

An ESR Study of Amino Acid and Protein Free Radicals in Solution.*

Part IV. An ESR Study of Gamma-Irradiated Amino Acids in Frozen Aqueous Solutions.

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Abstract An ESR study has been made on free radicals produced in frozen aqueous solutions (ices) of glycine, *DL*- α -alanine, *DL*-serine, *L*-cysteine, *DL*-leucine and *DL*-isoleucine by gamma-irradiation at dry ice temperature.

All free radicals induced were decayed concomitant to the successive annealing but the radical species which is believed to be dominant seems to be stable even near the melting point of the ice. These dominant species were found to be identical to those resulted from direct action of radiation in the solid at room temperature. Small but significant changes in the spectra of glycine and *DL*- α -alanine were observed by varying the microwave power. These results seem to support the view that the spectra obtained were composite consisting of more than two different resonances having different power saturation characteristics. The relative contribution of unidentified resonances to the composite spectra was greater for solutions of low concentration. These resonances are assumed to be induced by indirect effects, mainly hydrogen abstraction by radiation produced hydroxyl radicals and also C-N bond cleavage by hydrated electrons.

Introduction

In recent years electron spin resonance spectroscopy has been used¹⁻³ in the study of radiation-induced free radicals in frozen aqueous solutions containing amino acids at 77°K. Most of the spectra obtained in their work were found to be composite consisting of more than two different resonances which make their individual identification difficult. It is now believed, how-

ever, that the solute radicals produced in irradiated dilute aqueous solution are induced as the result of both direct and indirect action of radiation³. The indirect effect at 77°K is largely due to the action of water radicals³, consisting mainly of hydroxyl radicals², formed in a small sphere of bound water surrounding each solute molecule. The water resonance is relatively unstable and disappear rapidly in the temperature range 100° to 130°K but the indirect effects in aqueous solution were found to be increasing

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with increasing irradiation temperature, presumably owing to a greater contribution of diffusible water radicals formed outside the sphere of bound water³.

In the present work⁴ the ESR centers have been induced in frozen aqueous solutions of glycine, *DL*- α -alanine, *DL*-serine, cysteine, *DL*-leucine and *DL*-isoleucine by gamma-irradiation at dry ice temperature. At this temperature, the composite spectra obtained were exclusive of water resonance but the extent of relative importance of indirect effect to that of direct action might be greater than the case of irradiation at 77°K.

Since the indirect action of radiation in aqueous solution is associated with the effect of hydroxyl radical^{5,6}, and different resonances in the composite ESR spectra seem to have different microwave power saturation characteristics¹, it is hoped that our previous data^{6,7} obtained in the Ti-H₂O₂ flow experiments at room temperature and power variation technique could provide a useful information for the study of relative importance of direct and indirect effect of radiation on amino acid ices.

Experimental

All amino acids used in the present experiment were obtained as crystalline powders of the highest purity available from Sigma Chemical Company, St. Louis, Missouri, and used without further purification.

Solutions of 0.01, 0.05, 0.1, 0.5, and 1 molar glycine and *DL*- α -alanine, 0.5 molar *DL*-serine, 1 molar *L*-cysteine, 0.01 and 0.05 molar *DL*-leucine and *L*-isoleucine were prepared and examined.

After the solutions were withdrawn into a 3mm. i.d. quartz syringe tube to a liquid height of about 2 cm., they were rapidly frozen

in liquid nitrogen to give opaque crystalline solids. The ice cylinders of samples were pushed out into 4mm. i.d. pyrex irradiation tubes after carefully warming the outside of the syringe tube. The irradiation tubes had previously been placed into a dewar filled with dry ice. The tubes were sealed with rubber stoppers, centered in the dewar, and placed into irradiation facility.

The total absorbed dose was 3–4M rad. γ -ray from ⁶⁰Co source given at a dose rate of 3,400 rpm. as measured by replacing the samples by Fricke dosimetric solution taking $G(\text{Fe}^{3+})=15.5$.

After irradiation the ice cylinders were transferred, without allowing to warm up, into a V-4540 variable temperature controller accessory tube which fits into the cavity of an ESR spectrometer.

The ESR measurements were made using a Varian model V-4500 spectrometer. The spectrometer operates at a nominal frequency of 9.5 k Mc./sec., employs 100 kc./sec. modulation and phase sensitive detection, and records the first derivative of the absorption signal on Varian F-80 XY recorder.

With the frozen samples, the experimental modulation amplitude was chosen by decreasing the modulation to that value where no change was observed in the spectral line width. A stream of dry nitrogen gas entered one side of the cavity to prevent moisture condensation on the tube. During the experiments where the temperature was varied from 195°K to 265°K, the samples were annealed stepwise of 10 degree intervals for 3 minutes to assure temperature equilibration. After each annealing, the spectrum was recorded. Power of approximately 10 to 25 mw. to the cavity was used during all experiments.

Results and Discussion

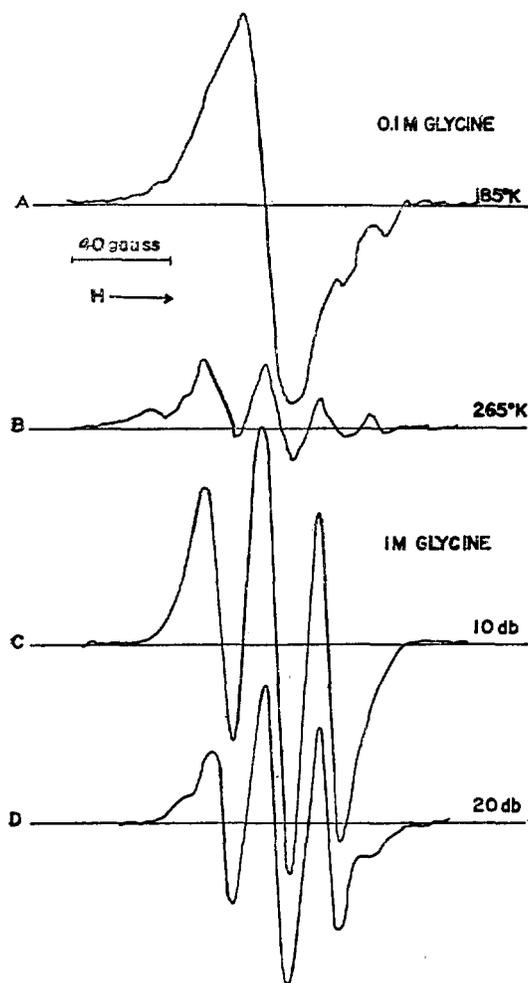


Fig. 1. Qualitative EPR spectra of glycine ices obtained after irradiation with γ -rays: (A) spectrum of 0.1M glycine ice observed at 185°K after irradiation at 195°K; (B) spectrum observed immediately after annealing (A) at 265°K and the same signal level; (C) spectrum of 1M glycine obtained after irradiation at 195°K with Klystron microwave power attenuated at 10 db; (D) spectrum of (C) after increasing the attenuation from 10 to 20 db with the other settings unchanged.

Glycine: The spectrum of a 1M glycine ice, irradiated with γ -rays at 195°K and observed

at 185°K, exhibits a triplet with 21 G hyperfine splitting, and a line width of 11 G (Fig. 1 C). The relative intensity distribution is about 1-2-1. This spectrum seems essentially the same as that observed in the range of 173°K-243°K following irradiation of 1.5M glycine ice with X-rays at 77°K^{8,9}. If the spectrum is compared to the well-known pattern of irradiated, polycrystalline glycine at room temperature,¹⁰⁻¹² it appears that the hyperfine splitting and intensity distribution are the same. The line width in the ice is about 8 G which is narrower than the line width observed in irradiated polycrystalline glycine. This difference may result from differences between the matrix fields.

It is interesting to note that when the relative microwave power is lowered from 10 to 20 db the line width becomes narrower and additional small resonances are unmasked on either side of the triplet. This spectrum is closely similar to that observed for crystalline solid glycine exposed to low doses of α -particles.¹¹ However, no plausible explanations as yet have been offered for these small resonances.

The signal height of the glycine triplet represents concomitant growing with successive heat treatments up to -20°C at a 10 db microwave power (Fig. 2A) whereas that of other amino acids investigated decreased. No reasonable explanation why glycine exclusively shows this unusual behavior can be given at the present time. However, the phenomenon was interpreted as due to a microwave power saturation effect. When the signal heights are plotted as a function of microwave power at two different temperatures, -90°C and -10°C (Fig. 2B), significant discrepancies appeared between these two curves. It becomes obvious from the curves (a) and (b) that within the temperature range of -90°C and -10°C at 10 db of relative microwave power, the signal height should increase

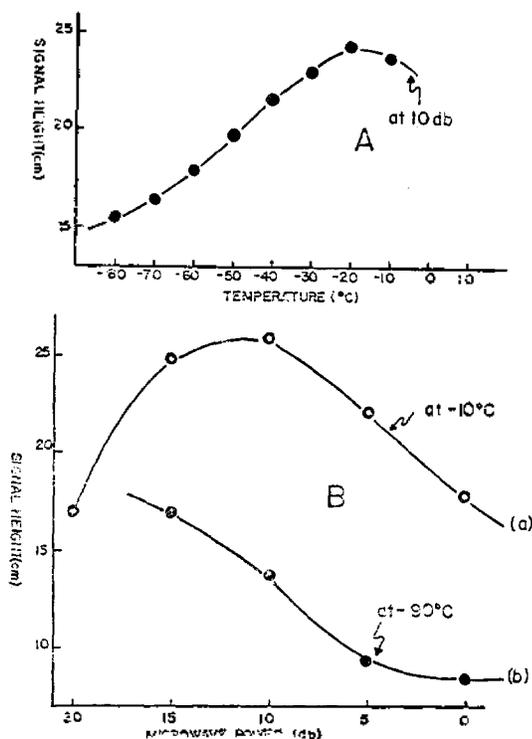


Fig. 2. Variation of signal height of the spectra of irradiated glycine ice as a function of temperature and microwave power: (A) signal height change due to temperature variation at 10 db; (B) signal height change due to microwave power variation at -10°C (a) and -90°C (b).

concomitantly with annealing.

The glycine radical was found to increase almost linearly with the concentration, which is consistent with a previous observation at 77°K . This result seems to indicate that the major fraction of the glycine radicals induced at 195°K as well as 77°K is the result of the direct action of radiation. However, when the glycine concentration is lowered to $0.1M$, the spectrum exhibits a singlet centered around the free spin value, with a 21.6G line width (Fig. 1A), instead of the usual triplet. This singlet changed when the glycine solution was annealed at 265°K ; the resonance of the glycine solution then consists of a triplet with some other resonance

superimposed, (Fig. 1B). This result implies that the singlet is the reflection of the radical species induced primarily by indirect action of radiation. This radical concentration could be so low relative to the species which gives rise to the triplet in solutions of higher concentrations that the spectrum of the singlet is masked by the triplet spectrum. In agreement with expectation the singlet obtained from further diluted solutions ($0.05M$ and $0.01M$) vanished after annealing to 205°K with no other resonance appearing.

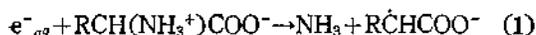
It is of interest that a similar broad singlet with little indication of structure was also observed following irradiation of polycrystalline glycine with a high dose ($129M$ rad.) of α -particles at room temperature¹¹. This was also subject to further modification upon storage in air. In addition, a similar spectrum was reported¹⁰ by heating the γ -irradiated polycrystalline glycine at 160°C for 45 minutes.

In all cases described above, the spectra were not identified. However, at this stage, it seems apparent that the radical species giving rise to the singlet in the ice are induced by an indirect effect. This radical produced by the indirect effect is not identical with that induced in solid state^{10,11}; although both exhibit singlets, they show quite different stability on heat treatment.

The indirect effect at 77°K was found to be largely due to the action of a water radical, believed to be OH radical, formed in a small sphere of bound water surrounding each solute molecule. OH radicals will abstract H-atoms from amino acid molecules and thereby convert these into amino acid free radicals. The radical $\text{CH}(\text{NH}_3^+)\text{COO}^-$ could be formed by H-atom abstraction when glycine is exposed to H-atoms^{12,13}. However, H-atoms in solution above 77°K do not seem to be significant^{2,8}.

Box et al¹⁴ reported that the principal inter-

mediate free radical results from cleavage of the C—N bond of crystalline glycine. A recent indication^{15,16} is that hydrated electrons, e^-_{aq} , in irradiated aqueous solution can also react with both the zwitterion and cation forms of the simple amino acids as well as their peptides to cleave the C—N bond of the primary amino group. This reaction takes place via the reductive deamination



Thus, after studies on glycine in the solid state as well as in ices, the ESR absorption has been variously assigned to $\dot{C}H(NH_3^+)COO^-$ (or $\dot{C}H(NH_2)COOH$), $\dot{C}H_2COO^-$ (or $\dot{C}H_2COOH$), $N\cdot H_2$, $\dot{C}H_2NH_3$ and $\dot{C}HCOO^-$ (at 77°K in solid). In view of the uncertainties which remain it would be premature to postulate detailed mechanisms for the various chemical reactions. However, the present indications are that in irradiated glycine ices greater than $10^{-1}M$, mainly radicals similar to those observed in solid glycine are formed. In irradiated glycine ices less than $10^{-1}M$ mainly unidentified radicals are formed which are different from those obtained in solid glycine.

DL- α -Alanine; Fig. 3A shows the spectrum obtained following γ -irradiation of 1M alanine ice at 195°K and observed at 185°K. The spectrum exhibits a broad single line with appreciable indication of structure. Upon warming to 213°K it was converted to the well-known five line alanine structure obtained at room temperature in the solid^{3,11}.

The same result was also observed for the solution of lower concentrations (0.01, 0.05, 0.1, 0.5M) but the total radical concentration was found in decreasing amounts almost linearly with the concentration and with lesser indication of structure.

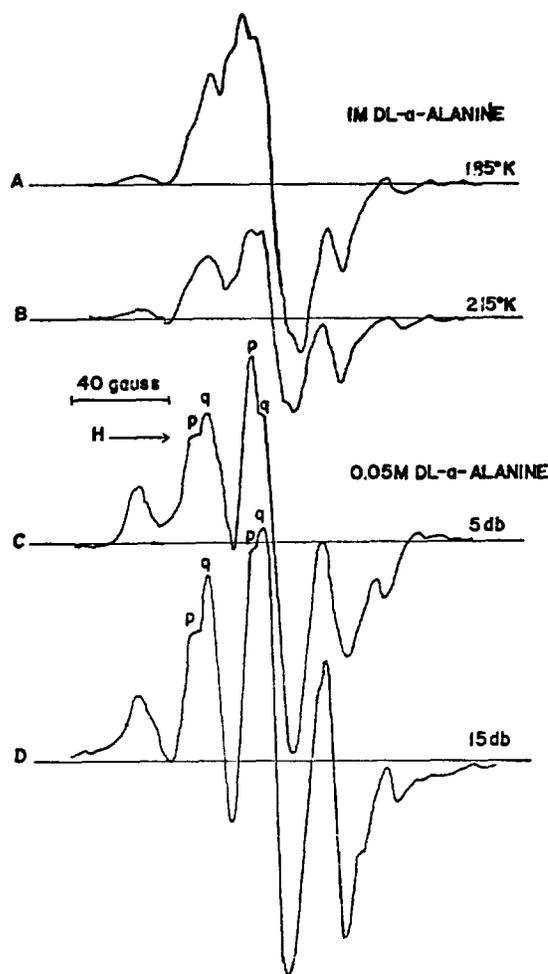


Fig. 3. Qualitative spectra of DL- α -alanine ice obtained after irradiation with γ -rays:

(A) spectrum of 1M DL- α -alanine observed at 185°K; immediately after irradiation at 195°K (B) spectrum obtained after the sample was annealed at 215°K with other settings unchanged; (C) spectrum of 0.05M DL- α -alanine observed at 185°K after irradiation at 195°K and with 5 db attenuation of the microwave power; (D) spectrum obtained from the same sample after the attenuation of the microwave power increased to 15 db.

In a previous study³ 10% alanine aqueous solutions were irradiated with X-rays at 77°K and subsequently subjected to successive heat-treatment for 3 minutes. They observed composite

spectra of the radicals stabilized in the solid and in the solution at 77°K . After the water resonance had been bleached by heat-treatment they found the spectrum which is similar to those found when alanine was irradiated in the dry state and subjected to similar heat-treatment.

The spectrum shown in Fig. 3A of course does not show the water resonance because it is not stable at 195°K . However, the composite spectrum of the principal radical giving rise to five lines and some other unidentified radical which was not observed by former workers³ during their heat-treatment at 77°K – 250°K , may be the reflection of the radical species induced by the indirect effect. A more relative contribution of the latter radical than the former in a lower concentrated solution may support this assumption.

Lately, it was indicated that the initial fragment formed at 80°K in the single crystalline state¹⁷ has the unpaired electron localized mainly on the carboxyl group and is converted to $\text{CH}_3\dot{\text{C}}\text{HCOOH}$ upon warming to 140 – 150°K . Thus the final free radicals produced by X -irradiation in solid alanine is known to result exclusively from the rupture of the $\text{C}-\text{N}$ bond¹⁴. In aqueous solution, in addition, reductive deamination of the primary amino group by the hydrated electron, e^-_{aq} , in alanine has been reported¹⁶.

In the present experiments, however, small but significant changes in the five line spectrum at 195° – 265°K were observed by varying the microwave power employed. As shown in spectrum C and D in Fig. 3, the peak q shows an appreciable increase when the power is changed from 5 to 15 db., while the remaining part of the composite line p remains constant. This result obviously demonstrates that at least two different radical species are involved in the irradiated alanine ice.

The findings in this experiment and the cited paper seem to indicate that the final free radi-

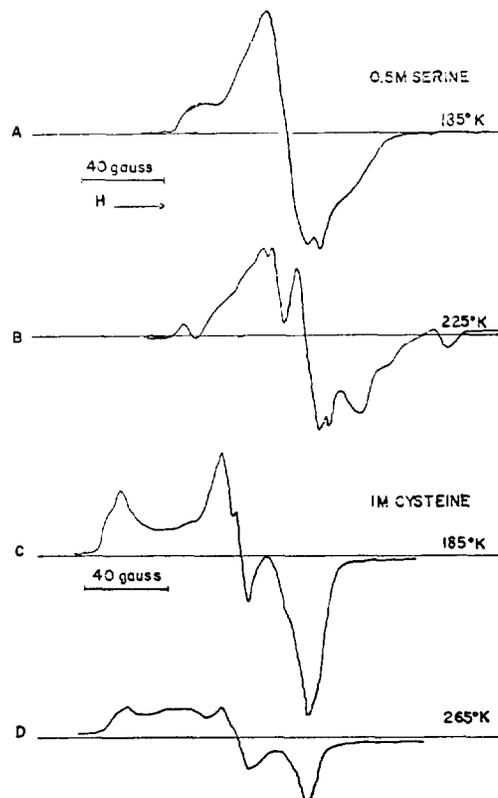


Fig. 4. Qualitative spectra of *DL*-serine and *L*-cysteine ices obtained after irradiation with γ -rays;

(A) spectrum of $0.5M$ *DL*-serine obtained immediately after irradiation at 195°K ; (B) spectrum of the same sample after annealing at 225°K ; (C) spectrum of $1M$ *L*-cysteine obtained immediately after irradiation at 195°K ; (D) spectrum of the above sample observed after annealing at 265°K and with the same signal level.

cals produced by X -rays as well as γ -rays in the solid and in the irradiated ices of alanine at around room temperature are mainly $\text{CH}_3\dot{\text{C}}\text{HCOOH}$. This radical will give a five line spectrum due to the hyperfine interaction of four protons. In addition, the experiments with ice seem to indicate some unidentified radicals co-exist in the solution and may be induced by the indirect effect of radiation presumably mainly due to action of e^-_{aq} .

***DL*-serine:** $0.5M$ serine ice irradiated at 195°K

and observed at 185°K, exhibits a broad singlet with little indication of structure (Fig. 4A). After the sample was warmed to 225°K, the spectrum changed appreciably and showed more indication of structure (Fig. 4B) yet not sufficient for its identification. This latter spectrum was found to be stable up to the melting point of the ice.

Because of the lack of symmetry in this molecule and the complexity of the ESR patterns, it could not be ascertained what free radicals are formed. However, it was apparent that there is an evidence for more than one free radical species at 195°K. Some of the radicals were probably induced by the indirect effect and decayed more rapidly than the others.

Observations of the solid serine at 77°K and 300°K by previous workers¹⁸ showed also the complexity of its ESR patterns which were not identified.

The spectrum in Fig. 4B, with less resolved hyperfine structures than that observed in polycrystalline state¹⁹, may be the reflection of the free radical being attacked by oxygen in the solution.

L-Cysteine: The qualitative spectrum of 1 M L-cysteine ice irradiated at 195°K and observed at 185°K, as well as the effect of heat-treatment are shown in Fig. 4C and D. This spectrum C is essentially the same as that obtained in the solid observed at 77°K after irradiation with X-rays at 295°K^{20,21}, and has been attributed to the "characteristic sulfur pattern." It is interesting to note, however, that 10% glutathione frozen aqueous solution failed to exhibit this spectrum in contrast to that spectrum observed in the irradiated solid at 370°K². No clear explanation has yet been given for this result.

It was suggested previously²¹ that none or very few radicals are formed at 77°K, but that

they are induced as the result of secondary processes at around 193°K and are observed as time dependent spectral changes. In addition, it was found that about 15% of the "water radicals", believed to be OH radicals, become effective in producing solute radicals in a 10% solution of glutathione². The H-atom abstraction by the OH radical, from the SH group in cysteine molecule was confirmed by photolysing H₂O₂ cysteine solution²². However, H-atoms induced in irradiated H₂O are assumed to be relatively ineffective in forming substrate radical². It is therefore reasonable to conclude that the secondary process has been almost completed in aqueous solution at 195°K after about 17 hours irradiation and results in the characteristic sulfur pattern, in part with the aid of the indirect effect. The radical has been attributed to be $\dot{S}CH_2CH(NH_3^+)COO^{-2,20,21,23}$.

DL-Leucine: 0.01 M DL-leucine ice, when irradiated at 195°K and observed at 185°K, gave an ESR pattern which appears to be a superposition of the signals from two distinct free radicals (Fig. 5A). The spectrum consists of a strong asymmetric singlet component and some broad weak lines spread over the total spectrum.

Previous workers¹⁸ observed quite well resolved hyperfine structure at 300°K in solid DL-leucine which was irradiated at 77°K in vacuum. After heat-treatment, the samples were cooled again to 77°K where equally spaced six lines were observed. This spectrum enabled them to attribute the dominant pattern to be a single free radical, $CH_3\dot{C}(CH_3)CH_2CH(NH_2)COOH$.

The following results, however, seem to demonstrate that the dominant free radical species induced in ice at 185°K and in the solid at 300°K are identical; the broad weak lines observed at 185°K (Fig. 5A) seem to originate from the same spectrum as the six lines observed

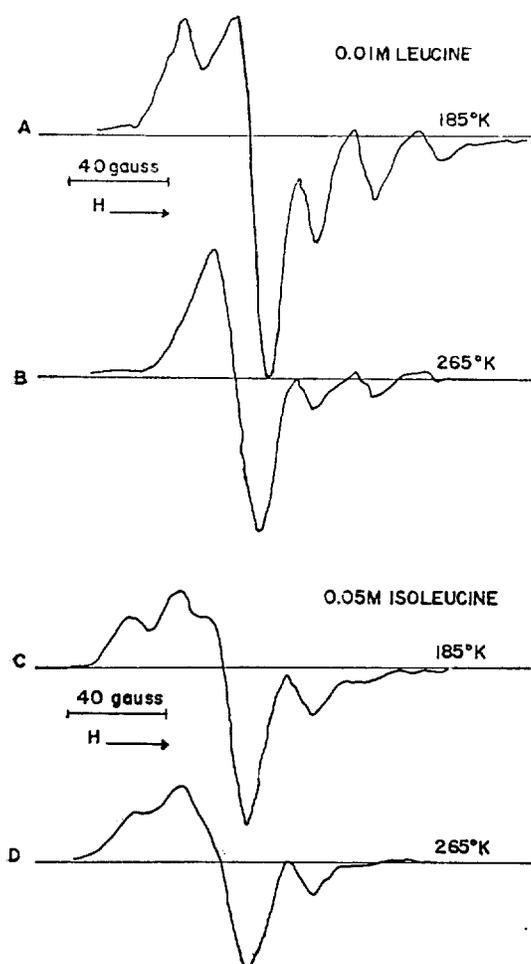


Fig. 5. Qualitative spectra of *DL*-leucine and *L*-isoleucine ices obtained after irradiation with γ -rays:

- (A) spectrum of 0.01M *DL*-leucine observed at 185°K after irradiation at 195°K; (B) spectrum of the above sample observed after annealing at 265°K; (C) spectrum of 0.05M *L*-isoleucine observed at 185°K after irradiation at 195°K; (D) spectrum of the above sample observed after annealing at 265°K.

in the solid¹⁸ after re-cooling back to 77°K from room temperature. This resonance appeared to be superimposed on a strong single line. When the solution was warmed to 265°K the spectrum shape changed significantly. The broad lines decayed rapidly while the strong singlet remains

unchanged. No further change was observed after recooling back to 195°K. This latter spectrum appears to be very similar to that observed at 300°K in the solid which was γ -irradiated at 77°K in vacuum and exposed to air at 300°K for 10 minutes¹⁸. This spectrum in the annealed solid then was attributed to the *DL*-leucine free radical after attack by oxygen¹⁸. These findings in the present experiments obviously indicate that the oxygenated *DL*-leucine free radicals are already formed at 195°K in the ices. This radical gives rise to a strong asymmetric singlet which is superimposed on the broad, weaker lines which were attributed to the dominant free radical, $\text{CH}_3\cdot(\text{CH}_3)\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$, in the irradiated solid. Upon annealing the ice, the weaker, multi-lined radical, unattacked by oxygen, gradually decays and the spectrum reflects mainly the oxygenated free radicals.

***DL*-Isoleucine:** 0.05 M *DL*-isoleucine ice gave a different spectrum (Fig. 5C) from that of *DL*-leucine at both 185°K and 265°K. The hyperfine structure observed by others¹⁸ in solid isoleucine at 300°K after irradiation at 77°K in vacuum was attributed to the free radical analogous to that discussed above for *DL*-leucine, $\text{CH}_3\text{CH}_2\dot{\text{C}}(\text{CH}_3)\text{CH}(\text{NH}_2)\text{COOH}$. Similar oxygen effects were observed in *DL*-isoleucine ices as were observed in *DL*-leucine ices. The electron spin coupling with protons in both *DL*-leucine and *DL*-isoleucine free radicals was suggested to occur through hyperconjugation¹⁸.

Thus, the present experiments on γ -irradiated amino acid ices have shown that all free radicals induced decay concomitant to the successive annealing. However, the radical species which is believed to be dominant in the solution seems to be quite stable even near the melting point of the ice. The signal intensity of the glycine spectrum showed a gradual growth with heat-

treatment up to around 255°K which was interpreted as due to microwave power saturation.

Most of the spectra studied in this work were found to be composite, in almost every case, consisting of more than two different resonances which make their individual identifications difficult. These different radicals, however, seem to have different power saturation characteristics, in other words relaxation time differences. Microwave power variation seems then to be a general technique for separating free radical mixtures.

Qualitative spectra and a linear relationship between spin number and amino acid concentration in a solution have indicated that the dominant radicals induced in ices are generally identical to those produced in the solid at room temperature. However, in certain amino acids, *DL*-leucine for example, the spectrum was attributed to the radicals which are believed to be attacked by oxygen in the solution. All samples investigated in these experiments were irradiated and observed in air. It is reasonable, therefore, to assume that in all cases the free radicals have more or less reacted with oxygen. *DL*-leucine-type signal (Fig. 5B) which is found to be somewhat similar to that of H₂O₂ ice, is ascribed tentatively to a peroxy-organic free radical. Appreciable amounts of unidentified resonances compared to the dominant resonance have been observed. These former resonances are assumed to be induced by the indirect effect. The main part of the indirect effect is believed to be H-atom abstraction by radiation-produced OH radicals, but cleavage of C—N bond by hydrated electrons, e⁻_{aq}, also appear to be significant. Direct action of radiation on amino acids, on the other hand, is generally known to result in the disruption of the C—H bond as well as the C—N bond.

The present results, therefore, could hardly

distinguish indirect effects from direct effects. Quantitative analysis on evacuated samples are necessary to improve the experimental results.

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Significant Structure Theory of Physical Adsorption

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Abstract A significant structure theory of monolayer physical adsorption is developed. The theory is tested with the adsorptions on graphite of gases Ar, N₂, CHCl₃, and CCl₄. A restricted rotation model is used for the polyatomic molecules N₂, CHCl₃ and CCl₄. The computed isotherms and heats of adsorption are in good agreement with experiment in all cases studied.

Introduction

In a previous paper¹, we showed that the significant structure theory of liquids was successfully applied to a two-dimensional liquid of hard discs. A further extension of the investigation is to show how the theory can describe the physical adsorption of gases on solids. In this paper only a theory of monolayer adsorption

on homogeneous surfaces will be discussed. For a basic understanding of the significant structure theory, the readers are referred to the literature² and the references cited therein.

Theory

According to the significant structure theory, the partition function for the adsorbed state is given by³

$$f_{ads} = \left[f_{2s} \left(1 + n_s e^{-\frac{\epsilon_s}{kT}} \right) \right]^{N' \frac{A_s}{A}} \left[f_{2g} \right]^{N' (1 - \frac{A_s}{A})} \quad (1)$$

where f_{2s} is the partition function for the two-dimensional solid-like structure, f_{2g} the partition function for the two-dimensional gas-like structure, N' the number of adsorbed molecules, A_s the molar area of the two-dimensional solid-like structure, and A the molar

area of the adsorbed state. Let θ be the fraction of the surface covered with adsorbed molecules, N_m be the number of adsorbed molecules when $\theta=1$, and A_m be the molar area of the adsorbed molecule at $\theta=1$. We then find that $A=A_m/\theta$ and $N'=N_m\theta$. Eq. (1) becomes,