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Studies on Ribonuclease Conformation and Racemization Using Tritium-Hydrogen Exchange and Optical Rotatory Dispersion

S. J. LEACH AND JULIE HILL

From the Division of Protein Chemistry, C.S.I.R.O., Parkville N2 (Melbourne), Victoria, Australia

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Procedures are outlined for following the kinetics of tritium-hydrogen exchange using liquid scintillation counting. The method is applied to a study of ribonuclease exchanging with tritiated water at 25° in the *p*H range 2.5 to 12. The number of slowly exchanging hydrogen atoms decreases from 100 at *p*H 2.5 to zero at *p*H 12. Similarly, the number of hydrogen atoms which exchange only on heating also decreases from 40 or 50 to zero over the same *p*H range. These results suggest extra hydrogen-bonding interactions at low *p*H and this is indicated also by measurements of optical rotatory dispersion. The presence of a large number of "hidden" hydrogen atoms is confirmed using pure ribonuclease-A. The lower numbers reported, using some other methods based on deuterium-hydrogen exchange, are attributed to differences in experimental procedures and/or the interpretation of the data they yield. Tritium-hydrogen exchange has been used to estimate the extent of racemization in alkaline solution. From measurements at *p*H 12, an estimated 28–35 of the residues in the ribonuclease molecule are racemized after 72 hours at 25°. Extensive racemization is indicated also by measurements of specific rotation on the denatured protein, though these are complicated by disulphide bond fission.

Of the hydrogen atoms in the protein molecule, only those attached at N,- or O,- sites normally exchange with the hydrogen atoms of the solvent medium. These exchangeable hydrogen atoms can be further subdivided experimentally into those which exchange at a rate which is too rapid to measure with existing techniques, i.e., within 1–3 minutes, and those which exchange at a measurable rate, i.e., within a few hours. Some proteins contain in addition a relatively small number of hydrogen atoms which will exchange only at elevated temperatures or under other denaturing conditions. The manner in which these rates and extents of hydrogen exchange have been measured and interpreted in terms of protein conformation have recently been reviewed (e.g., Leach, 1959; Scheraga, 1961).

In most cases, hydrogen exchange has been measured by substituting deuterated water for the aqueous medium and estimating the uptake or release of deuterium by the protein, using either the density-gradient technique of Hvidt *et al.* (1954), infrared spectroscopy (Lenormant and Blout, 1953; Haggis, 1957), or nuclear magnetic resonance spectroscopy (Wishnia and Saunders, 1962). Recently a method has been described for following the progress of hydrogen exchange using tracer amounts of tritium in water (Springell and Leach, 1962; Leach and Springell, 1962). The latter paper discusses some advantages of the tritium-hydrogen tracer technique over the deuterium-hydrogen methods.

The present paper describes the application of liquid scintillation counting to the study of tritium-hydrogen exchange and outlines some improvements in procedure. The rates and extents of exchange between ribonuclease and tritiated water have been measured in the *p*H range 2.4–12, and the number of hydrogens which fail to exchange below the transition temperature has been estimated at each *p*H value. The results provide evidence for conformational changes at both low and high *p*H and this interpretation is borne out by optical rotatory dispersion measurements. In addition, tritium exchange has now been applied to measuring the extent of racemization of ribonuclease in alkaline solution and the results compared with optical rotation measurements. The optical rotation of a native protein contains contributions not only from asymmetric carbon atoms but also from folded portions of polypeptide chain such as the α-helix. In order to evaluate changes in rotation in terms of optical inversions at the asymmetric centers, it was therefore necessary to convert the protein to a standard, fully dena-

tured state before measuring the optical rotation. In such a state, the rotation should be dependent only on the amino acid composition.

Measurements of optical rotation and hydrogen exchange in alkaline solution may be complicated by the occurrence of "side" reactions such as peptide bond hydrolysis and disulfide fission. Estimates of the extent of such reactions under the experimental conditions have therefore been made.

Materials and Methods

Ribonuclease.—Two samples of recrystallized bovine pancreatic ribonuclease were used. Armour (Lot EB2451) was used for most of the exchange experiments. This sample and also Sigma (Lot R100B-69, activity 49U) were used to prepare chromatographically pure ribonuclease-A by the procedure of Hirs *et al.* (1953). After isolation from the IRC-50 column, the pure fraction was dialysed against KCl (1 M) to displace phosphate, then against water for 2 days, and was finally freeze-dried.

Purities were calculated from Kjeldahl-N values, corrected for ammonium-N, assuming a value of 17.5% for pure ribonuclease. On a dry, ash-free basis, this provided a purity of 97% for the preparation of ribonuclease-A and the lower values quoted elsewhere (Leach and Springell, 1962) for the commercial samples.

Ammonium-N was determined by steam-distillation for 3 minutes in the presence of 20% sodium borate solution. This value was used to correct the Kjeldahl-N when estimating ribonuclease and also to correct the number of hydrogen atoms found per molecule for the presence of ammonium-H.

To provide an estimate of peptide bond hydrolysis occurring in alkaline solution it was required to measure the increase in amino-N. Amino-N was determined by reacting an aliquot of ribonuclease containing 0.2–0.8 μmole of amino-N with ninhydrin in citrate buffer (Moore and Stein, 1948) in the presence of *i*-propanol (2.5%) and pyridine (2.5%). After heating at 100° for 45 minutes and diluting 6-fold with ethanol (50%), the optical density at 570 mμ was measured and compared with a leucine calibration curve. Native ribonuclease gave 8.0 leucine equivalents per mole compared with the value of 7.7 found by Slobodian *et al.* (1962).

Thiol and disulphide estimations on ribonuclease were carried out by amperometric titration with methylmercuric iodide at the dropping mercury electrode as described by Leach (1960).

Optical densities of ribonuclease solutions were determined in 1-cm cells by running absorption spectra from 360 to 240 mμ using a Beckman DK-2 ratio-recording spectrophotometer. The optical densities at 275 mμ were roughly corrected for scattering by linear extrapolation of the curves between 360 and 315 mμ down to 275 mμ. Even with solutions filtered through a sintered glass disk (Jena G4) the corrections amounted to 5–10%. The concentrations calculated from optical densities corrected in this way showed excellent agreement with concentrations derived from Kjeldahl-N estimations on the same solutions. The validity of the scattering corrections was indicated also by the fact that when concentrations arrived at in this manner were used to calculate the total number of exchangeable hydrogen atoms, the latter values agreed with those calculated from the amino acid analysis of the protein (see Results). The consistently large magnitude of such scattering corrections emphasizes the importance of including this step and is probably due to the presence of molecular aggregates produced during evaporation or lyophilization and heating (see, e.g., Holcombe and van Holde, 1962).

Tritiated Water.—Tritiated water of approximate specific activity 5 c/ml, from the Radiochemical Centre, Amersham, England, was diluted to 1 mc/ml with deionized water and used as a stock solution. It was unnecessary to determine the specific activity of this solution, which was diluted approximately 2-fold on commencing an exchange experiment.

Radioactive Assay.—In adapting the procedure of Butler (1961) to the assay of tritium in proteins it was found that very small amounts of tetraethylammonium hydroxide could be used to solubilize proteins and amino acids in the scintillation mixture. This allows a higher counting efficiency than the large quantities of Hyamine hydroxide normally used (Steinberg *et al.*, 1958). The tetraethylammonium hydroxide was readily made by passing a solution of tetraethylammonium chloride (British Drug House, analytical reagent grade, 15 g in 150 ml water) through an anion-exchange column in the OH^- form (Dowex 2-X8, 200–400 mesh). The eluate was concentrated *in vacuo* to *ca.* 0.8 M and stored in a polythene bottle.

The scintillation mixture was made up by dissolving PPO(2,5-diphenyloxazole, 4 g), POPOP (*p-bis*-2-(5-phenyloxazolyl)benzene, 0.05 g) and naphthalene (120 g) in *p*-dioxane (1000 ml), all of scintillation grade. The scintillation mixture (15 ml) was added to water (2 ml) containing tritium (50–200 mμc) and ribonuclease (1–6 mg) in a 20-ml glass counting vial. Tetraethylammonium hydroxide solution (*ca.* 0.8 M, 25 μl) was then added, and after thorough mixing the vials were stored at −2° overnight in the dark. For counting, a Packard Tri-Carb liquid scintillation spectrometer (Series 314EX) was used, with samples, photomultiplier tubes, and preamplifiers at −2°. The counting efficiency for a calibrated tritiated water standard was 13%. The ratio of counts in the two counting channels was checked in all tritium assays to ensure that the degree of quenching was unchanged. The quantities of ribonuclease and tetraethylammonium hydroxide normally used were found to have no significant effect either on the count-rate or the degree of quenching, and in all cases the count-rate was linear with respect to the amount of tritium in the 0–10 μc experimental range.

Exchange Experiments.—To avoid complications due to specific ion effects on rates (Gally and Edelman, 1962), and to corrections for exchangeable hydrogen atoms in buffer salts, no buffers were used in rate measurements. In a typical experiment, a ribonuclease solution (72 mg in 4 ml water) was titrated with HCl (1 M, 50 μl) to *p*H 2.5, filtered under pressure through a G5 sinter and maintained at 25°. To commence the exchange, stock tritiated water (*ca.* 1 mc/ml, 4 ml) at 25° was added, the flask was closed with a spring-loaded stopper, and the contents was maintained at 25°. At timed intervals, samples (about 0.5 ml) were withdrawn, frozen, and freeze-dried in batches of six for 1 hour followed by heating for 3 hours at 40°. The glassware and vacuum system were those described in detail by Leach and Springell (1962), who also demonstrated that the stated drying conditions were adequate for removing excess tritiated water. After drying, the contents of each flask was immediately dissolved in water (about 4 ml), glacial acetic acid was added (1 M, 25 μl), and the solution was filtered through a G4 sinter. Optical densities were then measured as already described. It should be emphasized that up to this stage there was no need for precise weighings, pipettings, and transfers, or even the avoidance of losses during freeze-drying, since the required assays

of both protein concentration and radioactivity were conducted on the final solution. In early experiments, aliquots of these solutions were taken for Kjeldahl-N estimations, so that the optical density measurements could be used to calculate $E^{1\%}_{1\,cm}$ based upon a value of 17.5% N for pure ribonuclease.

A second exact aliquot (2 ml) was taken for tritium assay as described already and the count was compared with that of the reaction mixture. The latter was measured by diluting a portion of the reaction mixture 8000 times and assaying an exact aliquot (2 ml) for tritium. These counting standards were prepared from the reaction mixture three times during the course of each experiment, viz., after 2 hours, 24 hours, and after a further 24 hours at 60°. Each standard was used to evaluate the samples exchanging up to that time, so that any evaporation losses were compensated for. Normally, all three standards gave the same count. The number of hydrogen atoms exchanging at any time is given by:

$$\frac{2.22 \times 10^5}{D \cdot M} \frac{(S_t - B)}{(S_0 - B)}$$

where S_t is the count (cpm) for the sample (2 ml) taken at time t, S_0 is the count (cpm) for the reaction mixture (2 ml) after dilution D times, B is the count (cpm) for a blank vial containing all ingredients except tritium, and M is the amount of ribonuclease (μmoles in 2 ml) estimated from the optical density at 275 mμ assuming $E^{1\%}_{1\,cm}$ = 6.9. The numerical coefficient represents the number of μg-atoms of hydrogen in 2 ml water.

Exchange Caused by Racemization.—At *p*H 12, in addition to measuring the total amounts of tritium incorporated, samples exchanged for 24 hours or longer were tested for irreversible exchange at other than N,- and O,- sites by back exchanging with unlabeled water and measuring residual radioactivity. The tritiated and dried ribonuclease samples were neutralized with acetic acid (1 M, 5 μl, giving *p*H 4), heated for 24 hours with water (3 ml) at 60°, and freeze-dried. This procedure was repeated twice and the residue taken up in water for tritium assay in the usual way. The effectiveness of this procedure for removing tritium at N,- and O,- sites was checked on samples tritiated under nonracemizing conditions, i.e., at neutral and weakly acid *p*H values.

Optical Rotatory Dispersion.—Solutions of ribonuclease (*ca.* 0.5%) were titrated to various *p*H values and filtered when necessary through a G5 sinter, and optical rotations were measured in a 10-cm tube using a Stanley photoelectric polarimeter in conjunction with a Carl Leiss quartz prism monochromator and a mercury arc light source. Rotations measured at 365, 405, 436, 486, 546, and 578 mμ were plotted in the form suggested by Moffitt and Yang (1956) to evaluate the Moffitt parameter b_0, using a value of 212 mμ for λ_0, and concentrations calculated by optical density measurements on diluted and acidified aliquots. Values of $-b_0$ quoted in Table III are the mean of several measurements made on separate solutions. When urea (8 M) was included in the solution, values of $[\alpha]_D$ and b_0 were corrected for the change in refractive index (Foss and Schellman, 1959). Values of $-[\alpha]_D$ were estimated by extrapolating the specific rotations measured at the six wavelengths to 587 mμ.

Results

Ribonuclease Analysis.—Literature values for $E^{1\%}_{1\,cm}$ for ribonuclease vary from 6.9 to 8.3. For the commercial preparation used here, the value after applying scattering corrections and correcting also for the purity determined by Kjeldahl-N was 7.9. However, values calculated from corrected optical densities and Kjeldahl-N values on twelve solutions obtained from exchange experiments gave 6.9 ± 0.1, and this value was subsequently used in calculating the number of micromoles of ribonuclease present in each aliquot and hence the number of hydrogen atoms exchanged (H_e). The reason for the discrepancy is not clear but suggests the possibility that the vacuum drying procedure removes an ultraviolet-absorbing, volatile impurity which has a low nitrogen content. By using the $E^{1\%}_{1\,cm}$ value found on the exchanged and dried samples, the values obtained for H_e agreed with those calculated theoretically for each *p*H value (Table I, columns 4 and 5).

Table I

Number of H Atoms Exchanged per Molecule of Ribonuclease as a Function of *p*H, Time, and Temperature

	H Atoms Exchanged[a] (H_e)				
*p*H	1–3 Min at 25° (t_0)	22 Hr at 25° (t_1)	22 Hr at 60° (t_2)	Total H Atoms (calc) H_{theor}	H_e ($t_2 - t_1$)
2.4	153	220	259	256	39
3.9	162	212	261	255	49
6.0	197	237	260	250	23
9.1	210	236	243	237	7
12	213	220	[b]	222	2[c]

[a] Means of two or three experiments, corrected for NH_4^+-H at *p*H 2.4–6.0, but not at *p*H 9–12 where NH_3 would be removed during the freeze-drying procedure. [b] Precipitation occurred. [c] $H_{theor} - H_{t_1}$.

Chromatography of Sigma ribonuclease on an IRC50 column produced the pattern shown in Figure 1. Chromatograms of Armour ribonuclease were similar but with slightly more of the minor components. These preparations appear to be somewhat more homogeneous with respect to protein components than earlier commercial preparations (Hirs *et al.*, 1953; Rupley and Scheraga, 1962), but there are substantial amounts of a rapidly moving component with an absorption maximum at 257 mμ at *p*H 6.5. The identity of this component, which was recovered and found to be nondialyzable, is unknown. It does not give an orcinol color test for ribose, nor a diphenylamine color for DNA, but it contains 6–7% phosphorus. It is insoluble in diethyl ether and ether-alcohol mixtures

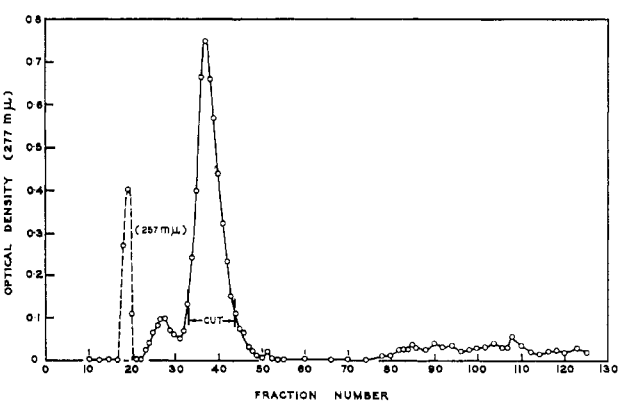

Fig. 1.—Chromatography of ribonuclease (Sigma, Batch R100, B-69, activity 49 U) on IRC 50 resin column at *p*H 6.5. The ordinate represents the optical densities of the effluent fractions measured at 257 mμ (broken line) or at 277 mμ (full line).

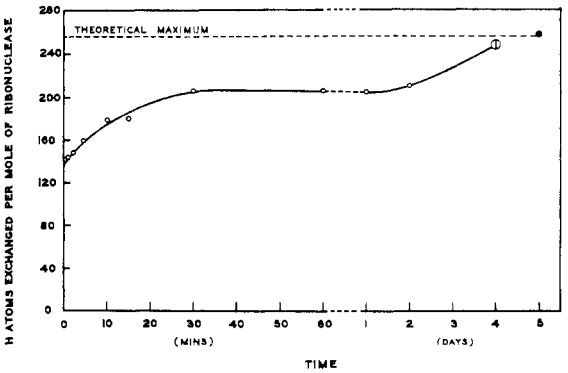

FIG. 2.—Rate of tritium-hydrogen exchange in ribonuclease at *p*H 2.4 (unbuffered) and 25°. The solid point represents the final value after heating the solution at 60° for 24 hours.

and there were no amino acids in HCl hydrolysates of the substance. To isolate chromatographically homogeneous ribonuclease-A, cuts were taken from several chromatographic runs at the points indicated in Figure 1.

Rates of Tritium-Hydrogen Exchange.—It was found that duplicate determinations of hydrogen exchange were usually within ±2 hydrogen atoms per mole and the scatter of experimental points during a kinetic run was small. Reproducibility of points between kinetic runs at similar *p*H was less satisfactory, being about ±5 hydrogen atoms per mole. This may be due to the fact that the solutions were unbuffered. The rate of exchange of ribonuclease with tritiated water at *p*H 2.4 and 25° is shown in Figure 2. Similar sets of data were collected for other *p*H values up to 12 at 25°. As observed elsewhere, there was a very rapid exchange, amounting to at least half the exchangeable hydrogen atoms, before the first measurements could be made at 1–3 minutes. This was followed by a less rapid exchange to a value which remained fairly constant from 1–24 hours. Except for the extremes of *p*H (2.4 and 12) it was then necessary to heat the solution to exchange the full number of hydrogen atoms expected from the amino acid analysis and from the titration curve of ribonuclease. These calculated values are shown in column 5 of Table I. The remaining columns show the number of hydrogen atoms exchanged after 1–3 minutes and 24 hours, respectively, at 25°, and after further heating for 24 hours at 60°. These three columns supply the data which are normally used for evaluating conformational changes from hydrogen exchange experiments. The proportion of hydrogen atoms which does not exchange within the first few minutes at 25° decreases from 40% at *p*H 2.4 to almost zero at *p*H 12.

In addition there were a number of hydrogen atoms which failed to exchange with tritium even after 24 hours at 25° and this number decreased from 40 to 50 in acid solution to zero at *p*H 12. In view of the heterogeneity of the ribonuclease used in all previous investigations it seemed important to establish the number of such "hidden" hydrogen atoms for a sample of chromatographically purified ribonuclease-A. For this purpose, exchange was carried out for 24 hours at two *p*H values and two temperatures using such a preparation. At *p*H 2.4 the numbers of hydrogen atoms exchanging at 25° and 60° were 225 and 267, respectively. At *p*H 6.15 the corresponding numbers were 199 and 246. Although these values do not agree well with those of Table I for the commercial sample, they confirm the presence of a comparatively large number

TABLE II

TRITIUM EXCHANGE OF RIBONUCLEASE AT *p*H 12 AND 25°

Time (days)	Total H Atoms Exchanged (H_e)[a]	H_e-222	H Atoms Trapped[b]
1	221	0	—
2	231	9	—
3	—	—	30
6	246	24	—
7	241	19	—
14	248	26	28
17	—	—	35

[a] No correction for ammonium-N is necessary at this *p*H. [b] Means of three experiments in which samples were back-exchanged with water at *p*H 4. After tritiation at *p*H 2.4, this procedure "trapped" fewer than three H atoms.

of unreactive hydrogen atoms, viz., 42 at *p*H 2.4 and 47 at *p*H 6.15. The fact that the purified sample had fewer unreactive hydrogen atoms at *p*H 6.15 than did the commercial sample suggests that either the stability of the unreactive regions increases on purification of the protein or, alternatively, that impurities are removed which have different exchange characteristics from native ribonuclease.

Although the number of hydrogen atoms exchanging at *p*H 2.4 increased only very slowly between 1 hour and 48 hours, prolonged storage beyond this time resulted in a somewhat more rapid exchange, so that after 4 days exchange was almost complete even at 25° (Fig. 2).

Tritium Exchange and Racemization.—The number of hydrogen atoms exchanging after 24 hours at *p*H 12 and 25° coincided with the total number of exchangeable hydrogen atoms calculated for this *p*H. However Table II shows that exchange continued slowly for several days beyond this point until a maximum of 246–248 hydrogen atoms had exchanged. This is 24–26 hydrogen atoms more than the theoretical maximum. When samples of such tritiated ribonuclease were acidified and back-exchanged with unlabeled water in order to remove tritium on N,- and O,- sites, there were still 28–35 tritium atoms left behind per mole of ribonuclease. The number of such irreversibly bound tritium atoms could be reduced slightly by omitting to acidify before back-exchanging with water. The back-exchange procedure was checked for efficiency of tritium removal by applying it to ribonuclease which had been fully N,O- tritiated for 7 days at *p*H 2.5. Of 253 tritium atoms incorporated, <3 tritium atoms failed to exchange back.

The extra tritium atoms incorporated on prolonged exchange at *p*H 12 were not associated with peptide bond hydrolysis, since the increase in the number of NH_2-end groups per mole was only 0.5 after 55 hours and 0.8 after 7 days at *p*H 12. In any case, new —NH_2 or —COOH sites would not account for *irreversible* binding of tritium. Nor is this extra uptake due to "hot-atom" labeling since (i) the latter would be expected to occur equally well at lower *p*H values and (ii) the values found are independent of the specific activity of the tritiated water over a 100-fold range of tritium concentration.

Optical Rotatory Dispersion.—Values of $-b_0$ and $-[\alpha]_D$ measured at various *p*H values are shown in Table III. As already shown (Imahori *et al.*, 1957; Weber and Tanford, 1959), the $-[\alpha]_D$ of *ca.* 75° and the $-b_0$ of *ca.* 90° suggest a low content of α-helix (*ca.* 14%) in aqueous solutions of ribonuclease. However there is evidence for an increase in the amounts

TABLE III
OPTICAL ROTATORY DISPERSION OF RIBONUCLEASE AS A FUNCTION OF pH[a]

pH	$-[\alpha]_D^0$	$-b_0^0$
1.6	78	95
1.8	—	84
2.5	76	125
3.9	74	96
6	80	90
6.9 (phosphate)	79	90
6.9 (phosphate, 8 M urea)	90	60
12.1 (8 M urea)	100	0
12.1 (zero time)	81	60
12.1 (1 day)	79	41
12.1 (2 days)	77	42
12.1 (7 days)	61	0
12.1 (20 days)	59	0

[a] These are mean values for two or three determinations at 20°.

of helical folding when the solution is titrated to pH 2.5, since the $-b_0$ value increases to *ca.* 125°. At lower pH values the b_0 falls again.

In order to obtain evidence of racemization at pH 12 it was intended to measure $-[\alpha]_D$ under denaturing conditions where there was no longer a contribution to the specific rotation from helical segments. Table III shows that at pH 6.9 the addition of 8 M urea was insufficient to destroy all folding, i.e., although $-[\alpha]_D$ increased to 90°, $-b_0$ was still 60°. The measurements at pH 6.9 were made in the presence of phosphate buffer which is known to stabilize ribonuclease against denaturation (Sela *et al.*, 1957). However, measurements in 8 M urea in the absence of any buffer ions gave similar high values for b_0. Similar values of b_0 were obtained even at pH 12.3 in the absence of urea. This bears out previous observations on the stability of ribonuclease to denaturative conditions (Brown *et al.*, 1959), and may explain why ribonuclease is still enzymically active even in 8 M urea (Anfinsen *et al.*, 1955; Anfinsen, 1956). In order to destroy the helix rapidly it was necessary to use 8 M urea at pH 12.1, when $-[\alpha]_D$ increased to 100° and b_0 fell to 0°.

We may expect that on prolonged exposure to alkaline conditions there will be several opposing influences on $-[\alpha]_D$, viz., a slow alkaline denaturation giving an increase, and a slow racemization or disulfide bond fission, both leading to a decrease. The observed values of $-[\alpha]_D$ after various periods at pH 12 (Table III) show a substantial decrease even though b_0 measurements indicate progressive denaturation. This indicates either extensive racemization or disulfide bond fission under the same conditions, viz., pH 12, 25°, as those under which there was tritium uptake at C-sites. After 7 days, when $-b_0$ had fallen to zero and there was no further α-helical contribution to $-[\alpha]_D$, the value of $-[\alpha]_D$ had fallen to 61°. This is to be compared with the value of 100° for the protein denatured with minimal racemization or disulfide bond fission. Disulfide estimations on the ribonuclease solution after 7 days showed that the disulfide content had fallen to only 30% of its initial value. The change in $-[\alpha]_D$ may therefore be attributed to disulfide fission as well as racemization.

DISCUSSION

The method outlined for following the progress of tritium-hydrogen exchange using scintillation counting is rapid and precise. It does not require the skill demanded by either the ionization-chamber procedure for tritium estimation (Leach and Springell, 1962) or the density-gradient method for deuterium estimation (Hvidt *et al.*, 1954). Furthermore, by estimating both radioactivity and protein concentration in each tritiated sample, the expected number of exchangeable hydrogen atoms is found in ribonuclease without recourse to the "normalizing" procedures which have formerly been necessary (Hvidt *et al.*, 1954; Ottesen and Stracher, 1960; Benson, 1959; Leach and Springell, 1962).

The new procedure has been used to confirm the observation (Leach and Springell, 1962) that the ribonuclease molecule contains 40 to 50 hydrogen atoms which fail to exchange below a critical temperature of about 45°, and this result has been checked using chromatographically purified ribonuclease-A. Previous estimates vary from zero (Hvidt, 1955) through 14 (Blout *et al.*, 1961), and 24 (Wishnia and Saunders, 1962) to 40 (Haggis, 1957), while Schildkraut and Scheraga (1960) quote a value of 20 but point out that this number varied with the crystalline modification used. Stracher (1960) found values of zero and 20 for two different samples of ribonuclease and stressed the effect of the previous history of the sample. Stracher's suggestion seems one likely source of the discrepancy between the various estimates. For example, a sample of ribonuclease most commonly used (Armour Lot 381-059) was manufactured in 1951 and might have aged in such a way as to alter its exchange properties. The methods of labeling could also affect the reactivity and accessibility of the replaceable hydrogen atoms. It may be significant that most of the low estimates of zero to 20 were obtained by the method of back-exchange, where the samples had been repeatedly heated, lyophilized, and dried at temperatures of 60°, i.e., above the transition temperature. This is known to affect subsequent exchange *rates* (Benson, 1959; Benson and Linderstrøm-Lang, 1959). The higher estimates of both Haggis (1957) and the present authors were obtained without such pretreatments, and it is reasonable to expect that the protein at the time of isotopic analysis was therefore in a state more closely resembling its native conformation. This difference in procedure may explain also the difference in the transition temperatures found between the back-exchange procedures (*ca.* 62°) and that found with the milder procedures of both Haggis (*ca.* 47°) and Leach and Springell (*ca.* 42°). It is possible that repeated drying at 60° will cause permanent changes in conformation affecting the more labile fraction of the unreactive hydrogen atoms. Any hydrogen atoms remaining unreactive after such treatment might be expected to have a higher transition temperature than those already exposed.

A direct comparison of the *rate* measurements made here with those of previous workers is not possible since pH values are not identical, and in addition there may also be specific buffer ion effects on such rates. However the general effect of pH on the rates agrees with the findings of Hvidt (1955) who observed that "qualitatively . . . exchange in the pH range from 3 to 8.3 is slower the lower the pH." This is in contrast to myoglobin, where exchange is slowest at the isoelectric pH of 7 and the protein appears to unfold on either side of this pH (Benson, 1959; Benson and Linderstrøm-Lang, 1959).

Scheraga (1957) and Hermans and Scheraga (1961) found evidence for conformational changes in ribonuclease at low pH from shifts in the ultraviolet absorption spectrum and from small increases in $-[\alpha]_{436}$. Such increases were not observed either in $-[\alpha]_{436}$ or in $-\alpha_D$ in the present work. Moreover, the conclusion

of Hermans and Scheraga (1961) that unfolding occurs at low *p*H is not supported by present measurements of b_0 and tritium-hydrogen exchange, both of which suggest that *increased* hydrogen bonding occurs.

Attempts have been made to evaluate the percentage of α-helical segments in proteins from the number of hydrogen atoms which fail to exchange "instantaneously" (Linderstrøm-Lang, 1958; Blout *et al.*, 1961). Using this approach, the large number of slowly exchanging hydrogen atoms in acid solution, viz., 100 at *p*H 2.4, might be explained by an increase in α-helical content from 20% at *p*H 6 to 80% at *p*H 2.4. Both estimates are considerably higher than those from optical rotation measurements (Table III). However, estimates of helical content derived from optical rotatory dispersion data are very sensitive to the values chosen for λ_0 and maximum b_0 (Urnes *et al.*, 1961) and assume that helix of only one type and screw sense is present, the remainder of the molecule being perfectly random and contributing nothing to the b_0 value. On the other hand, the number of "slow" hydrogen atoms derived from tritium-hydrogen or deuterium-hydrogen measurements may not be related in a simple manner to the amount of α-helix present. Where the α-helix is the only ordered structure present, this approach may be justifiable. However, there is an increasing body of evidence to suggest that other ordered but less well-defined structures are also present in proteins and these will also lead to slow exchange due either to hydrogen-bonding or merely steric inaccessibility. We cannot assume that all slowly-exchanging hydrogen atoms are located in —CONH— groups of the peptide backbone. Slow exchange may be expected for hydron-bonded side chains, particularly where these can be stabilized by proximate hydrophobic interactions.

Wishnia and Saunders (1962) have measured the exchange of deuterated water with ribonuclease using nuclear magnetic resonance spectroscopy (nmr). It may be assumed that in spite of overlap of DHO, NH, CH, and OH absorptions, the nmr data can be interpreted sufficiently quantitatively for the measurement of hydrogen exchange. For example, at *p*D 2.75 to 4.48, Wishnia and Saunders found that 60 to 70 hydrogen atoms exchanged during the course of an experiment at 25°, agreeing well with the results of Table I (67 at *p*H 2.4 and 50 at *p*H 3.9) where the interpretation of the results is much less complex. They also found an increase in rate of exchange with increasing *p*H and observed, as did the present authors, that the number of more "refractory" hydrogen atoms decreased to a value approaching zero at the isionic point (*p*H 9). However, in acid solutions Wishnia and Saunders found only 25 such refractory hydrogen atoms.[1] On the basis of exchange experiments with guanidinated ribonuclease, they made the interesting suggestion that while the 60 to 70 "slow" hydrogens are probably located in peptide groups, the additional 25 refractory atoms belong to the NH groups of the four guanidine residues rather than to peptide groups.

The idea that protein guanidinium groups fail to exchange with water even after 24 hours at 25° when all the peptide hydrogen exchange is ostensibly complete does not seem likely from experiments with simple model compounds. The interpretation is incompatible also with the results for β-lactoglobulin (Linderstrøm-Lang, 1955) and insulin (Linderstrøm-Lang, 1958), where the exchange results would suggest more and less nonexchangeable hydrogen atoms, respectively, then are expected from the arginine contents of these proteins. The numbers of unreactive hydrogen atoms in ribonuclease and myoglobin (Benson and Linderstrøm-Lang, 1959) do not vary with *p*H in the manner to be expected for the ionization of guanidinium residues.[2] The additional unreactive hydrogens observed by Wishnia and Saunders on guanidination of ribonuclease brought the total number to 54, a value close to that found by us, and might be explained by a subtle conformational change induced during the chemical treatment and resulting in a product with similar exchange properties to our sample.

Those hydrogen atoms which exchange only on heating are almost certainly located in α-helical segments or other well-ordered structures, stabilized either cooperatively by each other or by disulfide cross linkages. On the other hand, those hydrogen atoms which exchange at a measurable rate at 25° are most probably located in hydrogen-bonded side chains or in short helical segments, both being structures of very limited stability in aqueous solution unless stabilized by each other or by nearly hydrophobic interactions. The form of the temperature dependence of exchange and the effects of disulfide bond fission on the exchange kinetics of proteins are compatible with such an interpretation (Leach, *et al.*, in preparation).

The effect of increasing $[H^+]$ on the ribonuclease structure may be explained by increased side-chain interaction which leads to (i) the inaccessibility of larger domains and hence a decrease in the rate of tritium-hydrogen exchange, together with (ii) a minor increase in the size of the α-helical segments leading to a small increase in $-b_0$. Both effects might result from interactions between carboxyl groups which become protonated in mildly acid solution. These interactions may not withstand the electrostatic charge repulsions between chains in more strongly acid solutions. From the data of Figure 2 it appears that the extra molecular structures which occur in acid solution readily break down on standing. This weakness is compatible with the observed ease of thermal denaturation of ribonuclease in acid solution (Hermans and Scheraga, 1961).

This interpretation of the effect of *p*H on the rate of tritium-hydrogen exchange assumes that changes in rate due directly to changes in the extent of H^+ and OH^- catalysis *per se* do not appreciably affect the proportion of hydrogen atoms exchanging within the first 1–3 minutes. Such a possibility cannot be ignored. However, the observed *p*H dependence of the rate of exchange would then require that, in general, OH^- catalysis predominates over H^+ catalysis. While the existing data for myoglobin and bovine plasma albumin (Benson and Hallaway, 1962) do not support this contention, it is important to collect more data from proteins of different conformation and particularly from polyamino acids and simpler model compounds to clarify the mechanism of exchange. It also follows from what has been said that it is of limited value to quote the number of slowly exchanging hydrogen atoms for any protein, and then to calculate the degree of "folding" without reference to the *p*H at which the measurements were made and the effect of *p*H changes on the rate of exchange.

[1] It is noted however that Wishnia and Saunders (*ibid.*, p. 4238) did obtain a value of 41 for the number of hydrogen atoms remaining unexchanged after 24 hours at 25° if "only the aromatic protons" were deducted from the two overlapping nmr peaks, which represent absorption due to NH and aromatic protons, respectively. This seems a reasonable procedure and the result would then agree with that from tritium-hydrogen exchange.

[2] The 20 unreactive hydrogen atoms in myoglobin become reactive when the *p*H is raised from 7 to 8.8, a region in which the guanidinium cation remains fully protonated.

The 28–35 extra tritium atoms incorporated irreversibly on prolonged exchange at *p*H 12 are probably bound at carbon atoms, the most likely sites being the α-carbon atoms of the peptide backbone. Exchange at such sites would be facilitated at high *p*H values and could lead to racemization. Acidification after such exchange would "seal" the tritium atoms at their new sites and prevent their loss during back-exchange with water. The fact that the total number of these extra tritium atoms reaches a maximum after several days suggests that under the experimental conditions exchange and therefore racemization at such carbon-sites is limited to certain amino acid residues in the protein. The number of such tritium atoms means that these residues could amount to 28–35 of the 124 residues in the molecule. The residues most susceptible to alkaline racemization are considered to be serine, threonine, and cystine, which comprise 33 residues in the ribonuclease molecule.

If such measurements are to be used as a measure of racemization, the values obtained by back-exchange (e.g., Table II, column 4) are probably more reliable than the values from total exchange (column 2), which require subtraction of the theoretical total number of hydrogen atoms (here taken as 222), and this number is difficult to evaluate precisely in this *p*H range. The estimate of racemization from tritium-hydrogen exchange could be high since not every exchange event necessarily leads to an optical inversion. Nevertheless, the tritium-hydrogen exchange method gives a more reliable estimate of the number of residues racemized than do measurements of say $-[\alpha]_D$ on the denatured protein. While $-[\alpha]_D$ falls by 40–50%, this change cannot be used to evaluate the number of residues racemized since not all asymmetric centers contribute equally to the value of −100° to −120° in the denatured protein. In particular, the cystyl residues, which are particularly susceptible to racemization and disulfide bond fission in alkaline solution have an abnormally high optical rotation (see, e.g., Würz and Haurowitz, 1961). In view of the extensive disulfide bond fission (70%) observed under the experimental conditions, the fall in $-[\alpha]_D$ must be attributed largely to this factor and only in small part to the observed racemization.

The present measurements of tritium-hydrogen exchange and optical rotatory dispersion show that racemization and denaturation proceed at comparable rates at *p*H 12 in ribonuclease. One may speculate that irreversible alkaline denaturation could be due partly to the instability of —SS— linkages and partly to racemization at some of the α-carbon atoms which carry reactive side-chains. The role of racemization has not hitherto been considered in studies of the alkaline denaturation of ribonuclease (see, e.g., Brown *et al.*, 1959). Optical inversion will change the direction in which these side-chains point and might break some of the side-chain interactions which stabilized the protein in its unique native conformation.

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