Effects of γ Irradiation on the Structure of Ribonuclease*

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ABSTRACT: The effects of γ irradiation on aqueous solutions of ribonuclease A (polyribonucleotide 2-oligonucleotidotransferase, EC 2.7.7.16) have been studied using the techniques of viscometry and tritium–hydrogen interchange measurement. Irradiated samples showed a progressive increase in viscosity with increased enzymic inactivation; the most highly inactivated sample had a viscosity approaching that of native enzyme in 8 M urea. Ultracentrifuge patterns demonstrated the absence of aggregates in a heavily irradiated sample.

Tritium–hydrogen exchange studies, carried out using Bio-Gel P-2 column fractionation, also demonstrated the progressive loss of tightly bound, slow-exchanging hydrogens with increased radiation effect. Kinetic analyses of the exchange-out data confirmed that the loss of slow-exchanging hydrogens reflected the progressive unfolding of helical structure and the loss of intramolecular side chain hydrogen bonds. The results clearly demonstrate that irradiation causes extensive disorientation of the native ribonuclease A conformation.

This communication describes recent studies of the molecular configuration of ribonuclease A (RNAase) after γ irradiation of the enzyme in aqueous solution. Previous work showed that irradiated RNAase samples contained altered molecules which were chromatographically separable from the native enzyme, but which showed enzymic activity *vs.* RNA (Slobodian *et al.*, 1962). Subsequent analyses of highly inactivated samples (with only 15% residual activity) showed two sulfhydryl groups per mole and suggested that, on the average, at least one disulfide bridge had been cleaved by irradiation; amino acid analyses showed that a few residues had been destroyed. Further, unlike native RNAase A, these irradiated samples were digestible by trypsin (Slobodian *et al.*, 1965b), and had spectral characteristics which suggested the unmasking of "buried" tyrosine residues (E. Slobodian and W. Newman, unpublished data). From these observations, it was evident that irradiation had caused some damage to the primary structure of the molecule, and had also effected significant changes in the secondary and tertiary structure of the enzyme. The present study was undertaken in order to ascertain the magnitude of the latter radiation effect. Reports by others have shown that ionizing irradiation does indeed alter the molecular configuration of RNAase. Thus, Hunt and Williams (1964) found that X-irradiation of dry RNAase caused some rupture of disulfide bridges and of tyrosine hydrogen bonds. Ray *et al.* (1960) noted that X-irradiation of dry RNAase led to disulfide cleavage as well as to an increased susceptibility of the enzyme to proteolytic digestion. Hayden and Friedberg (1964) reported that γ irradiation of RNAase solutions caused destruction of certain amino acid residues; from studies of deuterium–hydrogen interchange it was found that there was a progressive loss in the number of hard-to-exchange amide hydrogens. The same workers have also reported that γ irradiation of dry RNAase causes an increase in the viscosity of the enzyme (Friedberg and Hayden, 1965).

In the work described here, the physical properties of irradiated RNAase have been investigated through measurements of viscosity and tritium–hydrogen interchange in a series of irradiated enzyme samples. The general method of Englander (1963) was applied to the study of tritium–hydrogen interchange kinetics.

Methods and Materials

Ribonuclease A was purchased from Worthington Biochemicals Co. (lot no. 6081 and 6094). The enzyme was obtained as a 1% solution in phosphate buffer, and was desalted on Sephadex G-25 (Fleisher, 1961). The desalted solution was lyophilized to dryness and the dry powder was stored at $-2°$. The enzyme had an E_m at 278 mμ of 9.6×10^3. Oxidized RNAase A was purchased as a dry powder from Sigma Chemical Co. (lot no. 75B-8550 and 85B-8830) and was used without further purification. The E_m at 275 mμ was 8.4×10^3 at pH 4.4. Tritiated water was obtained from Volk Radiochemicals Co. at a concentration of 100 mc/ml; this stock solution was diluted to 20 mc/ml for use in tritiation experiments. Bio-Gel P-2 was obtained from Calbiochem; the resin was swelled for 2 hr in 0.05 M NaCl, fines were removed by repeated washings, and a 2.5×12 cm column was poured from a slurry of the resin.

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Irradiated RNAase A. A solution of RNAase A was made up in glass-distilled water to a concentration of 1 mg/ml and was adjusted to pH 6; about 70 ml were irradiated in a Petri dish in a Keleket-Barnes ^{60}Co hectacurie unit (Rubenfeld and Slobodian, 1960). Doses[1] of 40,000–250,000 r were used to produce a series of samples of varying degrees of inactivation. Irradiated enzyme solutions were scanned in the Beckman DU spectrophotometer between 250 and 300 mμ to obtain the E_m at 278 mμ for each sample. Suitable aliquots were assayed for enzymic activity *vs.* substrate RNA (Slobodian *et al.*, 1962). Occasional checks of both enzymic activity and optical density were made during the course of subsequent experiments, and these showed no change in activity or spectrum with time. The major portion of each irradiated sample was lyophilized to dryness. Viscosity measurements and tritiation experiments were made with concentrated solutions of the dry powder.

Viscosity Determinations. Viscosity measurements were made with a Cannon-Fenske capillary viscometer (size no. 100) with an out-flow time for 0.1 M KCl of 65 sec. All measurements were carried out at 20° on solutions of native or irradiated enzyme samples made up to 5–10 mg/ml in 0.1 M KCl. Experiments were carried out to determine whether the reduced viscosities of irradiated samples were concentration dependent. Viscosity measurements were also carried out on selected samples made up in 8 M urea in 0.1 M KCl, at enzyme concentrations of about 5–10 mg/ml. No less than 10 estimates were made for each sample studied.

Tritium Exchange. To a given RNAase A sample (native, irradiated, or oxidized) were added tritiated water, 0.01 M sodium acetate buffer (pH 5), and sufficient 0.1 M NaCl to give a reaction mixture with a protein concentration of 5 mg/ml and a specific activity of about 6×10^8 cpm/ml. The reaction mixture was heated at 62° for 15 min and was then cooled. Determinations of optical density and of enzymic activity were made both prior to and after the heating period to ensure that no irreversible changes occurred during the heat treatment.

About 1.0–1.5 ml of the tritiation reaction mixture was applied to a Bio-Gel P-2 column (2.5 × 12 cm) which had been preequilibrated at 4° with 0.1 M NaCl. Elution with 0.1 M NaCl was started immediately after applying the mixture to the column, using a flow rate of 14 ml/min, and samples of 1 ml/tube were collected. The entire procedure was carried out at 4°.

After each column run, protein concentration in the effluent was determined from the optical density at 278 mμ in the Beckman spectrophotometer, using the E_m at 278 mμ for each enzyme sample. Aliquots of 0.4 ml were diluted with 10 ml of an appropriate phosphor in a 20-ml glass vial, and counting was carried out in a Packard Tri-Carb spectrometer Model 3003. The protein concentration in the vials never exceeded 0.05 mg/ml; it was found, by counting appropriate standards, that at this protein concentration, there was no quenching effect. For calculation of specific activity, a standard, prepared from an aliquot of the original tritiation mixture, was always counted with the samples of the column effluent.

In a typical fractionation, the tritiated protein peak appeared in the effluent at about 24 ml, in about 2 min; the free THO[2] did not elute until 30 ml. This rapid elution experiment was taken as a zero-time run, which is to indicate that it was the fastest satisfactory separation which could be obtained. During the 2 min before the protein peak appeared, there was an exchange-out of some of the tritium from the fully tritiated protein into the aqueous environment of the column. Therefore, a zero-time run gave a measure of the tritium retained by the protein at 2 min. The kinetics of the loss of tritium were followed by increasing the in-column equilibration time; this was done by allowing the sample to drain half-way through the column, stopping the column flow for a specified time, and then resuming rapid elution. It was found that an in-column incubation of up to 8 hr on Bio-Gel P-2 still afforded satisfactory separation of protein from THO, and that diffusion effects were negligible. Extremely long exchange-out times (in days) were obtained by pooling the protein effluent of a zero-time separation; this protein solution was stored at 4° for the desired time, and was then reapplied to the column[3] to separate protein from tritium which had exchanged out into the medium during the in-test-tube equilibration.

Results

Viscosity Studies. Viscosity measurements were made on 0.1 M KCl or on 8 M urea–0.1 M KCl solutions of various samples, and the reduced viscosity of each sample was calculated as $(\eta - \eta_0)/\eta_0 C$, where η is the flow time of enzyme solution, η_0 is the flow time of the solvent, and C is the protein concentration in g/100 ml. These viscosity values were corrected for water of hydration of the protein, using the value 0.695 reported for the partial specific volume of RNAase and assuming 20% water of hydration for all samples (Harrington and Schellman, 1956); additional corrections for urea binding were applied to values obtained from measurements carried out in 8 M urea (Harrington and Schellman, 1956). The observed and corrected viscosity values are shown in Table I. The viscosities obtained for native RNAase A, oxidized RNAase A, and native RNAase A in 8 M urea all agree well with values in the literature (Harrington and Schellman, 1956; Buzzell and Tanford, 1956). Determinations of reduced viscosity were made at several enzyme concentrations (not

[1] Absorbed dose rate was measured by ferrous sulfate dosimetry (Fricke and Morse, 1927), and was 505 roentgens/min.

[2] Abbreviations used: THO, tritiated water.

[3] Usually a duplicate volume was used for the second fractionation. Use of only one column would limit the number of experiments, since after each column run, it was necessary to wash the column with eluent for about 5–6 hr in order to remove the last traces of THO.

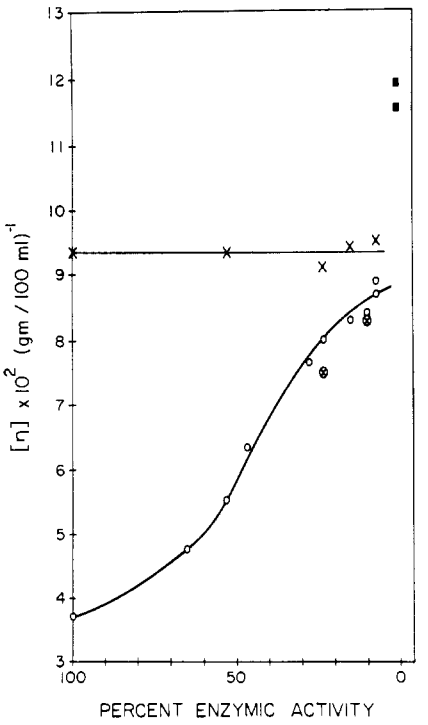

FIGURE 1: The correlation of loss of enzymic activity in irradiated RNAase A samples with change in viscosity. —O—O—, samples in 0.1 M KCl, the point at 100% enzymic activity corresponding to native enzyme. ×—×, the same samples in 8 M urea–0.1 M KCl; ⊗, samples in 0.1 M KCl after recovery from 8 M urea; ■, oxidized RNAase A in 0.1 M KCl.

exceeding 10 mg/ml). No concentration effect was observed for either native or irradiated enzyme, in either 0.1 M KCl or 8 M urea–0.1 M KCl solutions (Table II). Hence, the intrinsic viscosity (at infinite dilution) was taken to be equivalent to the reduced viscosity. The results obtained from viscosity measurements are plotted in Figure 1 as corrected intrinsic viscosity $[\eta]$ *vs.* residual enzymic activity.

The results shown in Figure 1 indicate a viscosity increase upon irradiation of RNAase A; the increase is not linear with respect to increased inactivation. At 8% residual enzymic activity the viscosity reaches 8.7, close to the value 9.4 found for native enzyme in 8 M urea. Of particular interest is the finding that dissolution of each irradiated sample in 8 M urea–0.1 M KCl caused a further viscosity increase to 9.4. The latter viscosity is that of native enzyme in 8 M urea, and was the maximum viscosity observed for any sample other than oxidized RNAase A. The urea effect was completely reversible, as was shown in experiments in which, after the urea was removed by extensive dialysis *vs.* 0.1 M KCl, the viscosity returned to the original value; furthermore, there was no net change in optical density profile or in enzymic activity.

The observed increase in viscosity upon irradiation is indicative of a loss of ordered structure in the protein.

TABLE I: Results of Viscosity Studies on Native, Irradiated, and Oxidized RNAase A Samples.

Sample (M)	$[\eta] \times 10^2$	v_1[a]	v_2[a]
Native RNAase A in KCl (0.1)	3.4	3.8	...
Native RNAase A in urea (8)–KCl (0.1)	9.5	(10.9)	9.4
Irradiated RNAase A			
65% active in KCl (0.1)	4.2	4.8	...
53% active in KCl (0.1)	4.8	5.5	...
53% active in urea (8)	9.5	(10.9)	9.4
47% active in KCl (0.1)	5.5	6.4	...
28% active in KCl (0.1)	6.7	7.7	...
24% active in KCl (0.1)	6.9	8.0	...
24% active in urea (8)	9.3	(10.6)	9.2
15% active in KCl (0.1)	7.2	8.3	...
15% active in urea (8)	9.6	(11.0)	9.5
10% active in KCl (0.1)	7.3	8.4	...
10% active in urea (8)	9.7	(11.1)	9.6
8% active in KCl (0.1)	7.6	8.7	...
Oxidized RNAase A, KCl (0.1)	11.6	13.4	...

[a] v_1 is the viscosity increment on the basis of 20% hydration. v_2 is the viscosity increment on the basis of urea binding.

The possibility was considered that, especially in highly inactivated samples, some aggregation might occur, and that part of the observed viscosity increase might be due to the presence of polymeric molecules. However, examination of the most viscous of the irradiated samples (with 10% residual enzymic activity) in the Spinco Model E ultracentrifuge showed a single peak, with a sedimentation constant of 1.9 S, the value reported for native RNAase (Anfinsen *et al.*, 1954; Harrington and Schellman, 1956; Ginsburg *et al.*, 1956). The sedimentation pattern (Figure 2) showed that, although the irradiated sample was heterogeneous, all of the molecules present were in the same molecular weight range. As the sample studied was the one most likely to contain aggregates, and as it proved to be essentially monomeric, it was concluded that all other samples in the irradiated series were similarly free of aggregates. The observed elevated viscosities must therefore be attributed to intrinsic changes in the configuration of the molecule.

As highly inactive irradiated samples gave evidence of the rupture of at least one, but not more than two disulfide bridges per molecule (Slobodian *et al.*, 1965b), this structural change may be regarded as having some influence on the shape of the molecule and hence upon the observed viscosity. At the same time,the magnitude of the increase in viscosity upon irradiation also suggests that the molecule is extensively unfolded. It is relevant to note that a large increase in viscosity occurs when native RNAase A is dissolved in 8 M urea. The latter effect involves an unfolding of the protein mole-

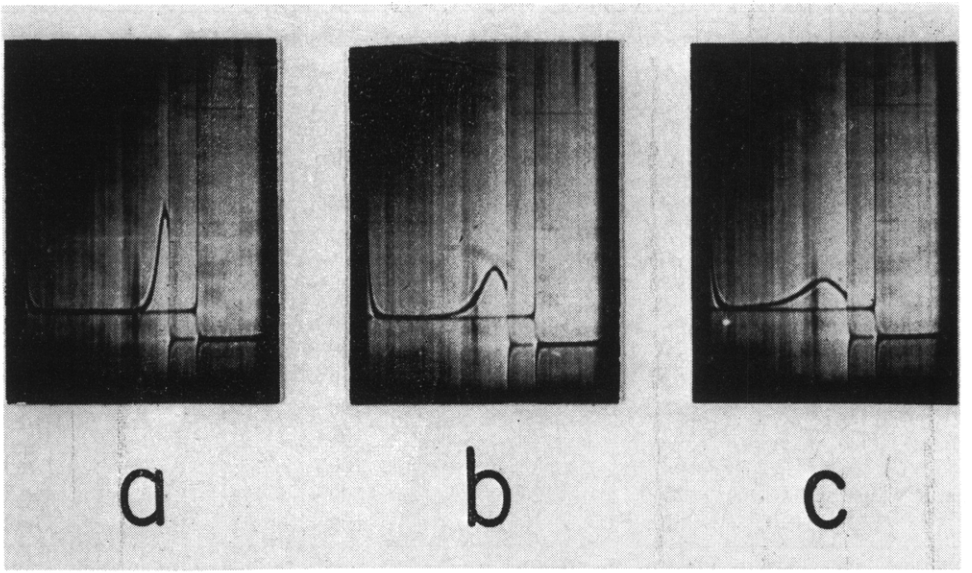

FIGURE 2: Ultracentrifugal patterns of irradiated RNAase A with 10% residual enzymic activity. (a), picture taken at 8 min, (b), at 24 min, and (c), at 44 min after the ultracentrifuge had reached full speed of 59,780 rpm. Protein concentration, 0.7%; temperature during sedimentation was 20°.

TABLE II: The Effect of Protein Concentration on Reduced Viscosity.

Sample	In 0.1 M KCl		In 8 M Urea–0.1 M KCl	
	Concn (mg/ml)	$\eta_{sp/c} \times 10^{2a}$	Concn (mg/ml)	$\eta_{sp/c} \times 10^{2a}$
Native RNAase A	9.5	3.5		
	7.9	3.4		
	6.2	3.5		
Irradiated RNAase A				
65% active	9.5	4.2		
	5.0	4.3		
53% active	10.0	4.8		
	6.0	4.7		
24% active	9.5	6.9		
	4.8	7.0		
15% active	9.0	7.2	10.0	9.6
	5.3	7.6	5.3	9.5
10% active	9.5	7.3	8.3	9.7
	7.0	7.2	4.2	9.8
	4.8	7.4		
8% active	9.0	7.6		
	7.0	7.5		
	5.0	7.7		

[a] Uncorrected for water of hydration or urea binding.

cule and has been attributed to the interaction of urea with the intramolecular hydrogen bonds and hydrophobic regions which stabilize the native enzyme (Anfinsen and Redfield, 1957). It is evident, from the data, that the viscosities of irradiated RNAase A samples also reflect a partial, albeit irreversible, unfolding of these molecules. Further and maximum unfolding is observed in 8 M urea, in which solvent maximum unfolding of native enzyme is also obtained; this urea effect is reversible, indicating that the further disorientation is occurring in undamaged portions of the molecule which still have enough structural integrity to permit refolding. Evidently, as in native enzyme, the limit on any further increase in viscosity is due to the spatial restrictions imposed by the remaining disulfide bridges.

Tritium Exchange Studies. A typical Bio-Gel P-2 column separation of tritiated protein from a tritiation reaction mixture is shown in Figure 3. It can be seen that constant specific activity was obtained throughout the major portion of the protein peak. The amount of tritium bound to protein was calculated as follows

$$\text{H/molecule} = \frac{E_m \times C/D \times 111}{C_0}$$

where E_m is the molar extinction coefficient at 278 mμ, C/D is the average specific activity (counts per minute per optical density at 278 mμ) of the protein, 111 is the molar concentration of hydrogen in water, and C_0 is the counts per minute per milliliter in the reaction mixture. The separation shown in Figure 3 is a zero-time run of an 8% active sample, and the calculated value for the number of hydrogens per molecule was plotted on an exchange-out time curve as a 2-min point, since the protein peak appeared at 2 min. In experiments involving longer exchange-out times, the time plotted was the total time elapsed between the start of exchange out and elution of the protein peak.

TABLE III: Effect of Reaction Conditions on Tritium Incorporation and Stability of RNAase A Samples.

Sample	Temp (°C)	Time (hr)	% Tritium[a] Incorp	% Enzymic Act. (after tritiation)	OD (after tritiation)
Native RNAase A	62	22	100	100	Unchanged
	62	0.25	100	100	Unchanged
Irradiated RNAase A					
63% activity[b]	25	24	80	63	Unchanged
	62	0.25	100	63	Unchanged
28% activity	72	1.5	100	...	...
	62	0.25	100	25	Unchanged
8% activity	25	24	100	8	Unchanged
	62	22	100	<2	10% high
	62	0.25	100	8	Unchanged
Oxidized RNAase A	25	24	100	0	Unchanged
	62	0.25	100	0	Unchanged

[a] Per cent tritium incorporation refers to the amount of tritium bound to a given protein sample under standard exchange-out conditions; 100% was taken as the maximum amount of tritium observed to be bound to the same sample under the most rigorous tritiation conditions used. [b] Activity refers to enzymic activity, compared to native RNAase A *vs.* substrate RNA.

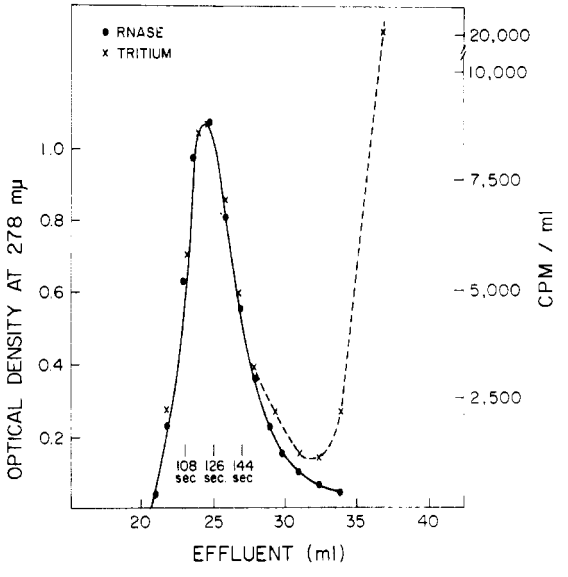

FIGURE 3: Bio-Gel P-2 column separation of tritiated-irradiated RNAase A (8% active) from a tritiation mixture. Column dimensions: 2.5 × 12 cm, eluent 0.1 M NaCl, flow rate 14 ml/min. Time inserts show time at which indicated tubes were collected after application of sample to column.

Tritiation Conditions. It was necessary, before studying tritium exchange out, to establish conditions which would effect complete tritiation of irradiated samples, but which would, at the same time, not produce irreversible changes in these samples. It was expected that highly inactivated samples would be unstable to prolonged heating. Several arbitrary tritiation conditions were tried, and as shown in Table III, a heating period of 15 min at 62° gave optimal results. It was found that the temperature threshold for complete tritiation of irradiated samples dropped with increased inactivation. Thus, whereas the 8% active sample could be tritiated at room temperature (25°), the 63% active sample gave only 80% of maximum tritium incorporation under the same conditions. Heating for 15 min at 62° gave, for both the 63% active sample and native RNAase A, as much tritium incorporation as could be obtained in a prolonged heating period. It should also be noted that 22 hr of heating at 62° destroyed the residual activity of the 8% active sample. Since heating for 15 min at 62° did not affect the activity of the 8% active sample, and as this sample was obviously the most labile in the series, all samples were, in the interest of consistency, tritiated under these conditions.

Tritium Exchange out. The curves in Figures 4 and 5 depict the tritium exchange-out behavior of native RNAase, oxidized RNAase, and a series of irradiated RNAase A samples. Each curve represents a series of experiments in which aliquots of each tritiated protein mixture were back exchanged with water, in column or in test tube, as described, for periods of time varying from 2 min to 24 hr. In each experiment, the protein was recovered free of THO, and the amount of tritium retained by the protein was measured. The results are plotted as the number of protons bound per molecule of protein *vs.* exchange-out time. The two uppermost curves show the exchange-out kinetics of two different preparations of native RNAase A. Although there is a slight difference between the two curves, both show clearly the existence of a significant number of hydrogens which exchange slowly with the medium and are

retained by the protein after hours or even days of equilibration. These tightly bound hydrogens are buried in the protein and reveal the secondary and tertiary structural characteristics of the native enzyme. In contrast, the two lowest curves, for oxidized RNAase A, show a rapid loss of tritium into the medium. The latter hydrogen interchange kinetics are typical of a random polypeptide chain, and fit very well to the known structural features of oxidized RNAase A. It is evident, from visual inspection of the exchange-out curves for irradiated RNAase A, that the irradiated samples had more rapid hydrogen interchange kinetics than native enzyme, and that this acceleration in exchange-out rate became more pronounced with increased degree of inactivation. The most highly irradiated samples studied (8–10% residual activity) gave exchange-out curves approaching those of oxidized RNAase A. Figure 5, which includes very long exchange-out times, further emphasizes the fact that irradiated samples showed a progressive decrease in content of tightly bound, slow-exchanging hydrogens with decrease in residual enzymic activity. These results show that irradiation disrupts both the helical and tertiary structure of the enzyme until, at high doses, the irradiated molecule has a structure approximating that of a random polypeptide chain.

The findings are consistent with viscosity data, which indicated that the irradiated samples were unfolded. Figure 6 demonstrates the relationship between loss of enzymic activity, change in viscosity, and loss of slow-exchanging hydrogens. In order to make this comparison, loss of hydrogens was plotted as the per cent of slow-exchanging hydrogens retained by each irradiated sample after 2 and 24 hr, taking 100% to equal the number of hydrogens found in native enzyme at the same exchange-out periods. The change in viscosity was evaluated as follows: as all samples whether native or irradiated had a viscosity of 9.4 in 8 M urea, this value was taken to represent a viscosity increase of 100% over 3.8, the viscosity of native enzyme in 0.1 M KCl. The per cent viscosity increase for each irradiated sample was then calculated as

$$\text{per cent viscosity increase} = \frac{[\eta]_{\text{irrad}} - 3.8}{9.4 - 3.8} \times 100$$

and was plotted counter to the other data presented in Figure 6. It is evident that there exists a striking parallelism between the change in viscosity and the decrease in the number of hydrogens involved in strong intramolecular bonds. The data obtained in hydrogen interchange studies were analyzed in the manner applied by Englander (1963) to the data for native RNAase A.

Thus, one may assign all the theoretically exchangeable hydrogens in RNAase A (245 at pH 5) to four distinct classes. Class 1 contains side-chain hydrogens (126 at pH 5) which exchange instantaneously, and class 2 probably represents unhindered amide hydrogens which exchange with a half-time of minutes at 4°. Class 3, with a half-time of hours, presumably represents amide hydrogens in α helix, and class 4 hydrogens, which are very slow exchanging, are probably side-chain hydrogens "buried" in hydrophobic regions of the molecule. It is also possible to estimate the size of each class, as well as the kinetic constants for those hydrogens which exchange at a measurable rate (Englander, 1963). Table IV shows the results obtained for irradiated RNAase A samples, as well as for native RNAase and oxidized RNAase A. For native RNAase A, the half-time for the hydrogens in classes 2–4 and the number of hydrogens in each class compare favorably with values in the literature (Englander, 1963; Schildkraut and Scheraga, 1960). In the irradiated RNAase A samples, the half-time for the hydrogens in classes 3 and 4 are similar to those of native enzyme, although fewer hydrogens were found in each class. However, the half-time for those hydrogens assignable to class 2 was only 4.5–5 min for the irradiated samples, as compared to 7.5 min for native enzyme. The half-time found for oxidized RNAase A

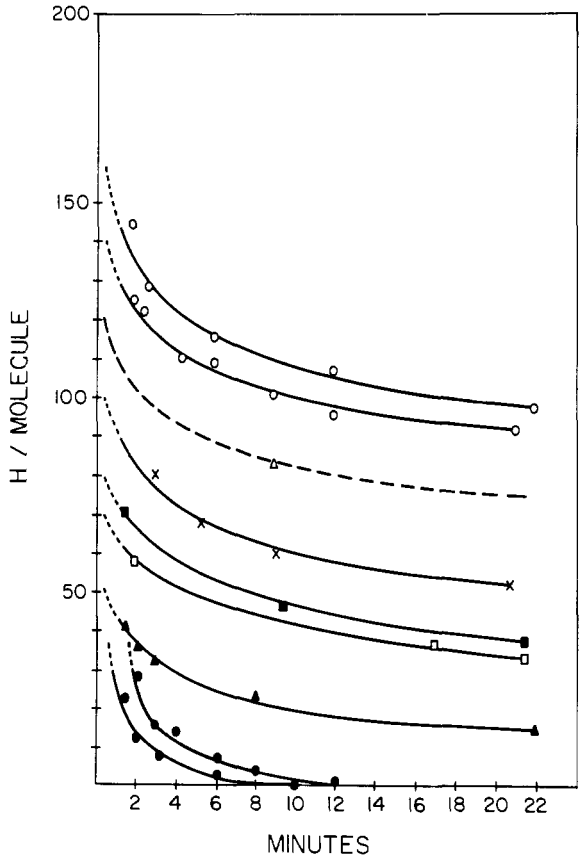

FIGURE 4: Tritium exchange-out curves for native, irradiated, and oxidized RNAase A *vs.* water at 4°, pH 5.0, 0.1 M NaCl. native RNAase A, O—O; irradiated RNAase A: 63% residual activity, Δ—Δ; 47% residual activity, ×—×; 28% residual activity, ■—■; 24% residual activity, □—□; 8–10% residual activity, ▲—▲; oxidized RNAase A, ●—●.

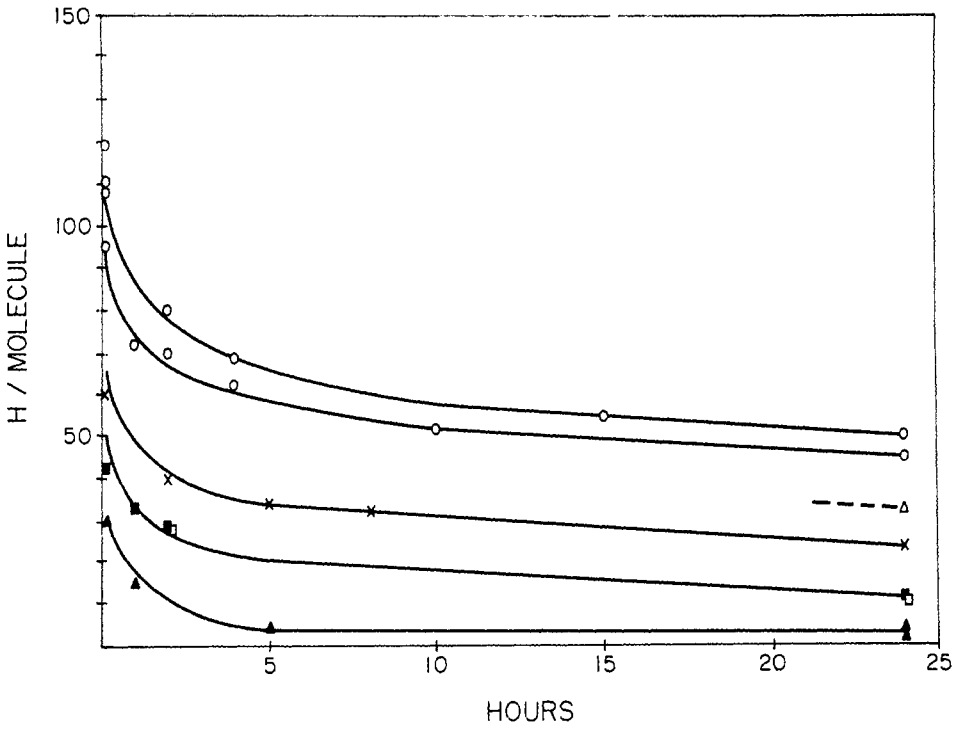

FIGURE 5: Tritium exchange-out curves for native and irradiated RNAase A *vs.* water at 4°, pH 5.0, 0.1 M NaCl; native RNAase A, O—O; irradiated RNAase A: 63% residual activity, Δ—Δ; 47% residual activity, ×—×; 28% residual activity, ■—■; 24% residual activity, □—□; 8–10% residual activity, ▲—▲.

was very fast, being only 1–2 min[4]; this half-time is close to that observed for poly-L-glutamic acid in its random chain conformation (Ikegami *et al.*, 1965), and is characteristic of amide hydrogens in a random-coil polypeptide chain.

It can be seen (Table IV) that irradiation disrupts the tertiary hydrogen bonding (class 4) and the helical content (class 3) of the molecule, and there is consequently a decrease in the number of hydrogens found in these classes. There is also a decrease, instead of an increase, in the apparent number of unhindered amide (class 2) hydrogens. This anomaly can only be explained on the basis that there are some amide hydrogens with kinetics so rapid as to preclude accurate measurement. In fact, the results strongly indicated the existence of a type of proton with exchange-out kinetics similar to the 1–2-min half-time observed for oxidized RNAase A, and which, of necessity, had to be relegated to class 1. For example, a theoretical analysis of the results for the 8% active sample (Table IV) would show the presence in class 1 of about 72 intrinsically fast-exchanging amide hydrogens. The 34 hydrogens found in class 2 are peptide hydrogens exchanging with measurable half-times of 4–5 min. The existence of different kinetic types of amide hydrogens probably reflects the restrictive influence of some residual ordered structure in certain portions of the irradiated molecule.

Discussion

The present findings support previous tentative conclusions that the aqueous irradiation effect on RNAase A involves extensive disorientation of the native configuration of this molecule. The magnitude of these physical changes is such as to suggest that the inactivation of the enzyme may be largely related to the observed loss of secondary and tertiary structure. Thus, it has been shown that the rate of loss of enzymic activity is almost the same as the rate of loss of structural integrity (Figure 6).

On the other hand, there is also some covalent damage to the molecule, of an order of magnitude less than the rate of loss of enzymic activity, but which may nevertheless be extremely important. For example, previous studies showed that 85% inactivated enzyme lacked about 25% of the SS bridges and, on the average, 25% of the methionine, tyrosine, histidine, and phenylalanine content; 10% of the lysine content was destroyed (Slobodian *et al.*, 1965b). It is possible that some of these chemical changes may be critical, either because they occur in the active site of the enzyme, or because they occur in regions which determine the three-dimensional conformation of the molecule. In

[4] This half-time is much shorter than the value of 4.7 min reported by Englander (1963). However, there is ample evidence in the literature of variations between preparations of oxidized RNAase A (Hvidt, 1955; Stracher, 1960).

TABLE IV: Size and Rate Constants of the Kinetic Classes of Hydrogens in Native, Irradiated, and Oxidized RNAase A Samples.

Sample	Class 1 Size (H/molecule) "Instantaneous"	Class 2 Size[a] (H/molecule)	Class 2 $t_{0.5}$[b] (min)	Class 3 Size[a] (H/molecule)	Class 3 $t_{0.5}$[c] (min)	Class 4 Size (H/molecule)	Class 4 $t_{0.5}$
Native RNAase A (lot 6081)	107	63	7.5	30	180	45	Slow
Native RNAase A (lot 6094)	97	62	7.2	35	180	50	Slow
Irradiated RNAase A							
47% active	144	52	5	26	180	23	Slow
28% active	170	45	5	18	180	12	Slow
8% active	198	34	4–5	10	180	3	Slow
Oxidized RNAase A (lot 75B-8550)	135	110	1.5–2	..		..	
Oxidized RNAase A (lot 85B-8830)	135	110	1–1.5	..		..	

[a] ±8%. [b] ±0.5 min. [c] ±15 min.

fact, even in dry irradiation of RNAase, where physical changes predominate, and destruction of amino acids is negligible (Hayden and Friedberg, 1964; Friedberg and Hayden, 1965), the irreversibility of the irradiation effect (*i.e.*, the unfolding) may be related to the simultaneous production of minute, but critical chemical lesions. Thus, Haskill and Hunt (1965) have recently shown the presence of "sublethal" lesions which become manifest only after the irradiated enzyme is put through a reduction–reoxidation cycle; these lesions prevent proper refolding so that the reoxidized sample does not return to its initial enzymic activity.

It must, however, be borne in mind that in the aqueous irradiation of RNAase A where free radical mechanisms predominate and where covalent damage is more evident, some lesions may be occurring in superfluous regions of the molecule and may be irrelevant to loss of enzymic activity. Evidence for this view has been obtained from studies of irradiated (Haskill and Hunt, 1965; Slobodian *et al.*, 1965b) or hydroxyl radical treated RNAase A (Slobodian *et al.*, 1962), in which it was observed that there were altered molecules which still possessed enzymic activity. Therefore, although it is evident that both physical and covalent changes are associated with loss of enzymic activity, it is not possible, at the present time, to evaluate the relative significance of these molecular alterations.

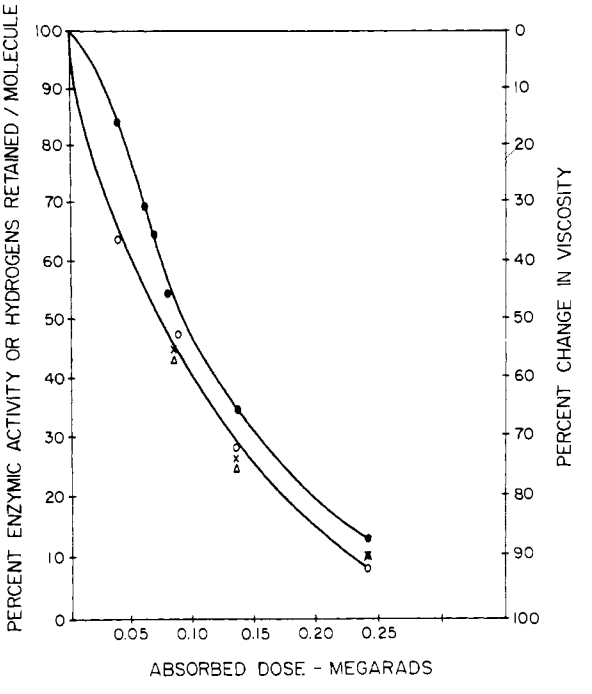

FIGURE 6: Correlation of loss of enzymic activity with loss of slow-exchanging hydrogens and change in viscosity in irradiated RNAase A; per cent residual enzymic activity, O—O. slow-exchanging, protein-bound hydrogens remaining: after 2 hr, ×—×; after 24 hr, Δ—Δ; change in intrinsic viscosity, ●—●. Note that the change in viscosity (right ordinate) is plotted in reverse.

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