 2.7 kGy, and plot D to 8.1 kGy.

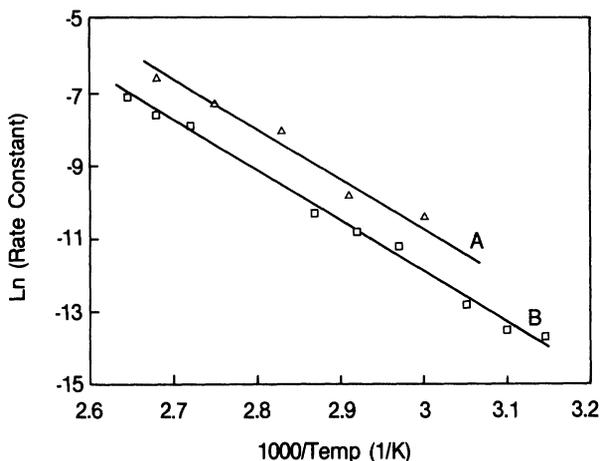


FIG. 7. An ln plot of rate constants as a function of reciprocal temperature for 1.5% L-cysteine hydrochloride samples exposed to 2.7 kGy (plot A) and 0.3 kGy (plot B).

centration of the cysteine hydrochloride is about 10^3 times greater than that of the carbon-centered radical. A plot of the rate constants as a function of reciprocal temperature is given in Fig. 7 for two samples of the same cysteine concentration but different irradiation doses. The rates for the two samples are different, but the slopes of the two curves are almost the same. The activation energy calculated from the slopes is 1.2 eV. This indicates that the activation energy for the rate controlling process is the same in the two cases.

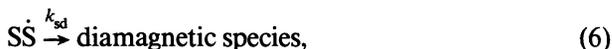
SULFUR-CENTERED RADICAL RECOMBINATION

After annealing at elevated temperatures for several hours (for example 100°C , see Fig. 4), the initial spectrum from an irradiated amino-butyric acid hydrochloride sample containing cysteine hydrochloride has completely changed from a carbon-centered radical spectrum to a spectrum from perthiyl radicals. On further annealing, the intensity of the spectrum from the perthiyl radicals slowly decays over a period of days. The decay follows a second-order rate equation. The rate constant at 100°C for a sample containing 2% cysteine and exposed to about 4.5 kGy is 0.014 molecule/(radical \cdot s). Dividing this constant by the concentration gives a pseudo first-order constant of $3.6 \times 10^{-5} \text{ s}^{-1}$. This decay of the perthiyl radical at 100°C is a factor of 10 times slower than the growth of the perthiyl radical resulting from the conversion of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radical (see Fig. 7).

DECAY KINETICS

The conversion of radicals from a carbon-centered radical to a perthiyl radical is a second-order process, but since the concentration of cysteine (about 1%) is much larger than the concentration of carbon-centered radicals (about 0.001%), the cysteine concentration remains nearly constant during the transfer, and the process may

be considered pseudo first-order. The reactions for the above processes are given schematically by the following equations:



where C represents the amino butyric acid molecule (carbon center), \dot{C} a carbon-centered radical, S the cysteine molecule (sulfur center), \dot{S} a thiyl radical, and $\dot{S}\dot{S}$ the perthiyl radical. The initial carbon-centered radical, $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$, is assumed to move from site to site in the crystal structure by abstracting a hydrogen from a neighboring molecule, see Eq. (2). Hydrogen abstractions in radical reactions are well known (15, 16). The structure of a carbon-centered radical formed by hydrogen abstraction may not be the same as that of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radical which results from deamination. However, the presence of a transient carbon-centered radical was not detected, and for the purpose of kinetics no distinction was made between $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radicals and those assumed to form by hydrogen abstraction. Through hydrogen abstraction, the carbon-centered radical is believed to walk through the lattice randomly until it encounters a site containing a cysteine adjacent to another cysteine, a site at which the perthiyl radical can be formed (17). In the random walk to the perthiyl site, the carbon-centered radical may encounter another carbon-centered radical and react according to Eq. (3) to form a diamagnetic species. Another possible encounter, described by Eq. (4), is with an isolated cysteine molecule, and the concomitant formation of a thiyl radical. Thiyl radicals are known to be unstable, and the random walk is believed to continue through the lattice until a stable perthiyl radical is formed Eq. (5). In these processes, carbon-centered radicals disappear and perthiyl radicals appear with some loss in the total number of radicals due to the formation of a diamagnetic species when two carbon-centered radicals encounter each other as in the decay of radicals in the butyric acid without the cysteine impurity.

The decay of the carbon-centered radical is described by the equation,

$$\frac{d[\dot{C}]}{dt} = -2k_{cc}[\dot{C}]^2 - k'_{cs}[\dot{S}\dot{S}][\dot{C}] \quad (7)$$

and the growth of the perthiyl radical is given as,

$$\frac{d[\dot{S}\dot{S}]}{dt} = k'_{cs}[\dot{C}][S]. \quad (8)$$

In Eqs. (7) and (8), $[\dot{C}]$ is the carbon-centered radical concentration, $[\dot{S}\dot{S}]$ is the con-

centration of cysteine on two adjacent sites, and $[\dot{S}S]$ is the concentration of the perthiyl radical. In these equations, the rate constant k'_{cs} is an effective constant representing the combined effect of the reactions postulated in Eqs. (2), (4), and (5). Since $[SS]$ is about 10^3 times greater than the initial $[\dot{C}]$, $[SS]$ remains approximately constant during the decay of $[\dot{C}]$, and $k'_{cs}[SS]$ may be replaced by k a pseudo first-order rate constant. With this approximation, the solutions for $[\dot{C}]$ and $[\dot{S}S]$ are

$$[\dot{C}] = \frac{k[\dot{C}_0]}{Ke^{kt} - 2k_{cc}[\dot{C}_0]} \quad (9)$$

and

$$[\dot{S}S] = [S\dot{S}_0] + \frac{k}{2k_{cc}} \ln \left[1 + \frac{2k_{cc}[\dot{C}_0]}{k} (1 - e^{-kt}) \right]. \quad (10)$$

In these equations, $[\dot{C}_0]$ and $[S\dot{S}_0]$ are concentrations at $t = 0$, k is $k'_{cs}[SS]$, the pseudo first-order rate constant for the perthiyl radical growth, k_{cc} is the rate constant for the $[\dot{C}]$ radical decay, and $K = k + 2k_{cc}[\dot{C}_0]$.

The percentage transfer of $[\dot{C}_0]$ to $[SS]$ is defined as

$$\%T = \frac{[SS_\infty]}{[\dot{C}_0]} \times 100. \quad (11)$$

Here $[SS]$ is the concentration of the perthiyl radical when the radical transfer from $[\dot{C}]$ to $[SS]$ is complete. An expression for the percentage transfer obtained from Eq. (10) is

$$\%T = \frac{1}{R} \ln(1 + R) \times 100, \quad (12)$$

where

$$R = \frac{2k_{cc}[\dot{C}_0]}{k'_{cs}[SS]} \quad (13)$$

and $[S\dot{S}_0]/[\dot{C}_0]$ is negligible. This approximation is good for all of the experimental conditions since the perthiyl radicals at $t = 0$ are mostly those formed directly by the irradiation. Equation (12) shows that the percentage transfer increases as R gets smaller and decreases as R gets larger. Both an increase in the initial carbon radical concentration and a decrease in the initial sulfur trap concentration, $[SS]$, will cause an increase in R , and therefore a decrease in the percentage transfer. Figure 8 shows the percentage transfer as a function of initial sulfur trap concentration for different initial concentrations of carbon-centered radicals. The concentration of the carbon-centered radicals was varied by exposing the samples to different amounts of X irradiation, and the concentration of the sulfur traps was varied by preparing samples with different concentrations of cysteine. The observed dependence of the percentage transfer on $[SS]$ and $[\dot{C}_0]$ agrees with the concentration dependence expressed by Eq. (12). Equation (10) reduces to a pseudo first-order equation for the growth of the perthiyl radical when R is less than 1,

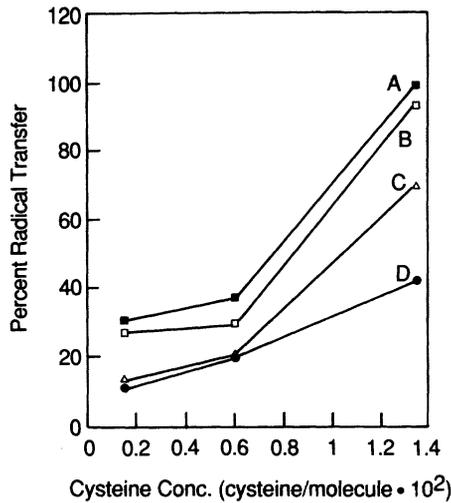


FIG. 8. Plots showing the percentage transfer as a function of cysteine concentration for different radiation exposures. Plot A was exposed to 0.3 kGy, plot B to 0.9 kGy, plot C to 2.7 kGy, and plot D to 8.1 kGy.

$$[S\dot{S}] = [S\dot{S}_\infty][1 - e^{-kt}]. \tag{14}$$

To obtain this equation, the final concentration of perthiyl radicals after extended annealing, $[S\dot{S}_\infty]$, is assumed equal to $[\dot{C}_0]$, and the approximation $\ln(1 + X)$ equals X , for small X , is also used. This first-order equation fits the perthiyl radical growth data very well. The rate constants as a function of temperature determined from this equation were found to vary with both $[\dot{C}_0]$ and $[SS]$. A plot of rate constants as a function of irradiation exposure (initial carbon-radical concentration) for a 1.5% L-cysteine sample annealed at 100°C is given in Fig. 9.

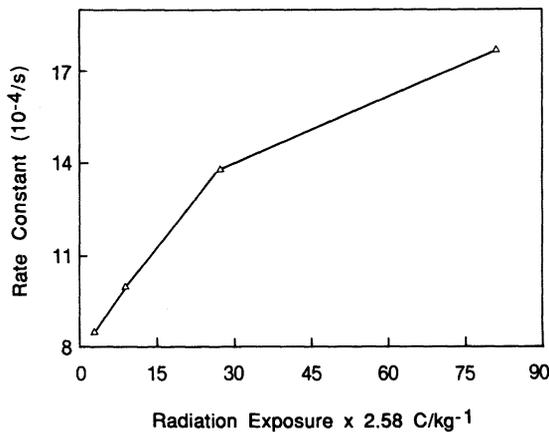


FIG. 9. A plot of rate constants determined from Eq. (14) for the growth of the perthiyl radical as a function of radiation exposure for a 1.5% L-cysteine sample annealed at 100°C.

DISCUSSION

The relatively stable radical, $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$, is formed in *L*- α -amino-*n*-butyric acid hydrochloride by X irradiation at room temperature. In this radical, the unpaired electron is localized on the α -carbon where there is a typical interaction with the α -hydrogen, and two β -hydrogens. The average β -hydrogen interactions are 10 and 27 G (9). The slow decay of the radicals at room temperature is accelerated when the sample is annealed near 100°C. This decay of the radicals follows a second-order diffusion-controlled rate equation more closely than a second-order rate equation. A slightly better fit of the data to a 2.5-order rate equation indicates that the decay process may be complex and not fully described by either a second-order rate or diffusion-controlled rate equation.

In *L*- α -amino-*n*-butyric acid hydrochloride containing a small percentage of *L*-cysteine hydrochloride, the radicals migrate to the sulfur-containing cysteine rather than decay. This radical transfer is a pseudo first-order process since the concentration of the cysteine is much greater than the number of $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radicals. The transfer is essentially 100% for low levels of radiation 0.3 kGy, decreasing to about 20% at a higher level of radiation 8.1 kGy. At the higher radiation level the density of the $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radicals is higher, with a concomitant higher probability that two carbon-centered radicals will combine to form a diamagnetic species before reaching the site of a cysteine molecule where a thiyl radical forms. The thiyl radical is unstable (17) and the carbon-centered radical continues to walk randomly in the crystalline structure until a cysteine adjacent to another cysteine is encountered. Here a perthiyl radical is formed (9). A decrease in the cysteine concentration causes a decrease in the percentage of $\text{CH}_3\text{CH}_2\dot{\text{C}}\text{HCOOH}$ radicals becoming perthiyl radicals. When the cysteine concentration is lowered, the number of cysteine-cysteine adjacent sites where the perthiyl radical forms is lowered, and the radical decay interaction between two carbon-centered radicals becomes the dominant process with a corresponding decrease in the formation of perthiyl radicals.

Hydrogen atom abstraction from a neighboring molecule is believed to be the mechanism by which a carbon-centered radical may randomly move from site to site in the crystal. When the carbon-centered radical reaches a site adjacent to two cysteine molecules, it interacts with one cysteine to form a neutral species and a thiyl radical which interacts with the other cysteine to form the perthiyl radical. Comparable activation energies for radical decay in pure *L*- α -amino-*n*-butyric acid hydrochloride (1.1 eV) and the formation of the perthiyl radical in *L*- α -amino-*n*-butyric acid hydrochloride containing *L*-cysteine hydrochloride (1.2 eV) indicate that the controlling mechanism is the migration of the carbon-centered radical through the crystal lattice.

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