from recent very high pressure mass spectrometry, that alkanes undergo many ion–molecule reactions[24]; this provides the necessary mechanism for immobilizing positive holes.

If the concentration dependence in Figs. 1–3 be taken as exponential, then the mole fraction X of solute required for an anion yield of half the limiting value leads to $0.69/PX$ for the average number of molecular encounters, where P is an efficiency factor. This number approximates $1500P^{-1}$ for these systems and indicates that the electron describes a fairly extended track. Since many solutes have efficiencies equal within a factor of two,[20] it is plausible that $P \sim 1$ for efficient solutes. Samuel and Magee[25] arrived at a number 10^3 using a model which is probably as applicable to these systems as to water. Both the preceding considerations and the fairly large quantum yield for bleaching solvated electrons are inconsistent with the assumption that electrons escape from the field of the cation. This is also indicated by the constancy of G(anion) for 0.01 M biphenyl in hydrocarbon at doses up to 6×10^{18} ev./l. The final concentration of holes was about 1.6×10^{-4} M. If electron capture cross sections by holes were 10 to 100 those for solute molecules, the ratio of hole to solute efficiencies for steady state kinetics would have been 0.16–1.6.

The facts reported here provide qualitative support for the view that ionic processes are important in the radiation chemistry of liquids.[9] Quantitatively, the G(anions) reported here rise much faster with concentration than do yields of chemical products in irradiated solutions at room temperature. We do find, however, that chemical yields from irradiated glassed solutions of ethyl iodide and phenyl acetyl chloride in methyl tetrahydrofurane, collected after thawing, increase about ten times more rapidly with solute concentration[26] than do the corresponding solutions in benzene.[9]

(24) S. Wexler, private communication.

(25) A. H. Samuel and J. L. Magee, *J. Chem. Phys.*, **21**, 1080 1953).

(26) J. R. Roberts, to be published.

[CONTRIBUTION NO. 1698 FROM THE DEPARTMENT OF BIOCHEMISTRY, DARTMOUTH MEDICAL SCHOOL, HANOVER, NEW HAMPSHIRE, AND DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

The Nature of the Slowly Exchanging Protons of Ribonuclease[1a]

BY ARNOLD WISHNIA AND MARTIN SAUNDERS

RECEIVED MAY 31, 1962

The nuclear magnetic resonance spectra of ribonuclease in D_2O solutions between pD 2.8 and 4.5 showed exchange of NH protons with time. 24 ± 5 protons exchanged much more slowly than the others. Guanidinated ribonuclease was shown to contain 54 ± 7 hydrogens exchanging at a comparable rate. When both proteins were heated to 72° exchange of all the NH protons was noted. It has been suggested that these very slowly exchanging protons are attached to the guanidinium groups of both proteins.

The rate of deuterium exchange between proteins or polypeptides and an aqueous environment has been the subject of many investigations since Linderstrøm–Lang proposed that those protons of the peptide backbone involved in the strongly hydrogen-bonded secondary structure might be distinguished from others by having a slower rate of exchange.[1b] Slow exchange has indeed been observed in many proteins.[2,3] Ribonuclease, typically, has been found to possess a distribution of NH and OH protons with widely differing exchange rates, both distributions and rates having a strong dependence on temperature, pH and other conditions.[3–7]

We were able to show[5] that the exchange of protons between ribonuclease and D_2O could be observed directly by nuclear magnetic resonance spectroscopy (n.m.r.), since the n.m.r. spectra of deuterons and protons are widely separated. The area of the ribonuclease absorption peaks in the region corresponding to aromatic and NH protons decreased with time, presumably because of the exchange of the NH protons with the solvent. The data were not precise enough to justify kinetic analysis, but it was clear that in solutions of pD between 2.5 and 5, the NH-aromatic peaks still contained about 25 more protons than could be attributed to the aromatic hydrogens alone, even after several days. In isoionic solutions the exchange had been complete, leaving a single low-field peak of the proper area for aromatic hydrogens.[8]

Using the Linderstrøm-Lang method, Linderstrøm-Lang, Schildkraut, and Scheraga[6] have observed that after 24 hours at pH 4.8 and 0, 38, or 60°, deuterated ribonuclease retained 49, 22, and 0 ($\pm$ 5) deuterons, respectively. More recently, Hermans and Scheraga[7] concluded that 20 refractory NH protons account for the residual absorp-

(1) (a) This work was supported in part by funds from National Science Foundation Grant C19973 and U. S. Public Health Service Grant RG8121. (b) A. Hvidt and K. Linderstrøm-Lang, *Biochim. Biophys. Acta*, **14**, 574 (1954). The precise suggestion had been foreshadowed by H. Lenormant and E. R. Blout, *Nature*, **172**, 770 (1953).

(2) Multiple references to the technique and results of determining exchange by the density-gradient method of analyzing the solvent for deuterium content are given in (a) K. Linderstrøm-Lang, in "Symposium on Protein Structure," ed. A. Neuberger, London, 1958, pp. 23–24. (b) H. A. Scheraga, in "Protein Structure and Function," Brookhaven Symposia in Biology, No. 13, 1960, pp. 71–88.

(3) References to infrared absorption studies are given in E. R. Blout, C. de Loze and A. Asadourian, *J. Am. Chem. Soc.*, **83**, 1895 (1961).

(4) A. Hvidt, *Biochim. Biophys. Acta.*, **18**, 307 (1955).

(5) M. Saunders and A. Wishnia, *Ann. N. Y. Acad. Sci.*, **70**, 870 (1958).

(6) C. L. Schildkraut and H. A. Scheraga, *J. Am. Chem. Soc.*, **82**, 58 (1960).

(7) J. Hermans, Jr., and H. A. Scheraga, *ibid.*, **82**, 5156 (1960).

(8) M. Saunders, A. Wishnia and J. G. Kirkwood, *ibid.*, **79**, 3289 (1957).

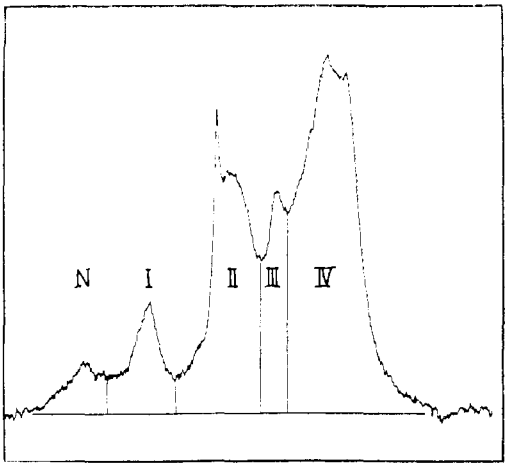

Fig. 1.—Nuclear magnetic resonance spectrum of ribonuclease. This sample still contains 90 protons in N + I.

tion at 1.5 μ (in the NH overtone region of the infrared) of ribonuclease exposed for 24 hours in D_2O at pD 4.8 and 38°. The results of studies[3] employing the decrease of absorption in the amide II region of the infrared relative to the amide I region appear similar (see below, however).

It has generally been concluded (with, perhaps, some reservations[2b,3]) that the most slowly exchanging protons correspond to the most strongly hydrogen-bonded peptide NH of the secondary structure. There is an alternative explanation which has not previously been considered, which, however, cannot be dismissed *a priori*. There are 20 protons on the guanidinium groups of the four arginine residues of ribonuclease. The observed n.m.r. chemical shifts of guanidinium protons[9] lie in the NH-aromatic region; the guanidinium group shows a strong absorption at 3200 and 3300 cm.$^{-1}$,[10] (the fundamental NH stretching frequency also observed in amides) and presumably in the overtone region as well. The possibility that guanidinium groups might exchange very slowly under the conditions of pH and concentration used in the studies of the globular proteins is suggested by the behavior of arginine[9,11] and guanidinium ions[11] in aqueous solutions and the known mechanisms of the relatively slow exchange of ammonium ions with water.[12]

Accordingly, we examined native ribonuclease and guanidinated ribonuclease (in which the amino groups have been converted to guanidinium groups by reaction with O-methyl isourea) under a variety of conditions.

Experimental

Materials.—Bovine pancreatic ribonuclease was Armour Lot 381-059, from several bottles. The 1958 sample of guanidinated ribonuclease was prepared according to Singer[13] and Klee and Richards,[14] with compromise concentrations. The sample used in 1961, the generous gift of Prof. F. M. Richards was the "95-guan-RNase" prepared and characterized by Klee and Richards,[14] containing 0.48 lysine residues per mole and having 33% of the specific activity of native ribonuclease.

Preparation of Samples.—The n.m.r. cells, designed for larger than customary volumes and for *in situ* lyophilization of samples, consisted of a 10 mm. o.d. tube sealed at one end (the cell proper) fused axially to a 25 ml. bulb having a short, ball-jointed neck opposite.

Stock solutions of RNase, after dialysis and deionization, were analyzed for protein concentration by dry weight (24 hours at 106°). These solutions contained 8–10% RNase. To establish the desired pH values, one aliquot was titrated with 0.592 *M* $HClO_4$, using a Beckman Model G pH meter. For each sample, 1.009 ml. of stock RNase and an appropriate volume of standard $HClO_4$ (constriction pipets[15]) were introduced into the bulb of an n.m.r. cell, mixed, shell-frozen, lyophilized, and sealed under vacuum. The initial pH of guanidinated samples was determined on the samples themselves, and their concentrations estimated by absorbance at 277 mu.[14]

In early experiments D_2O was sublimed into the cell. For most of the work reported here, 0.90 ml. D_2O was simply pipetted into the bulb, with zero time taken as the instant of contact. Dissolution by gentle rotation was rapid, usually not more than one or two minutes. For the kinetic runs, the cell was then immediately placed in the probe insert of the spectrometer, and spectra taken. The temperature in the probe insert was 26 ± 1°. The chief obstacle to precision during the first ten or twenty minutes is the drifting base line, probably arising from temperature equilibration, since a stable base line is obtained after longer periods. When not in the probe insert, the sample was thermostatted at 25.0°.

At the end of the experiment the apparent pH of each sample was determined on a Cambridge Research Model pH meter with a condenser-type glass electrode. The true pD^{25} was computed by adding 0.40 to these readings.[16]

Analysis of Nuclear Magnetic Resonance Spectra.—Spectra were obtained on a Varian 4300 B spectrometer at 40 Mc. as previously described[5] or at 60 Mc., with a phase-sensitive detector and a Varian recorder or a Moseley X–Y recorder.

The spectra were dissected by vertical cuts at suitable minima (Fig. 1). The area of each region was determined by weighing, or in some cases, by counting boxes. The distribution of CH protons among these regions was established using the spectra of sample which had been relyophilized and reexchanged with D_2O. The contribution of the residual HDO was substracted from peak II, assuming a Lorentzian shape for the water spectrum, or was eliminated by "saturating" the water line with sufficient RF power. Similar results were obtained in either case.

From the currently accepted amino acid analysis of RNase,[7] the empirical formula at the isoionic pH is $C_{575}H_{901}O_{193}N_{171}S_{12}$ (mol. wt. 13,683). There are 664 protons attached to carbon which are not exchangeable, and 237 attached to oxygen or nitrogen, which do exchange. The distribution of protons is given in Table I. Their chemical nature has been discussed.[5,8,9]

For most spectra the ratio of the area of the whole low-field region (peaks N + I, NH and aromatic protons) to that of the sum of peaks III and IV was measured. The number of NH and aromatic protons was computed from this ratio and the number of protons in peaks III and IV (429). Since in spectra of guanidinated ribonuclease peak III and the trough between peaks II and III are shifted from 3.90 to 3.60 and 3.38 to 3.23 p.p.m., respectively, the location of the cut is adjusted to maintain this number. This procedure obviates analyzing the complex RNase spectrum into Lorentian-shaped single lines. Use of the ratio of peak N to peak I is not satisfactory, both for technical reasons and because the spectral distribution of NH protons is too broad.

(9) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1958).

(10) E. M. Bradbury, W. C. Price and G. R. Wilkinson, *J. Mol. Biol.*, **4**, 39 (1962).

(11) M. Saunders and A. Wishnia, unpublished work.

(12) (a) T. M. Connor and A. Loewenstein, *J. Am. Chem. Soc.*, **83**, 560 (1961) give references to the publications intervening between their work and the first report. (b) E. Grunwald, A. Loewenstein and S. Meiboom, *J. Chem. Phys.*, **25**, 382 (1956).

(13) S. J. Singer, *Proc. Natl. Acad. Sci.* (U. S.), **41**, 1041 (1955).

(14) W. A. Klee and F. M. Richards, *J. Biol. Chem.*, **229**, 489 (1957).

(15) M. Levy, *Compt. rend. trav. lab. Carlsberg, ser. chim.*, **21**, 101 (1936).

(16) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960.)

(17) C. H. W. Hirs, S. Moore and H. W. Stein, *J. Biol. Chem.*, **235**, 633 (1960).

TABLE I

ANALYSIS OF N.M.R. SPECTRA

Sample	Peak	N	I	Solvent	II	III	IV
pD 3.6, 7 × 10^6 sec. reexchanged	Peak spacing, p.p.m. error, ±0.05 p.p.m.	−1.88	0	2.03	2.50	3.90	5.43
	Peak area, protons standard deviation	16 + 5	46 ± 3		190 ± 9		429 ± 7

TABLE II[a]

RESIDUAL PROTONS AT LOW FIELD

	Sample	N + I
1	RNAse, pD 4.8	
	20 hr. at 25°	74 ± 5
	18 hr. at 72°	50 ± 6[b]
2	95-guan-RNase, pD 4.8	
	20 hr. at 25°	104 ± 9
	18 hr. at 72°	51 ± 6[b]
3	Guanidinated RNase, pD 4.7	
	24 hr. at 26° (2.8.58)	106 ± 3

END POINTS OF DEUTERIUM EXCHANGE

	pD	Time, sec. × 10^{-6}	I	(S.D.)	N + I	(S.D.)	No. of spectra
1	4.48	4.0	49.1	3.0	78.5	5.0	6
		0.6	49.5	2.1	77.7	3.5	9
2	3.95	4.0	47.6	1.6	74.6	3.4	4
		0.6	51.3	1.9	82.6	2.5	7
3	3.59	4.0	46.5	1.6	72.3	2.8	10
		0.6	48.7	1.9	78.9	3.1	6
4	3.14	4.0	47.6	2.9	72.7	3.1	9
		0.6	49.5	3.0	77.2	2.9	7
5	2.75	4.0	47.4	3.2	67.0	3.0	10
		0.6	46.4	4.2	68.9	4.5	12

[a] These samples were relyophilized and redissolved in fresh D_2O. [b] Single peak. See text.

Results

Experiments of two kinds were conducted. Those in which the time course of deuterium exchange was the object of study so that the samples were not disturbed after addition of D_2O and those in which final, or at least stable, values of exchange were attained, by manipulation or otherwise. The latter will be considered first.

From Table II it is clear that the observed stable values of low-field protons in peaks N and I fall into three well-separated categories: (1) guanidinated ribonuclease, 104; (2) native ribonuclease, 68–77; (3) heated samples of either protein, 50. In the third case the residual peak is the single, symmetrical, aromatic peak, comparable to the aromatic peaks of the second class which contain 46–51 protons. There are 47 aromatic protons.[8]

When the contribution of the aromatic peak to the total low-field spectrum is subtracted, it is seen that native ribonuclease, under a variety of conditions, contains 20 to 25 NH protons which are refractory to exchange. We wish to emphasize that when the reaction described by Hermans and Scheraga are repeated, the number of residual NH protons in guanidinated ribonuclease is 54 and in ribonuclease 24; heating to 72° reduces both values to zero.

The estimates of the number of NH and aromatic protons in the low-field peaks is likely to be high rather than low, but by only a few protons. The excess probably arises from the C^{13} satellites of the larger peaks ($\lesssim 2$), some HDO tailing (not necessarily eliminated by exchange alone, since the HDO line broadens as the proportion of H decreases), and, less likely, from the equilibrium NH content of the moderately slowly exchangeable moieties (not more than 2–3 in any case) which should be removed by reexchange. Kowalski, in a personal communication, suggests that one of the CH of the four histidine residues may absorb at lower field, leaving 43 protons expected in peak I and adding four protons to peak N. It is not inconceivable that the OH proton of the three tyrosine residues which do not participate in the hydrogen-ion equilibria of native ribonuclease exchange slowly, and contribute to the low-field spectrum.

Kinetic data for duplicate experiments at pD 2.75 and 4.48 pD are shown in Fig. 2. Experiments at intermediate pD yielded, within the fairly large

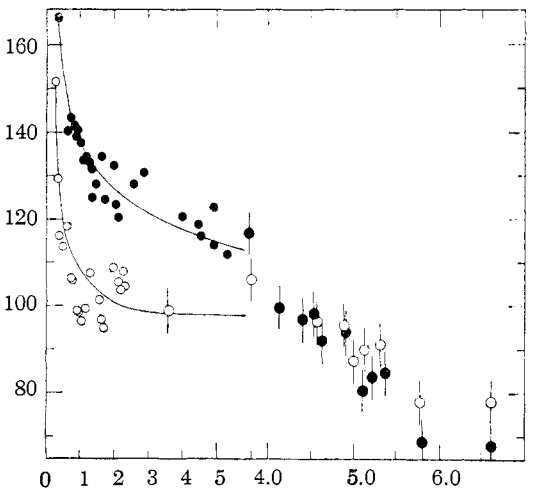

Fig. 2.—The time course of deuterium exchange of ribonuclease: ordinate, total NH and aromatic protons. Abscissa, seconds to t = 6000 sec., time, logarithmic thereafter: O, pD 4.48; ●, pD 2.75. Vertical lines indicate averaged values ±5 protons.

limits of error, intermediate results. Most of the 245–255 (depending on pD) exchangeable protons disappear before any measurements can be made; about 60 or 70 exchange during the course of the experiment; some 25 residual protons remain at the end. The rates of exchange during the first few hours may be said to increase with pD; afterwards, if anything, the reverse is true. As Linderstrøm-Lang remarks, "most kinetic data (and ours too) can be represented by such exponential series $\Sigma n_i \exp(-\beta_i t)$, and the number of constants β_i required is the smaller, the less accurate the experiments."[2a] While it is doubtless true that the

specific rate constants for different protons within the observable intermediate class are distributed over a range of several orders of magnitude, detailed analysis of the curves would not be fruitful. Even to specify the n_i, that is, to subdivide the group for which exchange is observed, would be essentially arbitrary.

Discussion

The results obtained using n.m.r. spectroscopy to study the exchange of H or D between ribonuclease and solvent are in approximate agreement with those obtained by other methods. We would also agree that the probability is high that the sixty-odd protons *seen* to exchange at moderate-to-slow rates, are, in fact, protons of the peptide backbone, if only because no other class of protons is sufficiently numerous. However, our observation that guanidinated ribonuclease retain 54 NH protons under conditions were untreated ribonuclease retains 24 suggests that the increased number retained by the guanidinated protein are held on the homoarginine residues which have been introduced. If these additional guanidinium residues exchange slowly, it is difficult to see why the guanidinium residues on ribonuclease are not also slow. Since these account quantitatively for the ribonuclease result, assumption of a special class of slowly exchanging peptide hydrogens is no longer necessary. We suggest, therefore, that in both cases all of these slowly exchanging hydrogens are guanidinium protons.

One possible alternative explanation of the increased number of slowly exchanging hydrogens in the guanidinated protein is an extensive structural change whereby, by folding or hydrogen-bonding, the fraction of non-exchangeable peptide bond protons increases from 24/119 to 54/119. On careful consideration and discussion with several people who are experts in the field we can find no other satisfactory explanation for our results. There are one and two points which require *ad hoc* explanations, at present, to make the unfamiliar notion of slowly-exchanging guanidinium groups acceptable, but there is little or no evidence for structural modification. The properties of 95-guan-RNase have been studied extensively.[14] $s_{20,w}$ is the same, the pH dependence of activity is the same, behavior in urea with respect to various substrates is essentially the same, as native ribonuclease. If the change in $\alpha^{25}D$ from -74 to $-64°$ is a possible indication of slightly higher helical content,[18] the change in absorptivity at 277 mμ is in the same direction as that caused by unfolding in urea. Probably the most significant data are that the heat stability is essentially unchanged and that the characteristic behavior of the tyrosine residues is unchanged: Three phenolic groups titrate normally, with unchanged pK, while three are not in equilibrium, and appear only upon denaturation at high pH.

The hypothesis that the guanidinium groups of proteins may exchange slowly near room temperature and pH 4 is consistent with work from other laboratories, and may sometimes reconcile conflicting reports. The data of Blout, *et al.*,[3] probably represent peptide NH only (there may be some interference from deuteriated guanidinium groups[10]). For ribonuclease, at pD 4.5 and approximately 25°, and without a second lyophilization, they found 35 ± 6% (42) "hard to exchange amide hydrogens" (HEAH) after 10 minutes and 12% (14) after 24 hours. In comparable circumstances we observe 115 ± 5 and 91 ± 3 protons in the low-field region. Only by subtracting the long-lived NH as well as the aromatic protons do we obtain 34 ± 4% and 13 ± 3% HEAH: if only the aromatic protons are deducted the results are 55 and 34%. The most nearly comparable experiments using the Linderstrøm-Lang method were done at 0 and 38°, while there is no obvious way to estimate a result at 25°, (an experiment with heated ribonuclease suggests a value halfway between) any reasonable interpolation between the observed numbers of NH protons not exchanged after 24 hours at the two temperatures (49 ± 5 and 22 ± 5) gives a result comparable to ours and requires the same interpretation.

The original cryosublimation procedure which gave anomalous results with ribonuclease,[4] has been modified.[2b,6] Since then, two other proteins have been studied by the solvent-analysis technique. In the case of myoglobin,[19] a change in pH from 7 to 8.8 causes the exchange of 20 ± 4 protons of the refractory variety (after 24 hours at pH 8.8 and 37° all protons have exchanged) without altering the rates of exchange of the moderately slow protons. There are four arginine residues (20 protons) per molecule of 17,830 mol. wt.[19] There is no evidence for the proposed structural change. Wilcox,[20] investigating the back exchange of deuteriated α-chymotrypsinogen by a procedure designed specifically to isolate "all-or-none" stable protons from those with moderate rates of exchange, found 21 ± 2 refractory deuterons when the initial deuteriation had been complete and 23 ± 5 such deuterons for the entire series of experiments. α-Chymotrypsinogen contains four arginine residues per 24,000 molecular weight.[21] In addition, the number of moderately exchangeable protons left after 24 hours, 20–30 (deduced from partial deuteriation studies) is comparable to the infrared value.[3] The older result for β-lactoglobulin, "in which about 100 hydrogen atoms out of 550 do not exchange at all during several days at 38° at pH 5.4"[22] is too high for the sum of arginine (30) and HEAH (30)[3] even after correction to 94 per 35,500 ml. wt., but the interval pH 5–5.5 is a region of complex interactions,[23] so that only experiments done on the same samples at the same conditions would be interpretable. Very recently, infrared studies have been made of films of protamine[10] and nucleohistone.[24] A large number of NH in nucleohistone

(18) C. Schellman and J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **30**, 463 (1958).

(19) E. E. Benson and K. Linderstrøm-Lang, *Biochim. Biophys. Acta*, **32**, 579 (1959).

(20) P. E. Wilcox, *ibid.*, **34**, 602 (1959).

(21) P. Desnuelle, in P. Boyer, H. Lardy and K. Myrbäck, ed., "The Enzymes," Academic Press, New York, N.Y., 1960, Vol. 4, pp. 102–103.

(22) K. Lindstrøm-Lang, Soc. Biol. Chemists, "India, Silver Jubilee Souvenir," p. 191, quoted by him in ref. 2a, p. 32.

(23) N. Timasheff and R. Townend, *J. Am. Chem. Soc.*, **83**, 3157, 3161, 3168 (1960).

exchange slowly; the unknown composition of this preparation precludes detailed discussion. The observation that the NH of protamine, which is about 50% arginine, exchange rapidly, is important; if the analysis below is correct, we would expect the predominant mechanisms of exchange of protamine and of the globular proteins to differ.

To begin with, while native and guanidinated ribonuclease contain 20 and 67.5 guanidinium protons, respectively, the observed values of residual NH are 24 and 54. Moreover, the exchange of arginine, while not instantaneous, occurs on a much shorter time scale. Since no studies of guanidinium ions have been published, we must discuss the extensive studies, using n.m.r. techniques,[12a] of the presumably analogous kinetics of exchange of ammonium and methylammonium ions with water. The observed rates increase monotonically, but not linearly, with pH, and in the interval $2 < pH < 8$ exhibit a striking dependence on RNH_3^+ concentration. The early report[25] that ND_4^+ has a half-life of several minutes in strongly acidic solutions has been confirmed,[26] but is now interpreted to represent only a part of the direct exchange $NH_4^+ + H_2O \rightarrow NH_3 + H_3O^+$, which is much faster at lower concentrations of hydronium ion.[12a,26] The direct exchange of methylammonium ion with water is, however, too slow to measure by n.m.r. methods[12a,27] and remains unknown. Guanidinium ion, being considerably less acidic, would be expected to have an even slower rate. The reaction $RNH_3^+ + OH^- \rightarrow RNH_2 + H_2O$ is assumed to be rapid, but negligible in the pH region studied.[27] Analysis indicates that the principal reaction proceeds through an amine-catalyzed termolecular mechanism; the observed rate depends on the specific rate constant, pH, and the pK_a and concentration of ammonium ion. Again, a similar effect would be expected in the exchange of guanidinium ions.

Preliminary investigations of guanidinium chloride solutions by n.m.r.[11] have indicated a somewhat slower exchange than would be predicted from extrapolation of the data for ammonium and methylammonium ion using the new pK_a. Whether the observed reaction is slow enough to account for the behavior of ribonuclease and guanidinated ribonuclease depends entirely on the relative contributions of the bimolecular and termolecular reactions, which remain to be sorted out. In this instance, isolation of the direct exchange at very low pH is precluded by the appearance of an acid-catalyzed exchange. We would interpret the (immeasurably) slow rate of exchange of approximately 20 protons of ribonuclease as the direct exchange of guanidinium protons with water. The rapid exchange observed upon heating the proteins above the transition temperature would be attributable to the termolecular mechanism, with the now-flexible chain giving high local (*i.e.*, intramolecular) guanidinium concentration. In 95-guan-RNase, presumably, two or three of the 13 or 14 arginine-homoarginine residues are near neighbors; in protamine films virtually all the arginine residues must have neighbors. We would expect hydroxide-ion catalysis to become significant at alkaline pH.

As Linderstrøm-Lang anticipated, studies of the exchange of the NH hydrogens of proteins with water can yield valuable information about protein structure. The analysis of the experimental data must be circumspect; in particular, the facile assignment of hydrogens which exchange with the greatest difficulty to a hard core of peptide backbone protons must be avoided.

(24) E. M. Bradbury, W. C. Price, G. R. Wilkinson and G. Zubay, *J. Mol. Biol.*, **4**, 50 (1962).

(25) L. Kaplan and K. W. Wilzbach, *J. Am. Chem. Soc.*, **76**, 2593 (1954).

(26) M. T. Emerson, E. Grunwald and R. A. Kromhout, *J. Chem. Phys.*, **33**, 547 (1960).

(27) E. Grunwald, B. J. Karabatsos, R. A. Kromhout and E. L. Purlee, *ibid.*, **33**, 556 (1960).