scavenges most of the H atoms with little change in color-center intensity.

Similarly we conclude that both $[ClO_4 \cdot H_2O]$ and $[ClO_4 \cdot H_2O]^{2-}$ and both $[HSO_4 \cdot H_2O]$ and $[HSO_4 \cdot H_2O]^{2-}$ contribute to the color centers in ClO_4^- and HSO_4^- ices. But in SO_4^{2-} and HPO_4^{2-} ices only the $[SO_4 \cdot H_2O]^-$ and $[HPO_4 \cdot H_2O]^-$ centers are formed. Bleaching of these centers does not produce any additional trapped H atoms. The $[SO_4 \cdot H_2O]^-$ and $[HSO_4 \cdot H_2O]$ centers are presumably interconvertible.

Evidence for formation of similar hole centers has been reported for H_2SO_4, $HClO_4$, and H_3PO_4 acid glasses at 77°K,[8] in sodium metaphosphate glass at room temperature,[9] and in a single crystal of KH_2PO_4 at 77°K.[10] In the acid glasses bleaching of the hole species gave rise to a new epr spectrum which was attributed to H_2O^+. Warming to 120°K reversed the process to re-form the color center. In the polycrystalline ices studied here, no epr spectrum attributable to H_2O^+ is seen although a small spectrum would have been obscured by the OH spectrum. More importantly, the color centers are not re-formed on warming. Although the original color centers are undoubtedly the same in the acid glasses and the polycrystalline ices the reactions they undergo upon warming or bleaching depend on the media.

In summary, our results indicate that H atoms are trapped in a variety of frozen oxyanion solutions, that a part of these H atoms arise from the reaction of a mobile electron with an oxyanion to form a radical anion which is trapped or which dissociates to give a trapped H atom, and that a positive hole may also react with the oxyanion to form a trapped color center. Both electrons and holes seem to react relatively readily with oxyanions in frozen solutions.

Acknowledgment. We wish to thank the U. S. Atomic Energy Commission for support of this research.

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Free-Radical Distribution in the γ-Radiolysis of Dry Ribonuclease[1]

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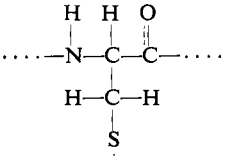
Contribution from the National Cancer Institute, National Heart Institute, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland. *Received September 7, 1965*

Abstract: A new method for determining the distribution of free radicals on carbon in irradiated dry proteins has been developed. γ-Radiolysis of ribonuclease in the absence of air and subsequent exposure to tritiated hydrogen sulfide leads to the formation of carbon–tritium bonds. The amount of such tritium incorporated per amino acid residue is very much greater for certain amino acids (lysine > methionine > proline > histidine) than for others (phenylalanine > isoleucine > valine); the maximum difference exceeds a factor of 70. Contrary to past interpretations of epr spectra, glycine radicals do not play a dominant role under these conditions. Analogous experiments with carboxymethylated reduced ribonuclease suggest that free-radical distribution depends markedly on molecular conformation of the protein.

When dry proteins are irradiated with γ-rays in the absence of air, trapped free radicals are generated. The question may be asked whether these radicals are distributed randomly along the polypeptide chair or whether they are preferentially located on certain amino acid residues.

The electron paramagnetic resonance (epr) patterns of proteins irradiated at room temperature have been found[2-5] to be mainly of two kinds. One of these has been interpreted[2] as due to a cysteine radical (I) and the other as predominantly due to a glycine radical (II). However, the assignment of the free radical II as the species responsible for the doublet observed with irradiated proteins is not without ambiguity because of the difficulties of unraveling superimposed spectra.



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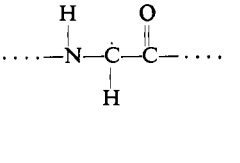

II

To identify the free radicals on carbon in irradiated proteins, a highly reactive scavenging agent is required which will tritium label the radicals produced by radiation without creating a new radical population by secondary reactions. Since hydrogen sulfide is known[6] to have a high rate of reaction with alkyl radicals in the gas phase and since sulfhydryl radicals will not abstract hydrogen from primary, secondary, and tertiary carbon–hydrogen bonds of hydrocarbons,[7] we have investigated the reaction of irradiated ribonuclease

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(RNase) with tritiated hydrogen sulfide (HST). The distribution of carbon-bound tritium among the various amino acids can be readily determined by hydrolysis of the protein, separation of the amino acids by ion-exchange chromatography, and scintillation counting of the individual amino acids.

Experimental Section

Materials Used. Ribonuclease A was obtained from Sigma Chemical Co.; only type IIIA was used in these experiments.

Carboxymethylated (CM) Reduced Ribonuclease. The reduction of ribonuclease was performed as already described,[8] except that the product was separated from the reduction mixture on Sephadex G-25.[9] After lyophilization, the reduced protein was carboxymethylated as described earlier[8] with the modification that the product was isolated on Sephadex as for reduction. The eluate was lyophilized.

Ribonuclease or CM reduced ribonuclease was deionized on a mixed-bed ion-exchange column as previously described.[9] The eluate was lyophilized and the fluffy product was packed by tamping with a glass rod in preparation for irradiation.

New England Nuclear Co. tritiated hydrogen sulfide (224 mcuries/mmole) was passed into a previously evacuated liquid nitrogen trap, and the hydrogen formed by self-radiolysis was removed before addition to protein.

Irradiation of Ribonuclease, HST Exposure, and Exchange. The deionized lyophilized protein samples (approximately 20 mg) were evacuated for 2 hr at 10^{-3} mm in a cylindrical Pyrex vessel (approximately 15 cm³, 1 cm i.d.) equipped with a break-seal side arm. After sealing off under vacuum, the samples were irradiated at 25° in a cobalt γ-source at a dose rate of 10 Mrads/day (1 Mrad = 10^6 rads). Dose rates were measured with the ferrous sulfate dosimeter in vessels identical with those used in labeling experiments.

Immediately after irradiation of the protein samples, any volatile radiation products were removed, and 3.0 ml of oxygen-free, dry HST containing 30.0 mcuries of tritium was added to the exposure vessel. After a suitable time, HST was transferred to a storage bulb and the protein sample opened to air. Exchangeable tritium bound to O, N, and S was removed by dissolving the sample in 40 ml of 0.1 *N* acetic acid and heating to 65° overnight, followed by lyophilization. This treatment was carried out two or three times until constant specific activity was obtained. After exposure to HST, the protein samples contained of the order of 10–20 mcuries of tritium; after the exchange procedure about 1–3 μcuries/mg of tritium bound to carbon remained.

Amino Acid Analysis and Counting. Acid hydrolysis of the protein was carried out with 2 mg in 2 ml of 6 *N* HCl at 110° for 18 hr under vacuum. The amino acids were separated on a Beckman-Spinco amino acid analyzer, and the effluent solution was collected in about 205 fractions each containing 1.7 ml. The tritium content of each fraction was determined by mixing with 15.0 ml of Bray's[10] solution and counting in a Tricarb scintillation counter. The counting efficiency for each fraction was determined by the addition of standardized HTO. Tritium content was determined by comparison with primary standards which agreed with one another to ±5%.

Tritium Exchange with Amino Acids. Experiments to determine the extent to which tritium exchanges into amino acids during hydrolysis were performed with approximately 5 mg of each amino acid (Sigma Chemical Co.), hydrolyzed with 1 ml of 12 *N* HCl and 1 ml of HTO (200 μcuries/ml), under conditions otherwise identical with the hydrolysis of the protein. The hydrolysate was transferred to a counting vial and dried under vacuum at room temperature over P_2O_5 and NaOH pellets. After addition of 1 ml of 6 *N* HCl and 4 ml of water, each sample was dried as before to remove exchangeable tritium on O, N, and S. The tritium content was determined by scintillation counting. Duplicate experiments were carried out for each amino acid.

Epr Measurements. Electron paramagnetic resonance measurements were carried out using a Varian V-4500 epr spectrometer with a 6-in. magnet which was regulated and scanned by "Fieldial." Changes in the sensitivity of the instrument were prechecked using a standard free radical sample (diphenylpicrylhydrazyl) over a period of several hours and were found to be negligible.

Results and Discussion

Some of the reactions to be considered in the HST labeling technique are shown in eq 1a–6.

$$\geqslant C{-}R \rightsquigarrow \geqslant C\cdot + R\cdot \quad (1a)$$

$$R\cdot + \geqslant C{-}H \longrightarrow \geqslant C\cdot + RH \quad (1b)$$

$$\geqslant C\cdot + HST \longrightarrow \geqslant C{-}T + HS\cdot \quad (2a)$$

$$\geqslant C\cdot + HST \longrightarrow \geqslant C{-}H + TS\cdot \quad (2b)$$

$$2HS\cdot \longrightarrow H_2S + S \text{ or } H_2S_2 \quad (3)$$

$$\geqslant C{-}H + HS\cdot \longrightarrow \geqslant C\cdot + H_2S \quad (4)$$

$$\geqslant C\cdot + HS\cdot \longrightarrow \geqslant C{-}SH \quad (5)$$

$$>C{=}C< \text{ or } -\text{phenyl} + HS\cdot \longrightarrow P\cdot \longrightarrow PT \quad (6)$$

Free radicals are produced in the γ-radiolysis of proteins by loss of hydrogen or various groups[11] (1a). Hydrogen atoms or other radicals may in turn abstract hydrogen atoms from the protein (1b). If the bond broken in reaction 1a is a C–H bond, it can be repaired by reactions 2a and 2b with introduction of a tritium label by the former. The resulting sulfhydryl radicals react predominantly by disproportionation and dimerization[12] since the abstraction reaction (4) has been shown not to occur with gaseous hydrocarbons.[7] This is to be expected in view of the low bond dissociation energy, 90 kcal,[13] of the HS–H bond. However, abstraction from the methylene group of phenylalanine and tyrosine may be possible; this reaction appears to be unimportant, since these two amino acids were found to contain very little tritium. The reaction of HS· radicals with trapped radicals on the protein will be suppressed, since the ratio of the H_2S concentration in the gas phase to the stationary state HS· concentration is extremely large. Products of reaction 5 would not contain any nonexchangeable tritium. Another possible reaction of HS· radicals is addition to olefinic double bonds, if these are produced by radiation, or to aromatic rings (6). Such thiol derivatives of phenylalanine or tyrosine could then contain carbon–tritium bonds as a result of reaction 2a.

In order to ensure that the observed tritium distribution corresponds closely to the free-radical distribution produced by γ-irradiation, the labeling experiments must be planned to fulfill three conditions. First, the time of exposure to HST must be sufficient to ensure that all of the free radicals on carbon react. Otherwise trapped radicals which might be located on amino acid residues in the interior of the molecule would remain unlabeled. Second, there must be no significant amount of exchange of tritium bound to carbon during removal of the exchangeable tritium bound to oxygen, nitrogen, and sulfur, or during acid hydrolysis of the protein. Third, the extent of tritium labeling induced by β-decay in the presence of HST (subsequently referred to as "β-labeling") must be small compared to tritium introduced into the protein as a consequence of radical formation by γ-radiolysis ("γ-labeling").

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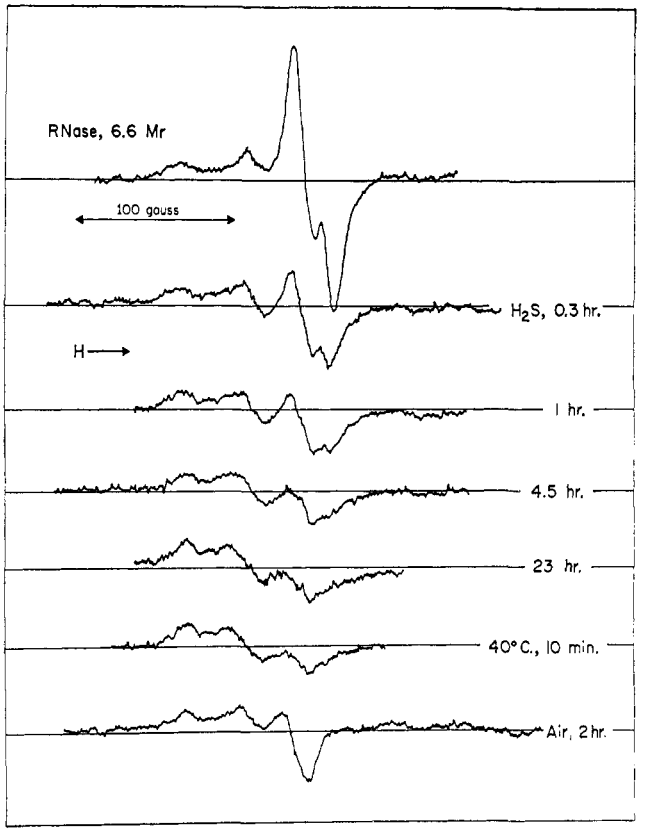

Figure 1. The reaction of free radicals on carbon in irradiated RNase with hydrogen sulfide, as studied by epr. The curves represent the first derivative of the absorption curves.

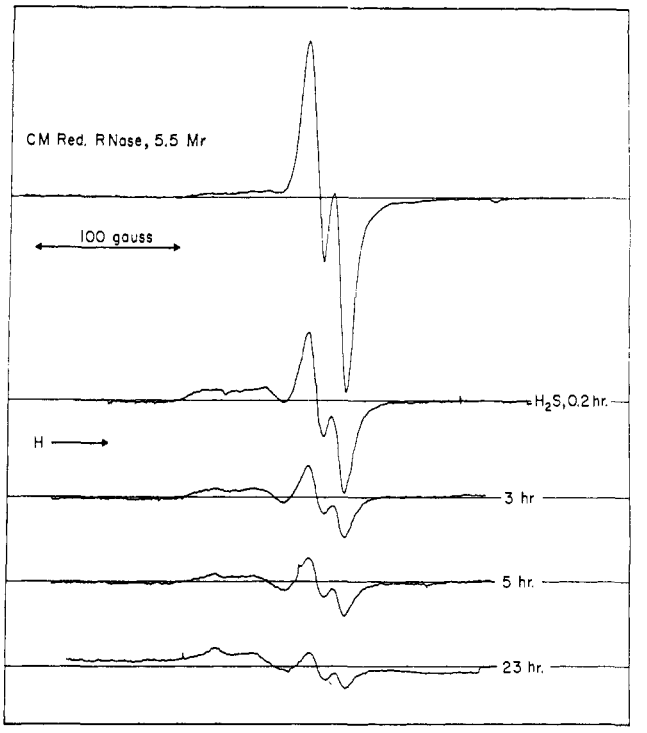

Figure 2. The reaction of free radicals on carbon in irradiated CM reduced RNase with hydrogen sulfide, as studied by epr.

Epr Studies. The reaction of free radicals with hydrogen sulfide can be conveniently followed by observing the electron paramagnetic resonance (epr) spectrum as a function of time. Figure 1 shows the results of a typical experiment in which 20 mg of lyophilized RNase A, γ-irradiated to 6.6 Mrads, was subsequently exposed to 3 ml of H_2S at a pressure of about 15 cm at room temperature. After 20 min a marked decrease of the doublet peak due to carbon radicals occurred. After 4 hr the epr spectrum approximated that of the "sulfur pattern" observed by Henriksen[4] from irradiated cysteine and cysteamine hydrochloride. There also appeared to be an increase in the total number of sulfur-type radicals as a result of H_2S exposure. The reaction responsible for this increase has not been identified at present and is being further investigated. No further significant changes occurred in the next 20 hr. Warming to 40° for 10 min after H_2S exposure produced virtually no change. This contrasts with an experiment in which ribonuclease was irradiated at 25°, not treated with H_2S, and then warmed to 40° for 10 min. These conditions resulted in a marked decrease of the "doublet" radical signal, and this change is consistent with the conclusion that almost all of the carbon radicals have reacted with H_2S. An analogous experiment, in which irradiated CM reduced RNase was exposed to H_2S, is shown in Figure 2. The exposure time of 4 hr was selected for γ-labeling experiments as a compromise to ensure almost complete reaction of carbon radicals and a reasonably small contribution due to β-labeling.

Exchange Studies. The exchange from carbon–tritium bonds during removal of tritium bound to O, N, or S was investigated by adding tritiated water to native RNase during this procedure. With 5 mcuries of tritiated water added to 20 mg of ribonuclease no carbon-bound tritium was incorporated.

The exchange of tritium between individual amino acids and the acid medium during protein hydrolysis was studied as described in the Experimental Section. The following percentages of exchange were calculated with the assumptions of a protium–tritium fractionation factor of 0.88[14,15] and exchange from all possible C–H bonds: lysine, 0.18%; methionine, 0.41%; proline, 0.40%; histidine, 1.7%; threonine, 0.14%; serine, 0.98%; cystine, 4.1%; arginine, 0.33%; glycine, 12.6%; leucine, 0.31%; tyrosine, 15.3%; alanine, 0.40%; phenylalanine, 1.2%; aspartic acid, 35%; glutamic acid, 14.7%; isoleucine, 0.18%; valine, 0.19%; and carboxymethylcysteine, 40%.

An exchange experiment in which 2 mg of native RNase was hydrolyzed under standard conditions in the presence of 1 mcurie of HTO, followed by amino acid analysis and scintillation counting, showed the amount of exchange occurring for aspartic acid, glutamic acid, and tyrosine was approximately the same as in the experiments with individual amino acids. This confirms the validity of determining the amount of exchange with individual amino acids.

Exchange losses of tritium are not significant for the present work except for aspartic acid, glutamic acid, tyrosine, and carboxymethylcysteine. If only one C–H bond of aspartic acid and of glutamic acid exchanges, tritium exchange would be almost 100 and 74%, respectively. Tyrosine is known[16] to have two

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labile positions *ortho* to the hydroxyl group, and thus exchange is 54%; for carboxymethylcysteine exchange is almost 100% if one assumes two exchangeable C–H bonds.

The amount of carbon-bound tritium exchanged into the amino acids represents the upper limit of tritium that could be lost from tritium-labeled RNase during acid hydrolysis, since the tritium may not be located at the exchangeable sites. For this reason the observed tritium content after protein hydrolysis cannot be quantitatively corrected for possible exchange losses. However, it must be remembered that the small amount of tritium found in the aspartic acid of HST-labeled RNase may be due to loss of tritium by exchange during hydrolysis.

Investigation of "β-Labeling." The amount of tritium introduced into the protein by "β-labeling" was determined by exposing nonirradiated RNase A to HST for 4 and 65 hr. The results of Table I show that the amounts of carbon-bound tritium incorporated, 0.073 and 1.03 μcuries/mg, respectively, were approximately proportional to the time of exposure. For experiments in which ribonuclease was γ-irradiated before HST exposure, the amount of tritium introduced by "β-labeling" was subtracted from the total tritium incorporated to obtain the amount of "γ-labeling." The percentages of "γ-labeling," defined as

$$\frac{\text{"}\gamma\text{-labeling"} \times 100}{\text{"}\gamma\text{-labeling"} + \text{"}\beta\text{-labeling"}}$$

for such experiments are listed in Table I.

Table I. *G* Values for Tritium Incorporation

Wt of protein, mg	γ-dose,[a] ev × 10^{-18}	β-dose,[b] ev × 10^{-18}	μcuries/mg[c]	% γ-labeling	$G(T)_\gamma$ × 10^{3}[d]
		RNase			
21.8	6.8	0.091	0.66	90	4.0
24.5	44.5	0.091	1.49	96	1.6
29.3	11.7	1.01	1.58	53	4.4
21.8	0	0.091	0.073	0	...
22.0	0	1.48	1.03	0	...
		CM Reduced RNase			
20.5	6.4	0.091	0.59	82	3.2
23.0	8.3	1.61	2.40	24	3.2

[a] Energy absorbed by protein. [b] Total energy available from β-decay of HST. [c] Tritium bound to carbon. [d] $G(T)_\gamma$ is the number of tritium atoms incorporated per 100 ev of energy absorbed by the protein from the Co^{60} γ-beam.

From the $G(T)_\gamma$ values of Table I, a rough estimate of the G value for radical formation on carbon can be made. The initial specific activity corresponds to a ratio for H_2S/HST of 129, but the effective specific activity for labeling carbon radicals might be decreased by a maximum possible factor of 3 if exchange on O, N, and S precedes reaction with radicals. The isotope effect for reaction 2a is not known but is probably small (<10), since the activation energy for the analogous reaction $CH_3\cdot + H_2S$ is only 2.6 kcal.[6] Assuming the initial ratio of H_2S/HST to be the effective one and neglecting intermolecular and intramolecular isotope effects, the G value for carbon radical formation, $G(C\cdot)$, is equal to $258G(T)$. Thus for ribonuclease A irradiated at γ-doses of 5 and 29.1 Mrads, $G(T)$ values of 2.0×10^{-3} and 0.81×10^{-3} correspond to $G(C\cdot)$ values of 1.0 and 0.4, or 0.08 and 0.18 carbon radical per molecule, respectively. These values are in unexpectedly good agreement with the results of Hunt and Williams.[5] In a careful epr study these workers found 0.076 and 0.142 carbon radical per molecule at 5 and 25 Mrads, respectively.

The geometrical arrangement of the exposure vessel is such that only a small fraction (<5%) of the β-particles generated in the gas phase is absorbed by the protein. The irreversible adsorption of H_2S was shown to be negligible under our experimental conditions. However, a substantial fraction of the tritium (approximately $^2/_3$ after 4 hr of exposure) is present within the solid protein due to exchange on O, N, and S. The energy released by the β-decay of this portion of the tritium is absorbed by the solid, leading to tritium labeling by processes analogous to those responsible for "γ-labeling." Tritium may also be incorporated into the protein as a consequence of radiolysis in the gas phase; the mechanism of these processes is being investigated.

Free-Radical Distribution. The distribution of tritium among the various amino acid residues was determined by hydrolysis of irradiated ribonuclease, after HST treatment, followed by amino acid analysis. The results of a typical amino acid analysis and counting experiment are shown in Figures 3 and 4. For every ninhydrin-positive peak with the exception of ammonia, there is a corresponding tritium peak. However, there are some tritium peaks which are eluted at positions other than those of the naturally occurring amino acids and without any corresponding ninhydrin-positive peaks. This is either due to the high specific activity of the radiation-produced compounds or, less probably, to the absence of ninhydrin-positive groups in these products.

The total amount of tritium from all of the observed peaks was found to be equal to the total tritium content in the sample after hydrolysis but before amino acid analysis. This proves that all of the tritium-containing components have been eluted from the ion-exchange columns of the amino acid analyzer.

The results of several tritium distribution experiments are summarized in Table II. Several salient features of the results of Table II may be noted.

(A) When ribonuclease is "γ-labeled" at 5 Mrads, only a small fraction (1.4%) of the total tritium is present in glycine. Hence it may be inferred that glycine radicals, whether made from glycine or by loss of the side chain from the α-carbon of other amino acids, do not play a dominant role under the conditions of these experiments. Patten and Gordy,[2] using the results of epr studies of several proteins and polyamino acids, concluded that the observed epr signals in collagen, gelatin, and histone are predominantly due to glycine radicals with minor contribution from alanine radicals. Similar interpretations of the observed doublet were offered for other proteins such as chymotrypsinogen, lysozyme, bovine albumin, and ribonuclease. Considering, however, the fact that a doublet pattern[2] could originate from amino acid residues other than those of glycine, and that the same amino acid radical could show different epr patterns according to the specific steric environment in a protein, it would be

Table II. The Distribution of Tritium in Irradiated Native and CM Reduced RNases

		RNase				CM reduced RNase	
γ-dose, Mrads		5.0	29.0	6.4	0	5.0	5.8
HST exposure, hr		4.0	4.0	44.5	65.0	4.0	70.8
% γ-labeling		90	96	53	0	81	23
Amino acid	No. of residues	% tritium per residue × 100[a]					
Lysine	10	373	379	400	294	102	77
Methionine	4	252	245	210	197	457	410
Proline	4	147	130	120	103	217	217
Histidine	4	113	101	189	427	87	90
Threonine	10	79	76	71	59	162	97
Serine	15	65	61	59	65	94	66
Cystine	4	64	64	108	78	...	...
Arginine	4	50	47	39	50	74	51
Glycine	3	46	63	58	72	139	73
Leucine	2	39	33	28	40	34	43
Tyrosine[b]	6	37	32	26	30	63	27
Alanine	12	24	19	22	22	11	21
Phenylalanine	3	21	24	19	20	43	29
Aspartic, asparagine[b]	15	18	13	17	36	38	9
Glutamic, glutamine[b]	12	17	16	14	13	11	13
Isoleucine	3	6	7	9	8	3	13
Valine	9	5	9	11	10	9	15
CM cysteine[b]	8	...	...	...	...	47	46
% T in unidentified compounds		4	9.8	5	2.3	2.8	25

[a] Values were obtained by calculating the percentage of the total carbon-bound tritium in the protein which is incorporated into a given amino acid, dividing by the accepted (C. H. W. Hir, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **235**, 633 (1960)) number of residues in the protein, and multiplying by 100. [b] Not corrected for possible loss of T during hydrolysis.

difficult to draw a definite conclusion from the composite epr spectra.

(B) Some amino acid residues contain much more tritium than others. For native RNase A irradiated to a dose of 5 Mrads, the tritium content of lysine is more than 70 times greater than that of valine. Such large factors rule out a correlation between tritium content and the number of primary, secondary, and tertiary carbon–hydrogen bonds. Moreover, there does not appear to be any simple correlation between tritium content and the polar character of the amino acid. It might be generalized from X-ray diffraction studies[17–19] that most polar groups lie on the surface of the protein, while nonpolar side chains are in general confined to the interior of the molecule. Location of an amino acid residue on the surface or in the interior of the protein thus does not seem to be the only decisive factor. A more detailed investigation of the distribution of tritium among the residues of a given amino acid is in progress.

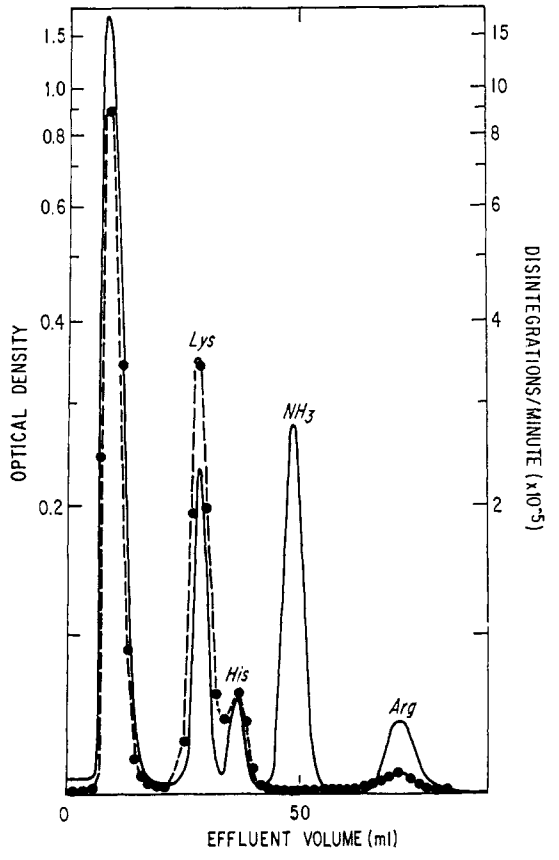

Figure 3. Basic amino acids from RNase A irradiated at 6.4 Mrads and exposed to HST for 44.5 hr: ———, optical density; - - - -, disintegrations/min.

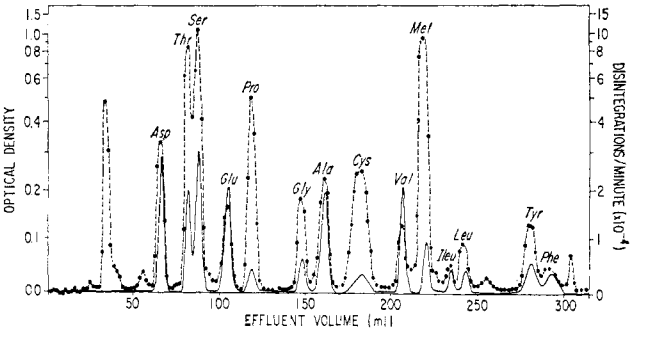

Figure 4. Neutral and acidic amino acids from RNase A irradiated at 6.4 Mrads and exposed to HST for 44.5 hr: ———, optical density; - - - -, disintegrations/min.

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(18) M. F. Perutz, *ibid.*, **140**, 863 (1963).
(19) C. C. F. Blake, D. F. Koenig, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Nature*, **206**, 757 (1965).

(C) The distributions of tritium in ribonuclease irradiated with γ-rays at 5 and 29 Mrads are almost identical with the exception of two amino acids. However, epr measurements indicate that radical production as a function of dose is strictly linear to only about 1 Mrad. The D_{37} does for the loss of enzymatic activity of ribonuclease irradiated in vacuum and subsequently exposed to H_2S has been reported[5] to be 44 Mrads, so that the percentage of molecules which has been subjected to two inactivating events can be calculated by simple target theory[20] to be 0.6 and 11% at doses of 5 and 29 Mrads, respectively.

(D) The tritium distributions obtained by "β-labeling" and "γ-labeling" are quite similar. When the amino acids are arranged in order of decreasing tritium content for "γ-labeling," the results of "β-labeling" can be brought into one-to-one correspondence by shifting only five amino acids, namely histidine, cystine, glycine, and aspartic acid, by a few positions in the vertical sequence of Table II. A similar result is found when β- and γ-labeled CM reduced ribonucleases are compared. These results can be understood, since a substantial fraction of the "β-labeling" results from direct energy absorption by the protein.

(E) The results of Table II show that only 4% of the total tritium incorporated into "γ-labeled" ribonuclease at 5.0 Mrads is found in unidentified compounds. Such products could arise either from breaking C–C or C–S bonds in amino acid side chains, or the $\geqslant$C–NH– bond of the polypeptide chain, or by addition of sulfhydryl radicals to aromatic rings. C–C bond cleavage could also lead to labeled glycine and alanine from many other amino acids, serine from threonine and valine from isoleucine, while alanine could be formed by breaking the C–S bond in cystine. Adding the tritium content of all of these residues to that of the unidentified compounds indicates that the total amount of C–C, C–S, and $\geqslant$C–NH– bonds broken by γ-radiolysis is at most one-fifth of the amount of C–H bond breakage and presumably much smaller.

No evidence for breaks in the polypeptide chain as a result of radiolysis of dry ribonuclease has been reported by other workers. Thus Friedberg and Hayden[21] have shown that the number-average molecular weight is not changed at 30 Mrads. It may be, however, that main-chain breaks do occur but that the intact disulfide bonds or the formation of intramolecular cross-links might have prevented their detection.

(F) A quite different order of tritium content is found when "γ-labeled" CM reduced ribonuclease is compared with native ribonuclease. The changes in the physical properties of enzymatically inactive material indicate[9] extensive destruction of secondary and tertiary structure. It may therefore be concluded that the free-radical distribution in γ-irradiated protein depends markedly on the conformation of the molecule.

Radiobiological Implications. The present results are of interest in connection with repair mechanisms in radiobiology. It is known[22,23] that certain SH compounds, which partly protect proteins against radiation inactivation when present with the protein in the dry state during irradiation, can also receive unpaired spins which were initially located on the protein. Two possible mechanisms for the intermolecular transfer of unpaired spins have been suggested.[22] One is hydrogen transfer according to eq 7. When RSH is cysteamine

$$\text{protein}\cdot + \text{RSH} \longrightarrow \text{protein-H} + \cdot\text{RS} \qquad (7)$$

or penicillamine, this mechanism would seem to require free radicals to be preferentially located on the surface of the protein.

The second possibility would be for a small diffusible radical (A·), formed from the protein, to react with the thiol, as shown in eq 8. The present experiments di-

$$\text{A}\cdot + \text{RSH} \longrightarrow \text{AH} + \text{RS}\cdot \qquad (8)$$

rectly demonstrate that process 7 takes place when RSH is hydrogen sulfide.

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