Primary Structure Effects on Peptide Group Hydrogen Exchange*

R. S. Molday, S. W. Englander,† and R. G. Kallen

ABSTRACT: Primary structure effects that influence hydrogen-exchange behavior of the peptide group were evaluated by studying, with proton magnetic resonance techniques, small molecule amide models. The effect of various amino acid side chains on peptide groups to their right and their left were measured. Only nearest-neighbor effects appear important and these are simply additive. Summed effects of different pairs of side chains on the included peptide group vary up to 300-fold, as for His^+ -His^+ compared to Ala-Ala, though all others fall within a 10-fold range of the value for Ala-Ala. Effects on the hydronium and hydroxide ion catalyzed hydrogen-exchange rate constants are qualitatively understandable, in the main, in terms of polar effects on the basicity and acidity, respectively, of the peptide group. The relative influences of different groups vary somewhat irregularly at a finer level of discrimination and remain as yet unexplained in detail. These conclusions and the quantitative results obtained were tested by comparing predictions made from them against the hydrogen-exchange behavior of peptide groups in other molecules. Accurate predictions were obtained for hydrogen-exchange behavior in small molecules, in random-chain homopolypeptides, in the random-chain heteropolypeptide oxidized ribonuclease, and in the exposed peptide groups of a globular protein, myoglobin. Thus, contrary to previous apprehensions that hydrogen-exchange behavior at the macromolecular level might be dominated by poorly understandable effects, such effects do not appear to be present. Stated more generally, it appears that aqueous chemistry at the surface of macromolecules is closely similar to that experienced by small molecule models.

The protein hydrogen-exchange (HX)[1] method was designed to provide information about macromolecular structure and conformational dynamics (Berger and Linderstrøm-Lang, 1957). Unravelling of the contribution of structure to HX behavior requires knowledge of the HX character of the unstructured polypeptide. Structureless *homopolypeptides* (Leichtling and Klotz, 1966; Ikegami and Kono, 1967; Englander and Poulsen, 1969) display satisfyingly simple hydrogen-exchange behavior characteristic of a single kinetic class of exchanging hydrogens (dashed line, Figure 1). By contrast, studies of oxidized ribonuclease, a random-chain *heteropolypeptide*, reveal the presence of multiple kinetic classes of peptide hydrogens as shown by the curvature in the rate plot of Figure 1 (see also Woodward and Rosenberg, 1970). Further, at pH below 3, random-chain poly-D,L-alanine exchanges faster than oxidized ribonuclease, while at higher pH it becomes slower.

We report here on studies of some factors that account for this perhaps unexpected hydrogen-exchange behavior. The solid line through the ribonuclease data points in Figure 1 represents a predicted curve for this polypeptide based on the results we have obtained. A variety of factors have been suggested to influence hydrogen-exchange rates of polypeptides: hydrophobic group mediated alteration in local water chemistry (Scarpa *et al.*, 1967), charge interactions (Sheinblatt, 1970), general acid–base catalysis by side chains (Klotz and Frank, 1965), and inductive effects (Leichtling and Klotz, 1966). In the present study, we have measured the effect of nearest-neighbor amino acid side chains and of adjacent peptide groups on the hydrogen-exchange behavior of peptide groups in small molecule derivatives. By use of nuclear magnetic resonance (nmr) techniques, the effects of substituents on both the specific acid and the specific base-catalyzed rates of peptide groups to the right (ρ) of the substituent and to the left (λ) were measured. The results provide a quantitative basis for consideration of the inductive and electrostatic influence of the various groups that occur in proteins.

These effects, measured in small molecule models, are shown to account for other small molecule data in the literature, for peptide-group hydrogen-exchange rates of some unstructured homopolypeptides, for the heteropolypeptide-oxidized ribonuclease, and for the exposed peptide groups of myoglobin, a globular protein. These successes in turn exclude the possibility of large and indeterminate contributions arising in the other factors mentioned in the above list.

The evaluation of the role of primary structure factors considerably enhances the utility of the hydrogen-exchange approach to problems in the secondary and tertiary structure of polypeptides and proteins.

Materials and Methods

Small molecule peptide derivatives were purchased (Cyclo Chemical Co.) or synthesized from the corresponding amino acid methyl ester. Oxidized ribonuclease samples used were purchased (Mann) or prepared from five-times-recrystallized bovine pancreatic ribonuclease (Worthington) using performic acid (Hirs, 1956). Poly-D,L-asparagine (DP ~10) was prepared from poly-D,L-aspartate β-methyl ester (Miles-Yeda). The various samples were checked by nmr, pH titration, and melting point analyses to establish identity and purity.

Hydrogen- (H–H) exchange rates of *N*-methyl and *N*-methylene peptides were measured by nmr total line-shape

* From the Department of Biochemistry, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104. *Received August 15, 1971.* Supported by Research Grants AM 11295 from the National Institute for Arthritis and Metabolic Diseases, and GM 13777 from the National Institute of General Medical Sciences, U. S. Public Health Service. This paper represents a portion of the thesis of R. S. M. submitted in partial fulfillment of the requirements for the Ph.D. degree at this University.

[1] Abbreviations used are: HX, hydrogen exchange; NMA, *N*-methylacetamide; NA-Ala-N′MA, *N*-acetylalanine-*N*′-methylamide ($CH_3CONHC(CH_3)HCONHCH_3$), and analogously for other amino acid derivatives; ρ peptide for peptide group to the right of the substituent, and λ to the left.

TABLE I: Effect of Neighboring Peptide Groups on H–H-Exchange Rate Constants (25°).

Peptide Derivative		k_H (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})	Log k^R − Log k^{NMA}	
				H_3O^+	OH^-
$CH_3C(=O)NHCH_3$		2.16×10^4	2.51×10^8	0.00	0.00
$CH_3C(=O)NHCHC(=O)NHCH_3$	ρ	1.60×10^3	6.25×10^8	−1.12	0.40
$CH_3C(=O)NHCH_2C(=O)NHCH_3$	ρ	2.70×10^3	1.04×10^9	−0.90	0.62
	λ	1.15×10^3	4.24×10^9	−1.27	1.23
$CH_3C(=O)NHCHC(=O)NHCH_2C(=O)NHCH_3$	ρ		9.24×10^8		0.57
	λ		1.64×10^{10}		1.82
$^+H_3NCH_2C(=O)NHCH_3$			2.94×10^{10}		2.07
$CH_3C(=O)NHCH_2C(=O)OH$[a]		1.77×10^3	1.85×10^8	−1.09	−0.13

[a] k_H is for the carboxylic acid; k_{OH} is for the carboxylate anion.

analysis (60 MHz Jeolco Model JNM C-60H) at 25° from the degree of coalescence of the spin–spin doublet (Jonas *et al.*, 1965). Especially the λ peptide of nonglycine derivatives was difficult to measure by this approach owing to additional coupling between the α- and β-carbon protons and to overlap with the dominant water peak. Deuterium- (H–D) exchange rates at 5° were measured, at pD values giving slow exchange, from the time-dependent change, following dissolution in D_2O buffer, of the nmr signal of the protons on the carbon adjacent to the peptide nitrogen. Tritium- (T–H) exchange rates of the polymers were determined at 0° by the previously described Sephadex (Englander, 1963) and rapid dialysis (Englander and Crowe, 1965) techniques. The molar extinction coefficient at 220 nm of oxidized ribonuclease is 1.35 ± 0.02 × 10^5 M^{-1} cm^{-1} by microkjeldahl analysis. In computation of oxidized ribonuclease T–H-exchange data, the factor 1.21 was applied (Englander and Poulsen, 1969) to correct for the peptide group T–H equilibrium isotope effect.

Procedures used are described in more detail elsewhere (Molday, 1971; Molday and Kallen, 1972).

Results

Effect of Neighboring Peptide Groups on Exchange Rate. The effect of neighboring peptide groups on the exchange rate of a central peptide is shown in Figure 2. These results and

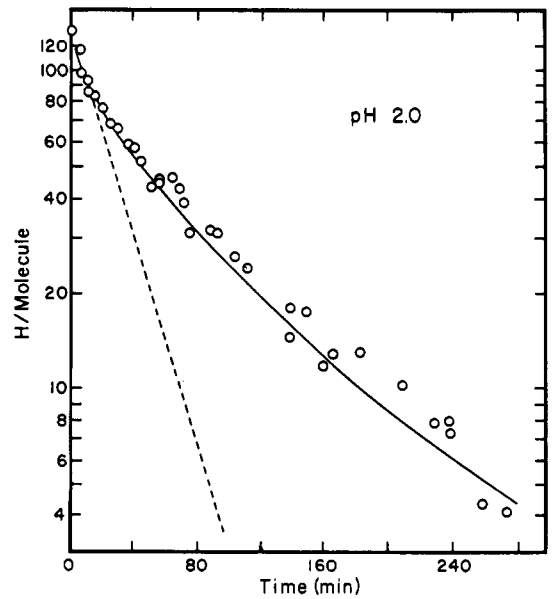

FIGURE 1: T–H exchange of oxidized ribonuclease at pH 2.0 and 0°. (----) The free peptide rate for random-chain poly-D,L-alanine under similar conditions. (——) The exchange curve predicted for oxidized ribonuclease taking into account effects of neighboring side chains on peptide hydrogen-exchange rate as summarized in Table IV.

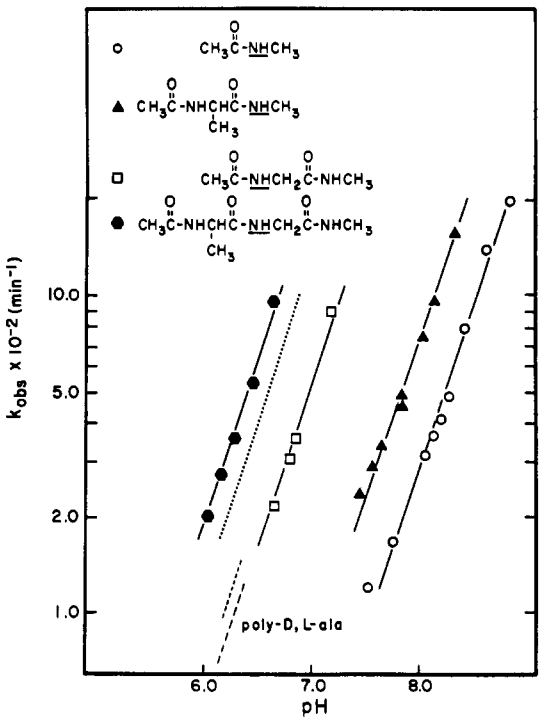

FIGURE 2: Effect of peptide groups on the hydroxide ion catalyzed HX rate of a neighboring peptide group (25°). Lines are drawn with unit slope. The upper dotted line indicates the summed effect of an alanyl peptide to the left and a glycyl peptide to the right of *N*-methylacetamide (individually measured in the derivatives shown) and can be compared to the rates measured in NA-Ala-Gly-N'MA. The lower dotted line represents HX rate expected for the central peptide of the derivative NA-Ala-Ala-N'MA and is compared to PDLA (---).

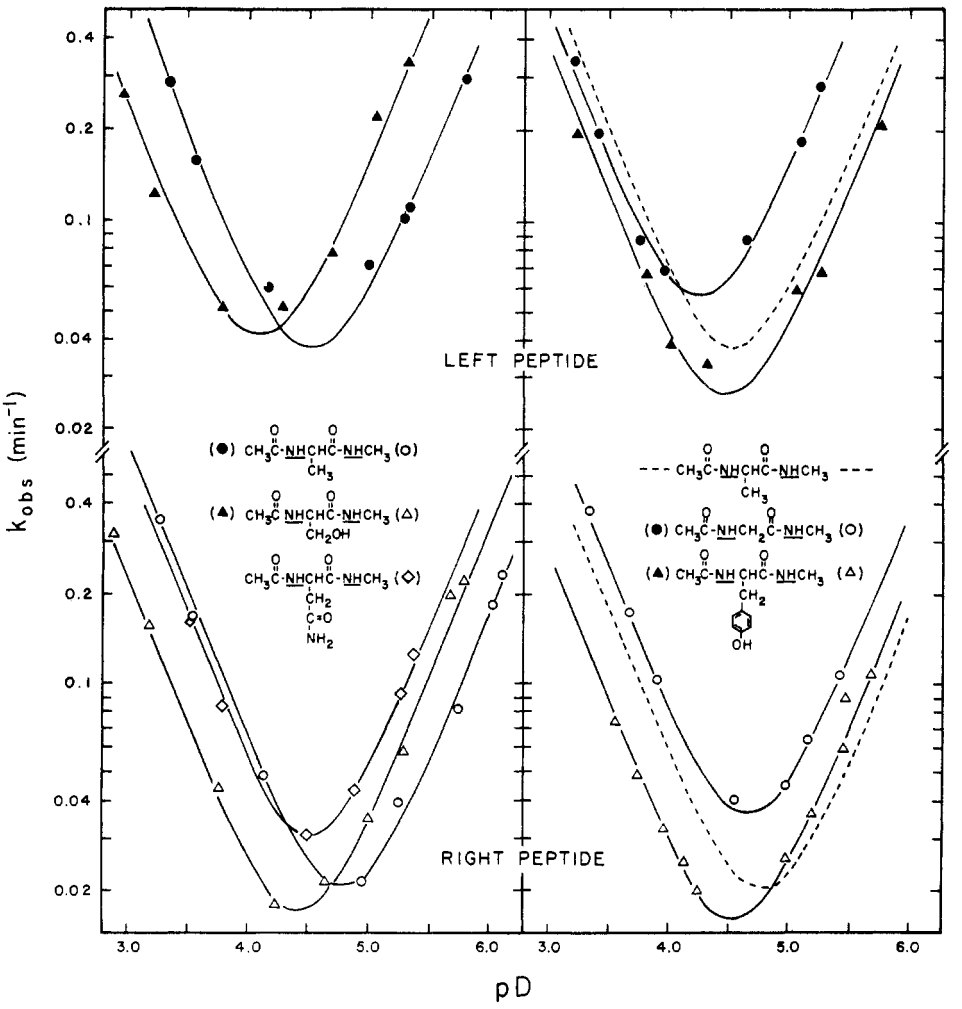

FIGURE 3: H–D-exchange data at 5° for peptide groups in derivatives with the neutral amino acid side chains shown. Solid lines represent theoretical curves for specific acid and base catalysis located to best-fit experimental points.

others are summarized in Table I, which shows the size of the effect, in the last columns, as the shift in log (rate) measured from *N*-methylacetamide (NMA).

Addition of a peptide group on the carbonyl (left) side of NMA, as in the derivative NA-Ala-N′MA, *increases* the specific *base-catalyzed* exchange rate of the *N*-methyl peptide by more than a factor of 2. Addition of a peptide group on the right side of NMA *increases the base-catalyzed* rate 17-fold (measured for the λ peptide of NA-Gly-N′MA). The combined effect of peptide groups on both sides is shown in Figure 2 for the central peptide of NA-Ala-Gly-N′MA. The effect measured is close to the computed sum (dotted line) of the inductive effects for a peptide on the left in NA-Ala-N′MA and for a peptide on the right in NA-Gly-N′MA. Thus, these effects appear largely additive.

Comparison of exchange rates of the ρ peptides of NA-Ala-Gly-N′MA (Δ log k = 0.57) and NA-Gly-N′MA (Δ log k = 0.62) indicates that more distant peptide groups have little if any effect on the hydroxide-catalyzed exchange rate (Table I).

Presence of a peptide group on either side of a given peptide *decreases* the specific *acid catalyzed* rate by about 10-fold relative to NMA as determined from the line shapes of the *N*′-methyl and *N*-methylene signals of NA-Gly-N′MA (Table I).

Other considerations of interest may be derived from Figure 2 and Table I by invoking the simple additivity relationship and the nearest-neighbor approximation. The summed effect of a peptide group to the left and to the right, compared to NMA, should act to *decrease* the acid-catalyzed rate by over two decades. This can be seen by adding the shifts in the acid rate shown in Table I for the λ and ρ peptides of NA-Gly-N′MA. The base-catalyzed rate is *increased* by almost two decades. Thus, if curves of log (rate) against pH were plotted for a central peptide group as in Figure 2, both acid and base limbs would fall to the left of NMA by about 2 pH units. The

TABLE II: H–H-Exchange Rate Constants for ρ Peptide (25°).

Peptide Derivative	k_H (M^{-1} min^{-1})	k_{OH} (M^{-1} min^{-1})
NA-Ala-N′MA	1.60×10^3	6.25×10^8
NA-Ser-N′MA	5.63×10^2	1.40×10^9
NA-Gln-N′MA	1.02×10^3	8.82×10^8
NA-Tyr-N′MA	6.37×10^2	7.39×10^8
NA-Gly-N′MA	2.70×10^3	1.04×10^9
NA-Lys$^+$-N′MA	8.10×10^2	1.02×10^9
NA-His-N′MA		1.07×10^9 [a]

[a] Unprotonated histidyl side chain.

TABLE III: H–D-Exchange Rate Constants for the λ and ρ Peptide (5°).

Peptide Derivative	k_D (M^{-1} min^{-1})		k_{OD} (M^{-1} min^{-1})[a]	
	λ	ρ	λ	ρ
NA-Ala-N′MA	6.12×10^2	6.60×10^2	3.30×10^9	9.30×10^8
NA-Ser-N′MA	2.30×10^2	2.30×10^2	1.07×10^{10}	1.85×10^9
NA-Asn-N′MA		5.82×10^2		2.60×10^9
NA-Tyr-N′MA	3.60×10^2	3.19×10^2	2.61×10^9	1.23×10^9
NA-Gly-N′MA	4.70×10^2	9.30×10^2	9.30×10^9	2.10×10^9
NA-Lys^+-N′MA	1.75×10^2	2.50×10^2	4.20×10^9	1.96×10^9
NA-His^+-N′MA	4.02×10^2 [b]	1.73×10^2	7.80×10^{10} [b]	1.30×10^{10}
NA-Asp-N′MA(COOH)	7.80×10^2	5.82×10^2	3.30×10^{10} [c]	4.65×10^9 [c]
NA-Asp-N′MA(COO^-)	4.20×10^3 [c]	1.99×10^3 [c]	4.19×10^9	1.04×10^9

[a] $pK_w(D_2O) = 15.74$ (at 5°). [b] Based on limited experimental data (see Figure 4). [c] Values to fit data in Figure 4 with eq 1.

acid and alkaline limbs of such curves intersect to define a pH value (pH_{min}) at which the minimum exchange rate occurs. It has long been known that pH_{min} for polypeptides falls close to pH 3 (Berger and Linderstrøm-Lang, 1957), 2 pH units lower than for simple primary and secondary amides. This rather extreme value, then, is explained by the summed inductive effects of the two nearest-neighbor peptide groups (see also Leichtling and Klotz, 1966).

Finally, these results allow a first comparison between polypeptide and small molecule model peptide rates. Published data for base-catalyzed HX in poly-D,L-alanine (Englander and Poulsen, 1969) fall on the dashed line in Figure 2 so identified. The small molecule model, NA-Ala-Gly-N′MA, provides a close analog in the peptide group underlined in the Figure. A prediction for the position of the HX curve for the middle peptide group of the (hypothetical) model, NA-Ala-Ala-N′MA, can be obtained by applying a correction for the glycine to alanine side-chain effect (Table IV), and is shown by the dotted line at the bottom of Figure 2. The agreement between the small molecule and the polymer curves demonstrates the absence of indeterminate polymer-based effects, and gives added support to the above deductions on additivity of local effects and on the adequacy of considering only nearest-neighbor contributions.

Effect of Amino Acid Side Chains on Exchange Rates of Neighboring Peptides. *N*-ACETYLALANINE-*N*′-METHYLAMIDE. H–D-exchange data at 5° for the λ and ρ peptides of NA-Ala-N′MA as a function of pD are shown in Figure 3. As found for all peptide derivatives (except those titrating in the region studied), exchange rates follow the simple rate law for specific acid and base catalysis represented by the theoretical curve (solid line) located to best fit the experimental data in these and following figures. H–H-exchange rates of the ρ peptide were also determined by the line-shape method at 25°. Second-order rate constants for hydronium and hydroxide ion catalysis are summarized in Table II for H–H exchange and in Table III for H–D exchange. In Table IV, the alanine derivative is taken as a base compound from which shifts in rate due to other side chains are measured.

N-ACETYLSERINE-*N*′-METHYLAMIDE. The pD–rate profile for the peptides of NA-Ser-N′MA are displayed in Figure 3 and compared with NA-Ala-N′MA. The OD group increases the deuteroxide ion catalyzed exchange rate of the λ peptide by threefold and decreases the deuteronium ion catalyzed rate by approximately the same factor. The net result is a shift of the pH–log rate curve to lower pD values by 0.5 log unit. An inductive effect of the serine side chain on the exchange rate of the ρ peptide is also apparent. Here, however, the acid-catalyzed reaction is more sensitive than the base reaction, and the rate minimum decreases.

Measurements by the two different methods, line-shape analysis at 25° (Table II) and H–D exchange at 5° (Table III), agree on the alanine to serine rate shift experienced by the ρ peptide, in both acid and base catalysis, to 0.10 log unit.

N-ACETYLASPARAGINE-*N*′-METHYLAMIDE AND *N*-ACETYLGLUTAMINE-*N*′-METHYLAMIDE. The effect of the primary amide side chain of NA-Asn-N′MA on the exchange rate of the ρ peptide is shown in Figure 3. An increase of 0.5 log unit relative to the analogous peptide of NA-Ala-N′MA is found for the OD^--catalyzed exchange rate, but little if any difference is observed for the D_3O^+-catalyzed exchange rate (opposite to the pattern for serine). A similar effect of the asparagine side chain on peptide-exchange rate was observed in T–H-exchange studies of poly-D,L-asparagine, *i.e.*, the base-catalyzed exchange rate of poly-D,L-Asn is 0.8 log unit larger than for poly-D,L-Ala while the acid-catalyzed rate is the same for both polymers. Thus, the increase in the base-catalyzed rate for the ρ peptide of NA-Asn-N′MA relative to NA-Ala-N′MA accounts for about half the difference (in log units) found in exchange rates of the respective homopolymers and suggests that the (unmeasured) influence of the primary amide side chain on the λ peptide accounts for the remaining effect.

Second-order rate constants for hydrogen exchange of the ρ peptide of NA-Gln-N′MA are listed in Table II. The primary amide of glutamine, displaced from the peptide groups by an additional methylene group, exerts a weaker inductive effect on the base-catalyzed exchange rate than does the primary amide of asparagine (Taft, 1956).

N-ACETYLTYROSINE-*N*′-METHYLAMIDE. The effect of the phenolic side chain is illustrated in the profiles in Figure 3. Only small differences are observed between the tyrosine and alanine rates in the base-catalyzed region. Larger differences are found in the acid-catalyzed rate. The rate shifts for the ρ peptide found by deuterium exchange (Table III) agree with the hydrogen-exchange results measured by line-shape analysis (Table II) to 0.08 and 0.05 log unit for the acid- and base-catalyzed rates, respectively.

N-ACETYLGLYCINE-*N*′-METHYLAMIDE. Although the glycyl and alanyl peptide derivatives differ only in the presence of a methyl group on the α carbon, their rates are significantly

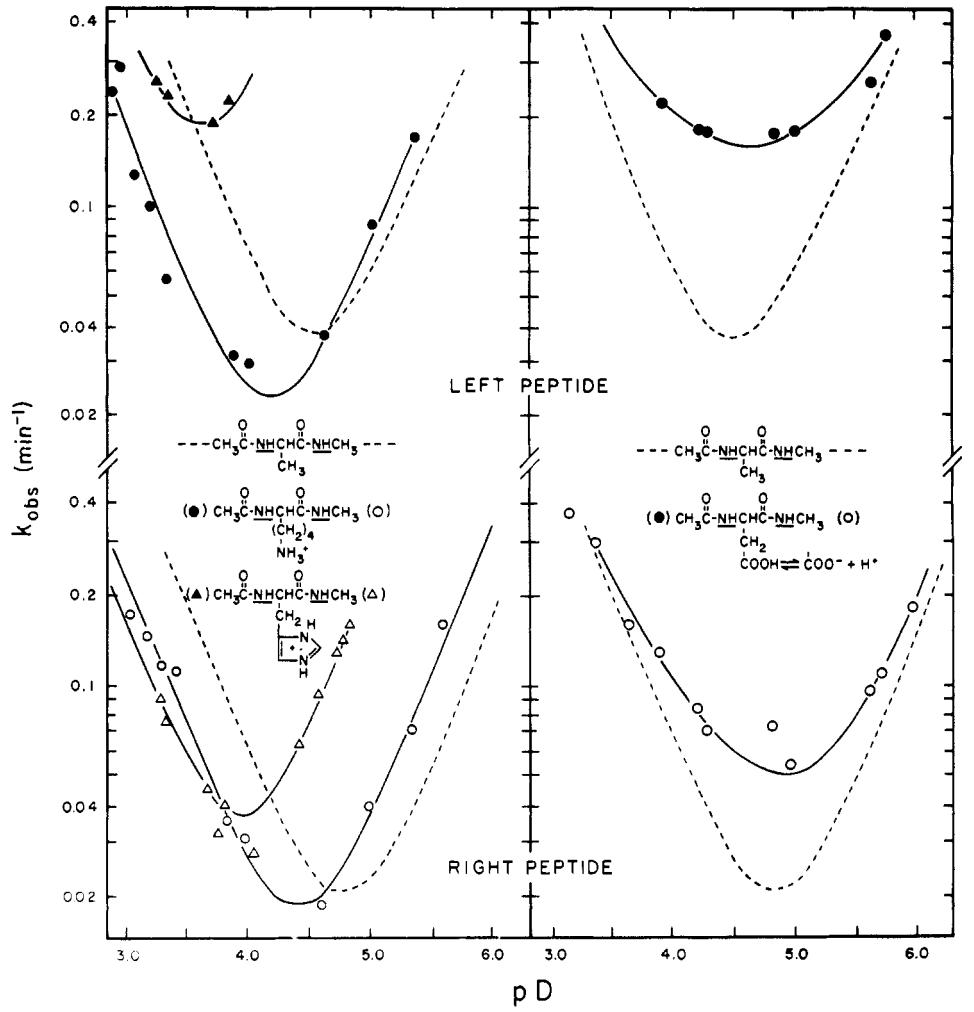

FIGURE 4: H–D-exchange data at 5° for peptide groups in derivatives with acidic and basic side chains. The broken curves for alanine are from Figure 3.

different as shown in Figure 3 and in Table II. Exchange rates are generally slower for the alanyl peptides. (A similar decrease in base-catalyzed H–D-exchange rate due to an additional alkyl group was reported by Klotz and Feidelseit (1966) for *N*-methylacetamide and *N*-ethylacetamide.)

The two methods (Tables II and III) applied to the ρ peptide of the glycine derivative differ by 0.08 log unit for the shift in acid rate and 0.13 for the shift in base rate relative to alanine.

N-α-ACETYLLYSINE-*N'*-METHYLAMIDE. The effect of the positively charged lysyl side chain is shown in Figure 4. The two methods (Tables II and III) applied to the ρ peptide of the lysine derivative differ by 0.13 and 0.11 log unit in the acid- and base-catalyzed shift relative to alanine.

The increase in the base-catalyzed rate of 0.30 log unit for the ρ peptide and 0.10 log unit for the λ peptide, when summed, accounts for the difference of 0.4 log unit between the hydroxide ion catalyzed exchange rates of poly-D,L-lysine and poly-D,L-alanine (Englander and Poulsen, 1969). Similarly the lysyl *vs.* alanyl difference in acid-catalyzed rate of 0.40 and 0.55 log unit for the ρ and λ peptides, respectively, when added, is in reasonable agreement with the difference of 1.1 log unit found for the analogous homopolypeptides.

The data shown (Figure 4) rule out significant general catalysis by the lysine side chain (0.3 M concentration).

N-ACETYLHISTIDINE-*N'*-METHYLAMIDE. An extreme effect is exerted by the protonated histidine side chain on base-catalyzed exchange of its vicinal peptide groups (Figure 4). Rates of both peptides are increased over 10-fold. A smaller effect is exerted on the acid-catalyzed reaction (opposite to lysine). Above pH 7.5, the histidine side chain is essentially neutral ($pK_a' = 6.5$, from C_2–H chemical shift) and hydroxide ion-catalyzed exchange of the ρ peptide of NA-His-N'MA is only 1.7 times faster than in the alanine derivative (Table II).

The enhancement in hydroxide ion catalyzed peptide HX by neighboring positively charged groups has been studied before (Sheinblatt, 1965, 1966, 1970) and has been related to electron-withdrawing effects on peptide group acidity.

In principle, general-base catalysis by the histidine side chain could contribute to HX rate of NA-His-N'MA above the pH minimum, but this seems unlikely in view of the failure to observe catalysis by imidazole in aqueous solution (Klotz and Frank, 1965; Englander and Poulsen, 1969).

N-ACETYLASPARTATE-*N'*-METHYLAMIDE. Data for NA-Asp-N'MA is given in Figure 4. In the region of the pD_{min}, the pD–rate profiles deviate from the simple theoretical curve for specific acid–base catalysis. This anomalous behavior can be explained by taking into account the titration of the carboxyl side chain in the region of the pD_{min} according to the rate law of eq 1.

$$k_{obsd} = [k_D^{COO^-}(\alpha) + k_D^{COOH}(1 - \alpha)]a_D + [k_{OD}^{COO^-}(\alpha) + k_{OD}^{COOH}(1 - \alpha)]K_W/a_D \quad (1)$$

Here the fraction of NA-Asp-N'MA with the side chain in the negatively charged carboxylate form is given by

$$\alpha = \frac{K_a'}{a_D + K_a'} \quad (2)$$

The dissociation constant, K_a' for the carboxyl side chain determined by potentiometric titration in D_2O, 0.1 M KCl at 25° is $10^{-4.33}$ ($pK_a' = 4.33$). The value of K_W for D_2O at 5° is $10^{-15.74}$. The second-order rate constants k_D^{COOH} and $k_{OD}^{COO^-}$ are estimated from the extremities of the pD–rate profiles for the λ and ρ peptides (Figure 4) and are essentially the same as the specific acid and base catalytic rate constants found for the respective peptides of the alanyl derivative. The effect of the neutral aspartyl (COOH) side chain on deuterium-exchange rates of the λ and ρ peptide is similar to the effect of the asparaginyl side chain (Charton, 1964; Taft, 1956). That is, relative to the alanyl peptide derivative, the base-catalyzed rate is increased significantly, but acid-catalyzed rate is unaffected. Values for the four rate constants used to fit the data are given in Table III.

T–H Exchange of Primary Amide Side Chains. Unlike the other polar side chains of proteins, the protons of which exchange at diffusion-limited rates (possibly excepting tryptophan), the primary amide side chains of asparagine and glutamine exchange quite slowly (Bovey and Tiers, 1963). Figure 5 shows T–H-exchange rates measured as a function of pH for poly-D,L-asparagine *side chains* at 0°. Exchange of the peptide groups and the primary amides in this polymer was distinguished by kinetic analysis. The analysis for amide side-chain rate was facilitated by the fact that the primary amides contribute two-thirds of the total exchanging H and by the large differences in HX rate between primary and secondary amides (for details, see Molday, 1971).

The expected behavior of the primary amide of glutamine is shown by the dashed line in Figure 5, and was surmised according to the following considerations. The acid-catalyzed rate of *N*-methylacetamide and the asparagine side chain are nearly identical ($k = 1.0 \times 10^3$ M^{-1} min^{-1} at 0°). Thus, the acid-catalyzed reaction of the side chain is insensitive to the backbone amides. Likewise, the side-chain amide has no net effect on the acid-catalyzed rate of main-chain amide (Figure 3). Therefore, we assume the acid-catalyzed rate of glutamine to be close to that for asparagine. By contrast, the *base*-catalyzed rate of the side-chain amides in poly-D,L-Asn differs by 1.1 log units from *N*-methylacetamide. Also, the *base*-catalyzed rate of the main-chain amide is sensitive to the amide side chain of asparagine (Figure 3). Thus we expect the alkaline limb of glutamine to fall below that for asparagine.

A quantitative interpolation for the glutamine position between the rates for *N*-methylacetamide and asparagine is made possible by the linear relationship shown to exist between HX rate of amides and p*K* value of analogous carboxyl groups (Sheinblatt, 1970; Molday, 1971). The p*K* values of pertinent model carboxyls have been measured by Nozaki and Tanford (1967). They find $pK_a' = 4.08$ for aspartate and $pK_a' = 4.50$ for glutamate (when amide groups are on both sides of the α carbon). As an acid analog of *N*-methylacetamide, we may take propionate with $pK_a' = 4.85$. Linear interpolation between the NMA and Asn rates (Δ log rate = 1.1) then suggests the HX rate of glutamine to be about 0.6 log unit slower than asparagine, *i.e.*, 1.1(4.50–4.08)/(4.85–4.08) = 0.6. The OH^--catalyzed rate constant for asparagine at 0° is 1.8×10^9 M^{-1} min^{-1} and for glutamine is 4.5×10^8.

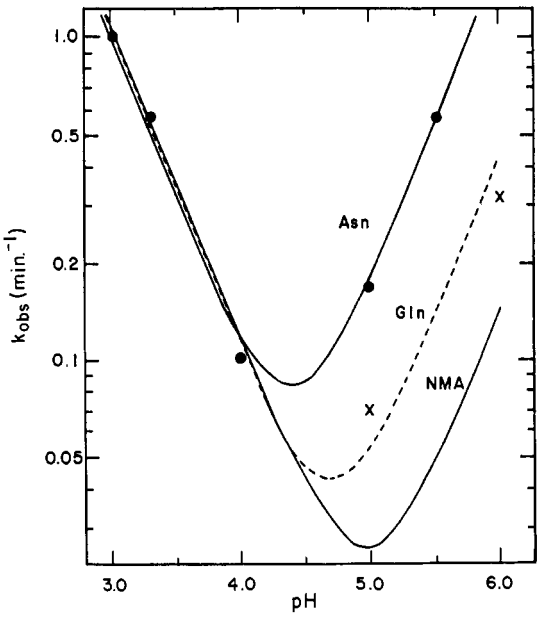

FIGURE 5: T–H-exchange data at 0° for the primary amide side chains of poly-D,L-asparagine (Asn). Shown for comparison are *N*-methylacetamide (NMA) and the interpolated behavior of glutamine side chains (Gln) (see text). The x's represent rates picked from the expected primary amide region of oxidized ribonuclease exchange curves (Figure 6).

The next section and Figure 6 present some HX data for random-chain oxidized ribonuclease. At pH 5 and 6, free primary amides exchange considerably more slowly than free peptides and should appear largely isolated at the bottom of the exchange curves. The slowest rates seen at higher pH in Figure 6 diverge somewhat from those expected for primary amides and are shown as x's in Figure 5. That is, when the HX data at pH 5 and 6 in Figure 6 are put on a semilog plot, a good first-order kinetic class is seen, closely equivalent in size to the total number of primary amides (Gln + Asn), and with an exchange rate close to that expected for glutamine alone, therefore significantly slower than the overall predicted curve.

Test of Results against Oxidized Ribonuclease. It is of primary interest to test the present results against some real polypeptide HX data. The present results together with the known amino acid sequence of oxidized ribonuclease allow predictions of the hydrogen-exchange behavior of this polymer. For the polymer predictions, we start with accurately known poly-D,L-alanine behavior (Englander and Poulsen, 1969) and apply factors from Table IV as required by the ribonuclease amino acid sequence. The predictions are based on and provide a reasonable test for results obtained and conclusions reached in this work; namely, the magnitude of individual side-chain effects, the simple additivity of these, the grouping of side chains not actually measured (Table IV), the predominance of nearest-neighbor effects, and nondependence on molecular size.

T–H-exchange data for oxidized ribonuclease at pH 2, 3, 4, 5, and 6 are shown in Figures 1 and 6. The solid lines present exchange curves predicted as described *i.e.*, by summing the predicted T–H-exchange curves for all the peptide and primary amide groups in the molecule. By any criteria, the agree-

TABLE IV: Relative Effect of Amino Acid Side Chains on Exchange Rates of Neighboring Peptides.[a]

		Log k^R − Log k^{Ala}			
		Acid Catalysis		Base Catalysis	
Side Chain, *R*	Class	λ	ρ	λ	ρ
Ala	Ala, Met Val, Leu, Ile, Pro	0.00	0.00	0.00	0.00
Ser	Ser, Thr, Cys	−0.40	−0.45	0.50	0.30
Asn	Asn	(0.00)	−0.05	(0.40)	0.45
Gln	Gln, Glu-COOH	(−0.20)	−0.20	(0.15)	0.15
Tyr	Tyr, Phe	−0.20	−0.30	−0.10	0.10
Gly	Gly	−0.10	0.15	0.45	0.30
Lys	Lys^+, Arg^+	−0.55	−0.40	0.10	0.30
His	His^+	−0.30	−0.60	1.40	1.15
	His			(0.20)	0.25
Asp	$Asp\text{-}COO^-$, $Cys\text{-}SO_3^-$	0.85	0.50	0.10	0.05
	Asp-COOH	0.10	−0.05	1.0	0.70
$Glu\text{-}COO^-$	$Glu\text{-}COO^-$			(0.0)	(0.0)
α-Amino	NH_3^+		(−1.65)		1.65
α-Carboxyl	COOH	0.05			
	COO^-			−1.35	

[a] The values listed indicate the effect of various side chains on the HX rate of neighboring left (λ) and right (ρ) peptides relative to that for alanine. Values given are in terms of Δ log (rate) and represent best estimates (±0.05) in light of available data. Parentheses indicate suggested values.

ment found seems rather remarkable. The previously measured data (Table IV) accurately predict a multiexponential kinetic curve over a multi-pH range through which rate–pH dependence is not even monotonic.

At pH 5 and 6, measured exchange is definitely slower than predicted, by up to almost threefold. Data in this region are expected to represent almost wholly the 34 primary amide protons. It thus seems possible that the quantitative results and conjectures for primary amides shown in Figure 5 are somewhat in error. Another possibility is that certain peptide groups and/or primary amides experience, in this pH region but not at lower pH, some tentative formation of structure having marginal stability.

The scaling of the ordinate in these figures depends on a factor in the data computation to account for the 21% equilibrium isotope effect found in T–H exchange of homopolypeptides. The good agreement between the true number of peptide H (119) and the number found confirms the accuracy of this isotope effect value.

Test of Results against Myoglobin. X-Ray studies have identified 27–32 free peptide H and 13 free primary amide H in myoglobin (Watson, 1969). We may expect these to exchange in the early part of measured myoglobin HX curves.

In previous T–H-exchange work, the faster exchanging free amide H of myoglobin were essentially isolated for study by labeling the protein in tritiated water only briefly, so that fast-exchanging groups were preferentially labeled and most of the slower (H-bonded) groups were not labeled at all (Englander and Staley, 1969). Figure 7 shows pH 4.7 exchange-out data for myoglobin initially labeled for 6 min at pH 5.50, 0°. This condition covers over 10 HX half-times for freely exposed peptides and over one half-time for free primary amides. A few very slow H, 5 in number, also became labeled, but their contribution to the early part of the exchange-out curve could be accurately obtained by back-extrapolation of their exchange, easily seen after the faster groups had already exchanged-out.

We can test the applicability of the results of the present work to the measured, free amide HX of myglobin by adding to the determined contribution of the slow H the simply predicted behavior of the free groups, just as for oxidized ribonuclease above. The two solid lines in Figure 7 show the predicted HX behavior for the cases of 27 and 32 free peptides (see Table V of Watson, 1969). Even with the experimental uncertainty in all three sets of data brought together here, the agreement found is very satisfactory. Thus, *free groups in proteins exchange at the same rates as do small molecule models* and random chain polypeptides.

Englander and Staley (1969) initially attempted to fit this data simply with the then known HX behavior of poly-D,L-alanine. This approximation is shown by the dashed line in Figure 7. These workers realized that an improved fit could be obtained by invoking the old smeared-charge model so that net positive charge on the protein could be pictured as generally speeding OH^--catalyzed exchange. However, more recent theoretical treatment (Tanford and Kirkwood, 1957) as well as penetratingly direct nmr data (Meadows *et al.*, 1968) convincingly argue against the reality of such a generalized charge effect. In agreement with this view, one implication of the good fit, that was obtained in Figure 7 without considering protein surface charges, is that their general, averaged out, net effect is very small.

Discussion

Effect of Side Chains and Other Groups on HX Rates. Quantitative results on the influence of side chains on the exchange rate of adjacent λ and ρ peptides are summarized in Table IV. Electron-withdrawing (inductive and positively charged) side chains generally increase the hydroxide ion catalyzed rate and

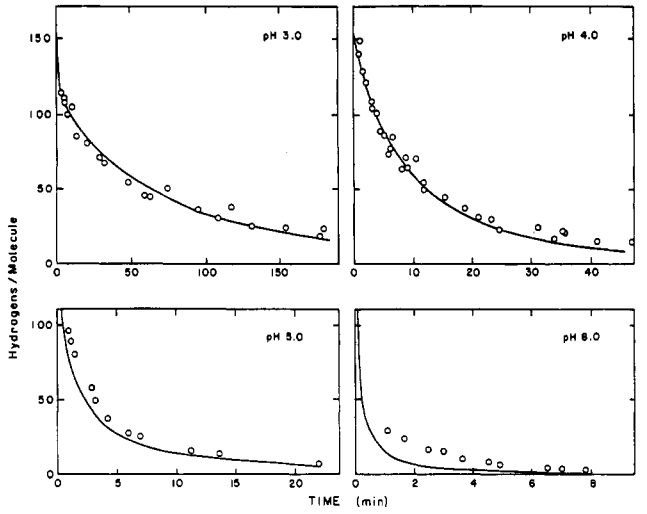

FIGURE 6: T–H-exchange data for oxidized ribonuclease at 0° and various pH. Solid lines represent exchange curves predicted for oxidized ribonuclease taking into account effect of neighboring side chains on peptide hydrogen-exchange rates as determined from model peptides in the present work.

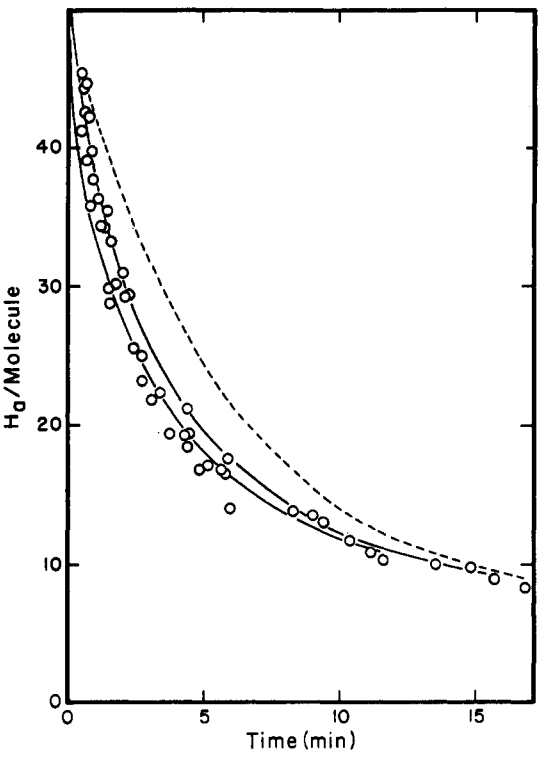

FIGURE 7: T–H-exchange data and predicted curves in the free amide region of myoglobin (pH 4.7, 0°). The data points were obtained in previous work by the exchange-in–exchange-out technique alluded to in the text. The solid lines represent predicted curves. These are based on the two extreme possibilities, 27 and 32 free peptides (together with the 13 free primary amides), found in the X-ray diffraction work, and exchange behavior for the free groups on the basis of results in the present work. The dashed line represents the prediction for the case of 32 free peptides all bounded by alanine residues. The ordinate is in terms of apparent hydrogens (H_a, uncorrected for the T–H equilibrium isotope effect) as in the original work (Englander and Staley, 1969).

decrease the hydronium ion catalyzed rate of peptides relative to the alanyl compound used as a reference. Adjacent peptide groups do the same. This effect is in agreement with previous studies (Leichtling and Klotz, 1966; Sheinblatt, 1970; Molday, 1971) and proposed mechanisms of specific base and specific acid catalysis (Berger *et al.*, 1959) as follows.

$$\begin{aligned} &\overset{O}{\overset{\|}{C}}NH + OH^- \underset{}{\overset{k_{OH}}{\rightleftharpoons}} \overset{O}{\overset{\|}{C}}N^- + H_2O \\ &\overset{O}{\overset{\|}{C}}NH + H_3O^+ \underset{}{\overset{k_H}{\rightleftharpoons}} \overset{O}{\overset{\|}{C}}N^+\!<\!\begin{matrix}H\\H\end{matrix} + H_2O \end{aligned} \qquad (3)$$

Thus, electron-withdrawing side chains favor formation of the amide anion in OH^--catalyzed exchange by increasing the acidity of the N–H, and impede the formation of the cationic amide intermediate in H_3O^+-catalyzed exchange. However, side chains do not in general increase k_{OH} by the same magnitude as k_H is decreased as was previously found for substituted amides in dioxane–D_2O solvent (Leichtling and Klotz, 1966). Thus, k_{min}, the hydrogen-exchange rate of peptides at the pH or pD minimum is not necessarily constant but varies with neighboring side chains.

Within the above generalizations, the detailed patterns observed are perplexing. Some side chains more potently affect the acid-catalyzed reaction (Lys^+, Asp^-), others the base reaction (His^+, Asn, Asp^0). The p peptide, more shielded from the side chain than is λ, often shows lesser sensitivity, but exceptions occur. Though these experiments were carried out at moderately high ionic strength (0.1 M KCl, 0.1–0.3 M peptide derivative), charge effects may account for some of these irregularities, but other factors also seem necessary for a complete explanation.

An estimate of the effect of side chains not studied can be made from similarity in nature and location of functional groups of particular side chains. Thus, the threonine side chain may be expected to alter HX rates of peptides to the same extent as serine since both side chains carry the functional hydroxyl group on the β carbon (Charton, 1964). Similarly, we place peptides containing only alkyl side chains in the same class as alanine. The aromatic phenylalanine side chain is grouped with tyrosine. These and other suggested groupings are given in Table IV.

The approximate effect of other groups of interest may often be inferred from these results. We briefly discuss α-NH_3^+ and α-COO^- groups as examples.

The effect of the positively charged, terminal α-amino group on the OH^--catalyzed hydrogen-exchange rate of the adjacent peptide can be estimated by comparing glycine-*N'*-methylamide ($^+H_3NCH_2C(=O)NHCH_3$; Molday and Kallen, 1972) and the p peptide of NA-Gly-N'MA. Data given in Table I show that the positively charged amine increases the base-catalyzed rate by 28-fold over that expected for an adjacent peptide group. If now the chain is further extended by adding a peptide group to the right, the peptide closest to the N terminus will exchange even faster, by another factor of 17 (NA-Gly-N'MA(λ)/NMA = $4.24 \times 10^9/2.51 \times 10^8 = 17$). These two effects together yield an estimate for the rate constant for $H_3N^+CH_2CONHCH_2CONH-$ of 4.98×10^{11} M^{-1} min^{-1} or 8.3×10^9 M^{-1} sec^{-1}. Sheinblatt (1970) measured for the *N*-methylene peptide of glycylglycinamide ($H_3N^+CH_2CONHCH_2CONH_2$) the rate constant 1×10^{10} M^{-1} sec^{-1}. Thus, the base-catalyzed exchange of peptide groups adjacent to N termini approach the diffusion-limited rate, suggested to be 2×10^{10} M^{-1} sec^{-1} by the results of Molday and Kallen (1972).

The effect of a terminal α-carboxyl group on the adjacent peptide relative to the effect of a neighboring peptide group can be estimated by comparison of the hydrogen-exchange rates of *N*-methylene peptide of *N*-acetylglycine and NA-Gly-N'MA (Table I). The negatively charged carboxylate group is expected to decrease the base-catalyzed peptide hydrogen-exchange rate constant for the adjacent (λ) peptide by about 20-fold (Table IV).

On the Simplicity of Primary Structure Effects on Peptide Hydrogen Exchange. The results obtained in this study show that amide hydrogen-exchange behavior adheres to some simple rules with respect to substituent effects. These are that only nearest-neighbor substituents play a significant role in determining amide-exchange rates, and that the net influence of amide adjacent substituents is closely the sum of the individually measurable effect of each.

A negative rule concerning the absence of new effects arising at the macromolecular level was also demonstrated. The evidence seems quite strongly against the concept that peptide group hydrogen exchange is likely to be greatly slowed by some generalized effect on water chemistry near polypeptides and proteins.

Another important simplifying principle confirmed by the present results is that the amino acid side chains do not act as general acid–base catalysts that contribute significantly to HX rates of amides in aqueous solution. This argument is even more conclusive when pH values away from the minimum rate are considered (see also Englander and Poulsen, 1969).

An exception to the nearest-neighbor rule may arise at the tertiary structure level if a charged group is held close to a peptide group during exchange. At the primary structure level, exceptions to the rule could occur for substituents having very strong effect. For example, when the exercise diagrammed in Figure 2 for prediction of base-catalyzed poly-D,L-alanine rate is carried through for its acid limb, the prediction falls short by just over a factor of twofold, suggesting that here a second nearest-neighbor peptide group may still contribute significantly. (This particular eventuality poses no problem for HX analysis of proteins since we can overstep it simply by accepting a polypeptide such as poly-D,L-alanine as defining our reference state. This was done for the oxidized ribonuclease and myoglobin exchange-out curve predictions.)

Implications for Macromolecular Studies. The value of the hydrogen-exchange method for study of macromolecular structure has been under challenge through the past decade. Indeed, initial doubts proved to be well founded, since all the early hydrogen-exchange data suffered from artifacts based in deficiencies in the techniques originally used. Also incorrect was the early association of slowly exchanging hydrogens with protein α helix. In time, problems with the technology of hydrogen-exchange measurement were remedied (see Englander, 1967), and data interpretation was modified, at least tentatively, to equate slow hydrogens with internally H-bonded amides (Englander and Staley, 1969).

At this time, standing challenges to the hydrogen-exchange analysis envision protein hydrogen exchange itself as being inherently capricious, unpredictable and uninterpretable, insofar as general catalysis by unfortunately placed side chains might greatly speed exchange of some protons, while on the other hand, alterations in local water chemistry owing to protein apolar groups might greatly slow others. The present work appears to rule out these phenomena as significant determinants for exchanging surface groups by demonstrating that HX behavior in macromolecules can be quantitatively equated with small molecule behavior. Thus, it may be stated as a major general deduction from this work that aqueous chemistry in the vicinity of polypeptides and at the surface of globular proteins appears very much the same as that experienced by small molecule models.

A final, fundamental uncertainty still exists concerning the mode(s) of exchange of hydrogens stably H bonded within a protein. If it is true that the operative HX mechanism for these involves a gross derangement of local structure that breaks the H bonds and brings these H fully into contact with water (Berger and Linderstrøm-Lang, 1957), the above conclusions must hold for them also. If, however, a more exotic physical situation obtains, then their HX chemistry might be more complex.

References

Berger, A., and Linderstrøm-Lang, K. (1957), *Arch. Biochem. Biophys. 69*, 106.

Berger, A., Lowenstein, A., and Meiboom, S. (1959), *J. Amer. Chem. Soc. 81*, 62.

Bovey, F. A., and Tiers, G. V. D. (1963), *J. Polym. Sci. Part A 1*, 849.

Charton, M. (1964), *J. Org. Chem. 29*, 1222.

Englander, S. W. (1963), *Biochemistry 2*, 798.

Englander, S. W. (1967), in Poly-α-Amino Acids, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 339.

Englander, S. W., and Crowe, D. (1965), *Anal. Biochem. 12*, 579.

Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7*, 379.

Englander, S. W., and Staley, R. (1969), *J. Mol. Biol. 45*, 277.

Hirs, C. H. (1956), *J. Biol. Chem. 219*, 611.

Ikegami, A., and Kono, N. (1967), *J. Mol. Biol. 29*, 251.

Jonas, J., Allerhand, A., and Gutowski, H. S. (1965), *J. Chem. Phys. 42*, 3396.

Klotz, I. M., and Feidelseit, P. L. (1966), *J. Amer. Chem. Soc. 88*, 5103.

Klotz, I. M., and Frank, B. H. (1965), *J. Amer. Chem. Soc. 87*, 2721.

Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.

Meadows, D., Jardetzky, O., Epanel, R., Rutejans, N., and Scheraga, H. A. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 766.

Molday, R. S. (1971), Ph.D. Dissertation, University of Pennsylvania.

Molday, R. S., and Kallen, R. G. (1972), *J. Amer. Chem. Soc.* (in press).

Nozaki, Y., and Tanford, C. (1967), *J. Biol. Chem. 242*, 4731.

Scarpa, J. S., Mueller, D. D., and Klotz, I. M. (1967), *J. Amer. Chem. Soc. 89*, 6024.

Sheinblatt, M. (1965), *J. Amer. Chem. Soc. 87*, 572.

Sheinblatt, M. (1966), *J. Amer. Chem. Soc. 88*, 2123.

Sheinblatt, M. (1970), *J. Amer. Chem. Soc. 92*, 2505.

Taft, R. W. (1956), *in* Steric Effects in Organic Chemistry, Newman, M. S., Ed., New York, N. Y., Wiley, p 559.

Tanford, C., and Kirkwood, J. G. (1957), *J. Amer. Chem. Soc. 79*, 5333.

Watson, H. C. (1969), *Progr. Stereochem. 4*, 299.

Woodward, C. K., and Rosenberg, A. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 1067.