References

Björk, I. (1963), *Exptl. Eye Res. 2*, 339.
Björk, I. (1964), *Exptl. Eye Res. 3*, 1.
Bloemendal, H., Bont, W. S., Jongkind, J. F., and Wisse, J. H. (1962), *Exptl. Eye Res. 1*, 300.
Bloemendal, H., Bont, W. S., Jongkind, J. F., and Wisse, J. H. (1964), *Biochim. Biophys. Acta 82*, 191.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
François, J., Rabaey, M., and Wieme, R. J. (1955), *Arch. Ophthalmol. 53*, 481.
Manski, W. J., Halbert, S. P., and Auerbach, T. P. (1961), *Arch. Biochem. Biophys. 92*, 512.
Ouchterlony, Ö. (1948), *Acta Pathol. Microbiol. Scand. 25*, 186.
Palmer, W. G. (1967), Ph.D. Thesis, University of Connecticut, Storrs, Conn.
Palmer, W. G., and Papaconstantinou, J. (1967), 154th National Meeting of the American Chemical Society, Chicago, Ill, Sept 11–15.
Papaconstantinou, J., Resnik, R. A., and Saito, E. (1962), *Biochim. Biophys. Acta 60*, 205.
Resnik, R. A. (1957), *Am. J. Ophthalmol. 44*, 357.
Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A. (1956), *J. Am. Chem. Soc. 78*, 756.
Spector, A. (1965), *Invest. Ophthalmol. 4*, 579.
Spector, A., and Katz, E. (1965), *J. Biol. Chem. 240*, 1979.
Svedberg, T., and Pederson, K. D. (1940), The Ultracentrifuge, Oxford, Clarendon Press.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.

Enzymic Hydrolysis of Synthetic Polypeptides under High Helical Content*

Wilmer G. Miller† and James Monroe

ABSTRACT: The effect of ethanol on the hydrolysis of polyglutamic acid and polylysine by papain was investigated. Under conditions favoring the helical conformation in aqueous solutions addition of ethanol to a concentration of 20 vol % lowered the rate by a factor of 10^2; in 40% ethanol the rate is lowered by a factor of at least 10^4. In the hydrolysis of polyglutamic acid by ficin and subtilisin, lowering the ionic strength shifts the alkaline limb of the rate–pH curve toward higher pH, while not affecting its slope. On the low pH (helix favoring) side of the rate–pH curve an appreciable rate of hydrolysis is observed at all ionic strengths. The rate data are considered in terms of the previously proposed model (Miller, W. G. (1964a), *J. Am. Chem. Soc. 86*, 3913) involving conformation and charge-state restrictions.

All data are consistent with the proposal that the helix is not amenable to enzymic hydrolysis. The high rate at low pH in aqueous solutions of polyglutamic acid is shown to be a result of residual random coil residues which are always present. The model assuming hydrolysis occurs only in random coil peptide bonds with adjacent side chains uncharged is found to be in qualitative agreement with all the experimental data, and in semiquantitative agreement with the ionic strength effects.

We have previously investigated the enzymic hydrolysis of high molecular weight PGA[1] (Miller, 1961, 1964a) and poly-α-L-lysine (Miller, 1964b) in 0.2 M NaCl by a variety of proteolytic enzymes. In most cases the rate of hydrolysis exhibited an unusually large pH dependence. The charge state and conformation of the substrate appeared to be of predominant importance. The pH dependence of the action of chymotrypsin, elastase, ficin, papain, and subtilisin on both polyglutamic acid and polylysine was accounted for in terms of a single mechanism. This mechanism assumed peptide bonds in helical regions of the polypeptide were not hydrolyzed, and that in random coil regions only those peptide bonds were hydrolyzed in which the adjacent amino acid side chains were uncharged. Several other model mechanisms were considered, but their predicted pH dependence was in poor agreement with the experimental data. Incorporation of some type of substrate charge dependence in the proposed mechanism had precedence in the work of Kimmel and Smith (1954), and was strongly supported by our data. The necessity for invoking a substrate conformational dependence was not as great, principally because low

* From the Department of Chemistry, University of Iowa, Iowa City, Iowa. *Received July 19, 1967.* This work was aided by a U. S. Public Health Service grant (GM08409).

† Present address: Department of Chemistry, University of Minnesota, Minneapolis, Minn.

[1] Abbreviation used: PGA, poly-α-L-glutamic acid.

solubility made it difficult to investigate hydrolysis under conditions of high helical content.

In 0.2 M NaCl there is only a narrow pH range between the region of association and insolubility of the helical polypeptide, and the onset of the helix–random coil transition. The enzymic hydrolysis of predominantly helical polypeptide could only be studied over a small pH range. As the ionic strength is lowered, polyglutamic acid becomes more soluble at low pH; in addition, the helix–random coil transition is shifted toward higher pH (Kono and Ikegami, 1966; Nagasawa and Holtzer, 1964; Iizuka and Yang, 1965). Thus as the ionic strength is lowered the enzymic hydrolysis of the helical polypeptide is more amenable to study.

An alternate approach is to study the hydrolysis in mixed solvents. Both polyglutamic acid and polylysine show appreciable solubility under helix-favoring conditions in alcohol–water solutions. Furthermore, there is considerable evidence that the helical content of these polypeptides increases as the alcohol content is increased (Cassim and Taylor, 1965; Hermans, 1966b).

Data on the rate of hydrolysis as a function of pH of polyglutamic acid and polylysine at several ionic strengths are reported in this communication. The rate of hydrolysis in 20 and 40 vol % ethanol is reported and shown to be markedly different from hydrolysis in aqueous solutions.

Different methods of predicting the rate data using the previously proposed theoretical model are also considered. We show that the ionic strength dependence of the enzymic degradation can be semiquantitatively accounted for within the framework of the previously proposed model. Of more importance is the finding that the rate of enzymic degradation may be nearly maximal at high helical content of the substrate, even if helical portions of the polypeptide do not act as substrates. The large reduction in rate upon adding alcohol is consistent with the proposed model but the observed effect is shown to be larger than can be sensibly accounted for without a more elaborate model.

Experimental Section

Poly-α-L-glutamic acid and poly-α-L-lysine hydrobromide were obtained from Pilot Chemicals, Inc. Polymers having weight-average molecular weights of 60,000–110,000 were used. Papain (papaya latex), twice crystallized, and subtilisin (*Bacillus subtilis*) were obtained from Worthington Biochemical Corp. All enzymes were used as received without further purification. Enzyme concentrations, $(e)_0$, were calculated on a molar basis with no correction for inactive material.

Enzyme stock solutions were prepared shortly before use. When ficin or papain was the catalyst, the reaction solution was 0.005 M in cysteine. All kinetic measurements were made at 25.0 ± 0.05° in Ostwald viscometers and rates calculated from the viscosity measurements as described previously (Miller, 1961, 1964a,b). Except for the very slow reactions in 40% ethanol, the reactions were routinely followed until at least an average of one bond per molecule was hydrolyzed.

In calculation of rates from the viscosity data, it is necessary to know the Mark–Houwink constant in the expression relating the intrinsic viscosity to the molecular weight. This constant was determined for polyglutamic acid at various pH and salt concentrations by studying the viscosity of samples of different molecular weights. Above pH 5.2, the Mark–Houwink constant is independent of pH (within ±1%) for a given ionic strength, varying from about 0.9 in 1.0 M NaCl to about 1.1 in 0.04 M NaCl. As the pH is lowered below 5.2, a rapid increase sets in at about the midpoint of the helix–random coil transition. The constant rises to about 1.4 before association and solubility problems occur. Similar determinations were not made for polylysine nor for either polymer in alcohol–water solutions. For alcohol–water solutions in pH regions where the polymers were predominantly random coil, a value of 1.0 was assumed. Inasmuch as the helical content of polyglutamic acid appears to increase at low pH as the alcohol content increases (Cassim and Taylor, 1965; Hermans, 1966b), it might be expected that the polymer would exhibit more nearly rodlike behavior. On this assumption the Mark–Houwink constant was taken as 1.5 at low pH in 20% ethanol, and as 1.7 at low pH in 40% ethanol. Errors in these assumptions are unlikely to introduce an error of more than a factor of two in the rate constants.

Theory

The Model. Peptide bonds in the α-helical conformation are not considered hydrolyzable. In the disordered or random coil portions of the macromolecular substrate only those peptide bonds whose adjacent side chains are uncharged are considered susceptible to hydrolysis. The rate of hydrolysis under conditions of substrate-saturated enzyme $[k_{cat}(e)_0]$ can be expressed mathematically as

$$k_{cat}(e)_0 = k f_{oo} f_{rc} f_{ec} (e)_0 \qquad (1)$$

where f_{rc} is the fraction of peptide bonds in disordered or random coil conformations, f_{oo} is the fraction of random coil peptide bonds which have adjacent side chains uncharged, f_{ec} is the fraction of the enzyme molecules in a catalytically active charge state, and k is a pH-independent constant characterizing the hydrolysis of a peptide bond in a random coil section of the polymer. Compared to f_{rc} and f_{oo}, f_{ec} is of minor importance over most of the pH range of interest.

This model assumes that the binding of the substrate to the enzyme is not significantly affected by the charge state or conformation of the substrate. Although we have had difficulties in determining precise values for the Michaelis constants, the justification for this assumption lies in the fact that we observe no drastic change in the Michaelis constant with pH for the enzyme–substrate systems used in this study (Miller, 1961, 1964a,b). The rate is consequently considered to be proportional to the fraction of bound peptide bonds in a catalytically hydrolyzable conformation and charge state of adjacent side chains.

The factors f_{oo} and f_{rc} are properties of the macromolecular substrate. They must be determinable either experimentally or theoretically in order to predict the pH dependence of k_{cat}. Three methods will be considered and compared. The first is to evaluate f_{rc} from either optical rotation or ultraviolet absorption data, and f_{oo} from the polymer titration curve. The second method differs in that f_{rc} is calculated using the experimentally determined thermodynamic parameters for the helix–random coil transition in a simple Ising model description of the transition. In the third method a statistical thermodynamic framework is set up to calculate the polymer conformation and charge. Electrostatic interaction parameters are adjusted until the theoretical titration curve agrees with the experimental one. The same statistical thermodynamic framework is then used to compute $f_{oo}f_{rc}$.

Previous Treatment of $f_{oo}f_{rc}f_{ec}$ (Miller, 1964a,b). The factor f_{rc} was estimated from optical rotation and ultraviolet absorpion data. The values of the optical parameters at the pH extremes were assumed to characterize the polymer at 0 and 100% helicity. A linear scale was assumed for helical content at intermediate values of the optical parameters.

The factor f_{oo} is a function of the fraction of the random coil side chains charged (α_c) and was calculated from the equation

$$f_{oo} = (1 - \alpha_c)[1 - 2\alpha_c/(1 + \beta)] \quad (2)$$

where β is a biasing parameter which takes into account nonrandomness in the charge distribution as a result of electrostatic interactions of charged side chains. Using a one-dimensional Ising model for an infinite chain with nearest neighbor interactions, β is given by

$$\beta = [1 - 4\alpha_c(1 - \alpha_c)(1 - e^{-w/kT})]^{1/2} \quad (3)$$

where w is the pairwise electrostatic interaction energy for nearest neighbor interaction. When $w = 0$ the charge distribution is random and f_{oo} becomes $(1 - \alpha_c)^2$. The value of w was treated as an adjustable parameter. It was further assumed that at any pH the degree of ionization of the side chains in helical and in random coil regions was the same. Consequently, α_c was determined from the titration curve of the polymer. This was known to produce some error under conditions of high helical content, as the titration curve of helical and of random coil residues is not the same.

The factor f_{ec} was taken as

$$f_{ec} = \frac{1}{1 + K_a/(H^+) + (H^+)/K_b} \quad (4)$$

where K_a and K_b are dissociation constants for ionizable groups on the enzyme.

Ising Model Treatment of Both f_{rc} and f_{oo}. The optical rotatory dispersion studies of Cassim and Taylor (1965) give good evidence that in water solutions polyglutamic acid and polylysine are never completely helical at any pH. Calculations based on the free energy (Nagasawa and Holtzer, 1964; Snipp *et al.*, 1965; Hermans, 1966a,b) of the helix–random coil transition utilizing Ising models (Zimm and Bragg, 1959; Zimm and Rice, 1960) are entirely consistent with this viewpoint. Thus arbitrarily scaling optical data from 0 to 100% helix will lead to serious underestimation of f_{rc} at high helical content. Inasmuch as the Zimm–Rice formulation for the helix–coil transition in ionizable polypeptides is an adequate framework to describe the physical properties of such polymers (Zimm and Rice, 1960; Snipp *et al.* 1965; Hermans, 1966b,c), it will be used to calculate f_{rc}. For the case of the infinite chain-length polymer the fraction of residues in random coil conformations is given by (Zimm and Rice, 1960)

$$f_{rc} = 0.5\{1 - (s' - 1)/[(1 - s')^2 + 4\sigma s']^{1/2}\} \quad (5)$$

The initiation parameter σ may be determined from the titration curve or from optical measurements (Zimm and Rice, 1960; Rifkind and Applequist, 1964; Snipp *et al.*, 1965) and is pH and ionic strength independent (Snipp *et al.*, 1965), at least for polyglutamic acid. The parameter s' is the equilibrium constant related to extending a helical sequence. It is a function of pH and may be determined from the titration curve of the polymer (Zimm and Rice, 1960; Snipp *et al.*, 1965). In the limiting pH corresponding to uncharged polymer it becomes the Zimm–Bragg parameter s. In the limit of uncharged polymer f_{oo} approaches unity and $f_{oo}f_{rc}$ is given by eq 5 with $s' = s$.

As in the previous treatment f_{oo} will be calculated by use of eq 2 and 3, using w as an adjustable parameter. However, instead of obtaining α_c from the titration curve of the polymer without regard for conformation, α_c will now be determined from the titration curve of the random coil residues. In the region of appreciable helical content it is obtained from the extrapolated titration curve for the random coil (Nagasawa and Holtzer, 1964; Hermans, 1966a).

Matrix Treatment of $f_{oo}f_{rc}$. Use of eq 2 is unsatisfactory in that the parameter w is arbitrarily adjusted to give the best fit of eq 1 to the experimental rate data. Furthermore, eq 5 is not easily modified to allow for the fact that bonds near the end of the polymer chain may not be as susceptible to hydrolysis as interior bonds (Miller, 1961) or to allow calculations for chains of finite length. A matrix formulation of $f_{rc}f_{oo}$ will alleviate these problems although creating new ones.

An $m \times m$ matrix $\mathfrak{V}_i$ is constructed. Its elements are statistical weights taking into account the conformation and charge of the ith residue and any interactions with its neighbors. The size of $\mathfrak{V}_i$ depends on the number of interactions considered. For a chain composed of n_p peptide bonds a semigrand partition function may be constructed

$$z = \mathbf{R}\mathfrak{V}_1\mathfrak{V}_2 \ldots \mathfrak{V}_{n_p}\mathbf{C} = \mathbf{R}\prod_{i=1}^{n_p}\mathfrak{V}_i\mathbf{C} \quad (6)$$

where $\mathbf{R}$ is a $1 \times m$ row matrix and $\mathbf{C}$ is a $m \times 1$ column

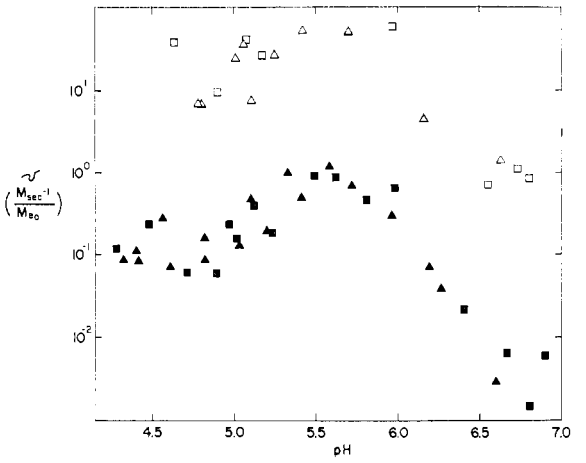

FIGURE 1: Rate as a function of pH for the hydrolysis of peptide bonds in polyglutamic acid in 0.005 M NaCl by subtilisin (filled symbols) and by ficin (open symbols). Substrate concentration (grams per liter): 6.4 (triangles) and 3.2 (squares). Rates for the ficin-catalyzed reactions have been multiplied by 40 in order to separate the two sets of rate data.

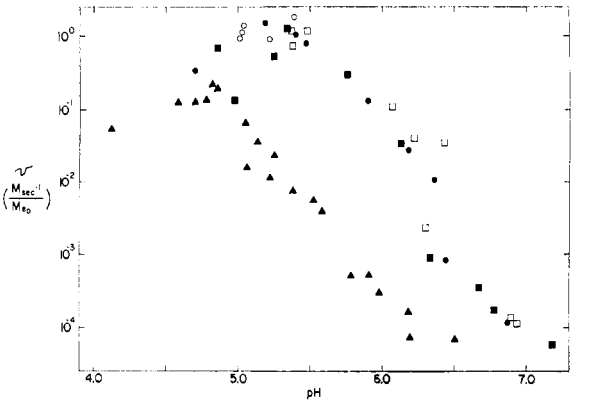

FIGURE 2: Rate of hydrolysis of peptide bonds for the subtilisin-catalyzed degradation of polyglutamic acid in 1.0 M NaCl, substrate concentration: 6.4 g/l. (▲); in 0.040 M NaCl, substrate concentrations (grams per liter) 1.8 (■), 4.5 (●), 6.4 (○), and 7.4 (□).

matrix. Average values ($\langle f \rangle$) for the fractions of polymer residues in various states are given (see Flory and Miller, 1966) by

$$\langle f \rangle = (1/n_p z)[\mathbf{R0}] \prod_{i=1}^{n_p} \begin{bmatrix} \mathfrak{V}_i & \mathfrak{V}_i' \\ \mathbf{0} & \mathfrak{V}_i \end{bmatrix} \begin{bmatrix} \mathbf{0} \\ \mathbf{C} \end{bmatrix} \tag{7}$$

where **0** are null matrices of sizes to conform. The submatrix $\mathfrak{V}_i'$ will depend upon the average of interest.

In the simplest case where the conformation and charge of the ith residue is considered influenced only by the charge and conformation of the $(i-1)$st residue, $\mathfrak{V}_i$ is given by

$$\mathfrak{V}_i = \begin{array}{c} \\ i-1 \\ \text{c o} \\ \text{c} \pm \\ \text{h o} \\ \text{h} \pm \end{array} \begin{array}{c} \begin{array}{cccc} i & & & \\ \text{o} & \pm & \text{o} & \pm \\ \text{c} & \text{c} & \text{h} & \text{h} \end{array} \\ \begin{bmatrix} 1 & \lambda_c & \sigma s & \sigma s \lambda_h \\ 1 & W_{cc}\lambda_c & \sigma s & \sigma s W_{ch}\lambda_h \\ 1 & \lambda_c & s & s\lambda_h \\ 1 & W_{hc}\lambda_c & s & s W_{hh}\lambda_h \end{bmatrix}_i \end{array} \tag{8}$$

where an uncharged random coil residue is arbitrarily taken as the reference state, and σ and s are the Zimm–Bragg parameters for the uncharged polymer. The W_{ab} are Boltzmann factors given by $W_{ab} = e^{-w_{ab}/kT}$ where the w_{ab} are electrostatic interaction parameters. The factor λ_c is given by $K_c/(H^+)$ for a glutamic acid side chain and by $(H^+)/K_c$ for a lysine side chain, where K_c is the intrinsic dissociation constant for the ionizable group in a residue in a disordered conformation. For a residue in the helical conformation λ_h has an analogous form. The matrix **R** is here given by [1000] and **C** by [1111]^T. The desired quantity $f_{oo}f_{rc}$ is then given by

$$f_{oo}f_{rc} = (1/n_p z)[10000000] \prod_{i=1}^{n_p} \begin{bmatrix} \mathfrak{V}_i\mathfrak{V}_i' \\ \mathbf{0}_4 \quad \mathfrak{V}_i \end{bmatrix} [00001111]^T \tag{9}$$

where $\mathbf{0}_4$ is a null matrix of order four. If all random coil peptide bonds with adjacent side chains uncharged are considered equally susceptible to hydrolyis

$$\mathfrak{V}_i' = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 \end{bmatrix}_i \tag{10}$$

If any bond in the chain is considered nonsusceptible to hydrolysis, $\mathfrak{V}'$ for that bond is a null matrix of order four. Inasmuch as we will assign the values of the electrostatic parameter w_{ab} by matching the theoretical with the experimental titration curve, the fraction of the side chains charged is given by an equation similar to eq 9 except that

$$\mathfrak{V}_i' = \begin{bmatrix} 0 & \lambda_c & 0 & \sigma s \lambda_h \\ 0 & W_{cc}\lambda_c & 0 & \sigma s W_{ch}\lambda_h \\ 0 & \lambda_c & 0 & s\lambda_h \\ 0 & W_{hc}\lambda_c & 0 & s W_{hh}\lambda_h \end{bmatrix}_i \tag{11}$$

As there are $(n_p + 1)$ ionizable groups, we have ignored the charge state of the first side chain in the molecule. Although the equations can be recast to take it into account, the error is completely negligible for a long-chain polymer. Equations for other averages, such as the average fraction of helical residues at any pH, may be formulated in a manner analogous to the ones already discussed.

Other $\mathfrak{V}_i$ matrices may be constructed to take into account more than nearest neighbor interactions. Two in particular will be useful. A 32 × 32 matrix will be

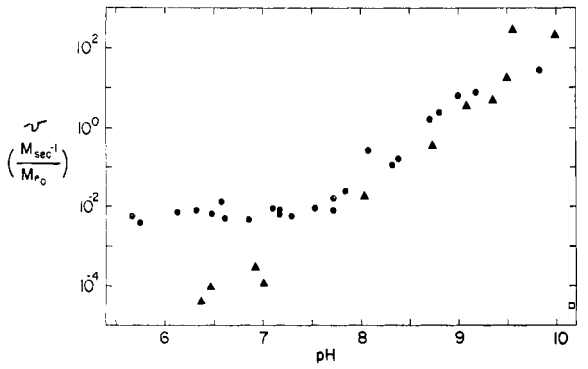

FIGURE 3: Rate as a function of pH for the hydrolysis of polylysine. Catalysis by subtilisin in 0.040 M NaCl (●) at a polylysine concentration of 6.4 g/l., and in 0.20 M NaCl (▲) (Miller, 1964b). Estimated maximum rate for hydrolysis by papain in 40% ethanol–0.1 M KCl (pH 10.2) is also given (□).

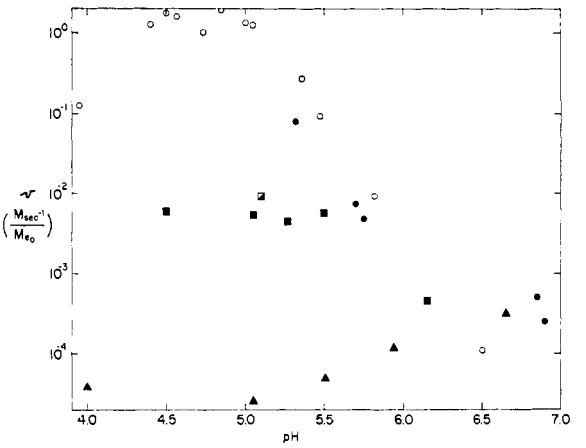

FIGURE 4: The rate of hydrolysis of peptide bonds in polyglutamic acid by papain in 40% ethanol–0.1 M KCl (triangles), 20% ethanol–0.1 M KCl (squares), and 0% ethanol–0.2 M NaCl (circles). Substrate concentration (grams per liter): 6.8 (▲), 7.3 (■), 3.6 (◣), and 6.7 (●). Open circles (○) are k_{cat} (Miller, 1961). Rates in 40% ethanol are maximum values.

constructed taking into account the conformation of the $(i - 1)$st residue and the charge state of the $(i - 1)$st through the $(i - 4)$th residue in the chain. This is the matrix used by Zimm and Rice (1960) in their original calculations. The matrices **R** and **C** are scaled to conform, and $\mathfrak{V}_i'$ is constructed in the same manner as in eq 10 and 11.

For reasons which will become apparent when discussing the mixed solvent results, it will also be of interest to construct a 16 × 16 $\mathfrak{V}_i$ matrix where the charge of the $(i - 1)$st residue is considered together with the conformation of the $(i - 1)$st through $(i - 3)$rd residue. Construction of all other matrices and formulation of averages follow from the previous examples. To include both a three-neighbor conformational dependence and a four-neighbor charge dependence leads to matrices too large to handle by computer.

Results

Experimental. The rate of hydrolysis of polyglutamic acid by subtilisin in 0.005 M NaCl is shown in Figure 1 and in 0.04 and 1.0 M NaCl in Figure 2. The hydrolysis of PGA by ficin in 0.005 M sodium chloride is also shown in Figure 1. Rates less than 10^{-3} M sec^{-1} $M_{e_0}^{-1}$ were determined under conditions such that the weight concentrations of enzyme and substrate were comparable. The substrate concentrations were varied by a factor of four in the 0.04 M NaCl data. In the concentration range studied the rate is, within experimental error, zero order with respect to substrate. The high pH limb of the rate–pH curve has essentially the same slope at all ionic strengths, but is displaced toward lower pH as the ionic strength is increased. At the lowest ionic strength studied rate data extend to approximately 1.5 pH units below the midpoint of the helix–coil transition. Upon reaching a maximum near the midpoint of the transition the rate drops as the pH is lowered and then appears to reach a limiting value.

The hydrolysis of polylysine in 0.04 M NaCl and 0.20 M NaCl (Miller, 1964b) is shown in Figure 3. In the region above pH 7.5 there appears to be only a small effect of ionic strength. At the low pH end the 0.04 M data appear to approach a limiting value in contrast to the 0.2 M data.

The effect of adding ethanol on the rate of hydrolysis of PGA by papain is shown in Figure 4. At low pH the rate is dramatically reduced with increasing concentration of ethanol. The rates are so low in 40% ethanol that they are barely perceptible. In a typical experiment in 40% ethanol an average of one-tenth of a bond per molecule was hydrolyzed in 3–5 hr. Only a single rate for the papain-catalyzed hydrolysis of polylysine in 40% ethanol was determined, as is shown in Figure 3.

Calculated Rate–pH Curves. All calculated curves are relevant to PGA and were made using the matrix formulation of $f_{oo}f_{rc}$, unless otherwise noted. The initiation parameter σ was taken as 0.003 (Snipp *et al.*, 1965). The Zimm–Bragg parameter s was determined at various ionic strengths from the data of Nagasawa and Holtzer (1964), and in 20 and 40% ethanol from the data of Hermans (1966b). The intrinsic dissociation constants K_c and K_h were obtained from the extrapolated titration curves. The electrostatic interaction factors were considered to be adjustable parameters. They were adjusted until the experimental and theoretical titration curves, plotted as pH − log $(\alpha/(1 - \alpha))$ *vs.* α, came within satisfactory agreement. In 0.2 M NaCl, for instance, with $w_{hh}/kT = 2.0$, $w_{hc}/kT = w_{ch}/kT = 0.8$, and $w_{cc}/kT = 0.7$ and using eq 7, 8, and 11, the calculated titration curve shown in Figure 5 was obtained. Except for w_{hc} the calculated titration curve is relatively sensitive to all of the parameters including s, σ, K_h, and K_c. When extending the electrostatic interactions to include up to fourth-neighbor dependence the electro-

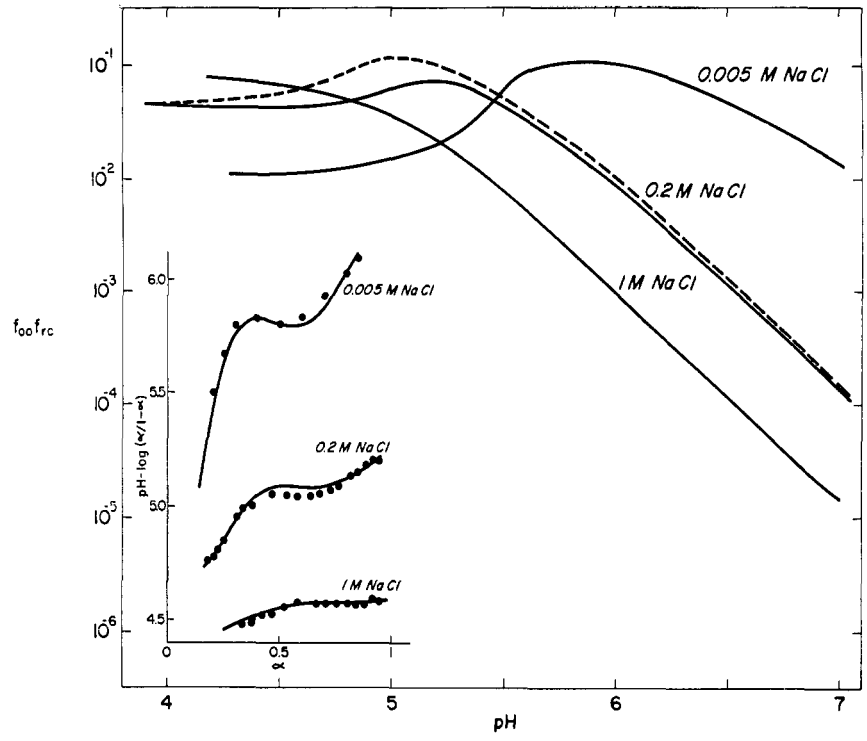

FIGURE 5: Predicted ionic strength dependence on the rate of hydrolysis of polyglutamic acid of $n_p = 512$. Theoretical (solid line) and experimental titration curves are shown in inset. Statistical parameters used in calculating the theoretical titration curves were used in calculating the corresponding $f_{oo}f_{rc}$ curves. In 0.2 M NaCl calculations based on first neighbor charge (solid line) is compared to four neighbor charge (dashed line) calculations.

static interactions in the helix were distributed according to Zimm and Rice (1960) and the coil interactions as a declining function of distance. Again the mixed helix–coil electrostatic interactions were not important. Once the theoretical and experimental titration curves were in reasonable agreement, the experimental and theoretical helix–coil transition curves were in good agreement. Equation 9, or its appropriate analog, was then used to calculate $f_{oo}f_{rc}$.

The calculated curves for three ionic strengths are shown in Figure 5. In 1.0 M NaCl calculations based on first neighbor and first-through-fourth neighbor charge interactions give essentially identical results. In 0.2 M NaCl there is a difference in the calculated curves in the pH region of the helix–coil transition, which becomes more pronounced as lower ionic strengths are considered. The calculated curve for 0.005 M NaCl in Figure 5 is based on first-through-fourth neighbor charge interactions and first neighbor conformation.

The titration curves in 20 and 40% ethanol (Hermans, 1966b) indicate s increases as the alcohol content increases. However, in plots of pH $-$ log $(\alpha/(1-\alpha))$ *vs.* α, the slopes of the curves compared in the helical regions and in the random coil regions are effectively parallel. Thus, the electrostatic interactions are not much changed in up to 40% alcohol. The electrostatic parameters were adjusted to fit the 0.1 M KCl, 0% ethanol titration curve, and assumed to be appropriate in making calculations for 20 and 40% ethanol. Although the value of σ is unknown in ethanol–water solutions, assignment of 0.003 gave a reasonable fit of the theoretical titration curve to the experimental results. These statistical weights were then used in calculating $f_{oo}f_{rc}$, as shown in Figure 6, with the added restrictions given below. Inasmuch as the fraction helix approaches unity at low pH in 40% ethanol, $f_{oo}f_{rc}$ becomes very sensitive to the model used in the calculation. In order to make the model conform as much as possible to the α helix, a 16 × 16 $\mathbf{U}_i$ matrix considering first-through-third neighbor conformations and first neighbor charge interactions was employed. Conformational sequences ...hch... and ...hcch... were suppressed. In addition, $\mathbf{U}_1'$, $\mathbf{U}_2'$, $\mathbf{U}_{n_p-1}'$, and $\mathbf{U}_{n_p}'$ were taken as null matrices, thus suppressing hydrolysis of the two bonds at either end of the chain (Miller, 1961).

Discussion

In 1.0 M NaCl the subtilisin-catalyzed rate of hydrolysis of PGA monotonically increases as the pH is lowered, while at lower ionic strengths the rate appears to go through a maximum and then level off. At any ionic strength the helical content of PGA increases as the pH is lowered. From these data one could easily conclude that the helical portions of the polymer are susceptible to hydrolysis. However, by the proposed model as expressed in eq 1 the rate of reaction should be proportional to $f_{oo}f_{rc}$, if all other factors are held constant. Comparing the calculated values of $f_{oo}f_{rc}$, as in Figure 5, with the experimental rates, as in Figures 1

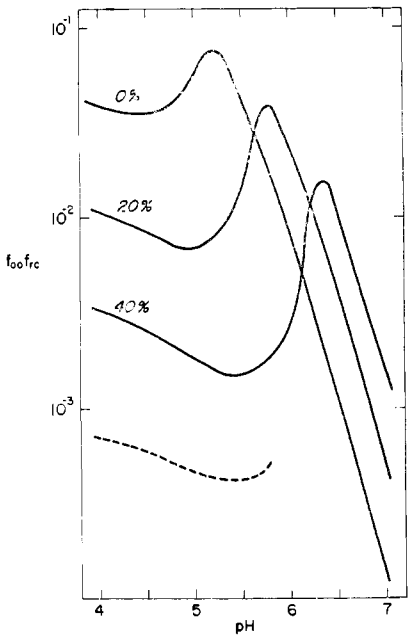

FIGURE 6: Predicted pH dependence of the rate of hydrolysis of polyglutamic acid in 0.2 M NaCl–0% ethanol, and in 0.1 M KCl–20 and 40% ethanol, using eq 7. Dashed curve: effect of changing the initiation parameter from 0.003 to 0.00001 in 40% ethanol.

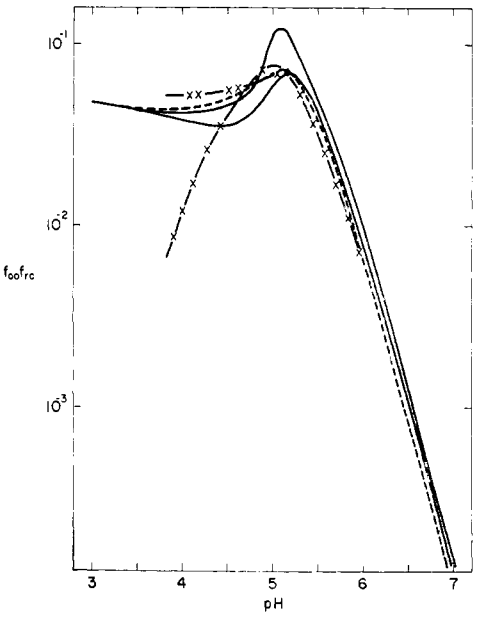

FIGURE 7: Comparison of various methods for calculating $f_{oo}f_{rc}$ for polyglutamic acid in 0.2 M NaCl. (—×—) Based on eq 2 and 3 for f_{oo} and optical data for f_{rc}, assuming maximum helical content equals 100%. (—××—) Based on eq 2 and 3 for f_{oo} and optical data for f_{rc}, assuming maximum helical content equals 93%. (- - -) Based on eq 2, 3, and 5. (—) Lower: based on eq 8, 9, and 10. (—) Upper: based on eq 7 with electrostatic interactions extended to fourth neighbor.

and 2, the similarity of the effect of ionic strength tends to substantiate the proportionality between the rate and $f_{oo}f_{rc}$. Acceptance of this proportionality is concomitant to concluding that helical portions of the polymer are not susceptible to hydrolysis. This seeming paradox is easily resolved when one considers the helical content of the polymers. The maximum helicity occurs when the charge on the polymer is reduced to zero and theoretically depends on the values of s and σ. Using the experimental values of s and σ and either eq 5 or 7, the *minimum* amount of random coil residues is calculated to be about 14% in 1.0 M NaCl, 7% in 0.2 M NaCl, and 2% in 0.005 M NaCl. It is this small amount of uncharged random coil residues always present in PGA which leads to the relatively large rate of hydrolysis at low pH, and not enzymic attack on the helical residues.

Recent experimental evidence substantiates the lack of complete helicity of PGA at low pH. Cassim and Taylor (1965) found the Moffitt b_0 parameter went to a more negative value as a variety of organic solvents were added to the polymer–H_2O system, thus strongly indicating an increase in helical content. The major increase appears to occur in going to about a 50:50 aqueous–organic solvent system with smaller changes occurring in going to more organic-rich systems, suggesting perhaps that essentially complete helicity was obtained.

If indeed it is the small amount of uncharged random coil present in water which accounts for the rate of low pH, hydrolysis in alcohol–water mixtures should lower the rate at low pH. Experimentally the reduction in rate was vividly observed, being more than four orders of magnitude in going to 40% ethanol. The calculated reduction in rate as seen in Figure 6, though having the correct trend, was not as large as the effect observed experimentally. Although σ is unknown in ethanol–water solutions and was arbitrarily taken to be the same as in water solutions, lowering it to 10^{-5} (dashed line, Figure 6) did not give a large enough effect.

In the mixed-solvent studies other factors might also be important, such as the effect of dielectric constant, effect of organic solvent on protein conformation, and the availability of water at the peptide bond. In the hydrolysis of benzoylargininamide by papain Stockell and Smith (1957) found the catalytic constants did not change more than a factor of three in going to 50% methanol or to 30% isopropyl alcohol. In a study of the hydrolysis of methyl hippurate by papain in the presence of alcohols Lake and Lowe (1966) found a linear relationship between the reciprocal of the catalytic constant and the molar ratio of alcohol to water. Extrapolation of their results to 20% (3.47 M) and to 40% (7.1 M) ethanol gives a reduction by a factor of two and three, respectively, in the rate constant. The correlation of Lake and Lowe takes into account the change in water concentration. However, in a mixed-solvent system the concentration of water around the polymer may differ from the bulk composition. From data presently available the titration curves give the only information about solvent composition near the polymer. Inasmuch

as the titration curves in 20 and 40% ethanol indicate little change in the electrostatic interaction between side-chain carboxylates upon addition of ethanol, the effective dielectric constant in the vicinity of the polymer must not be significantly different from that of the pure aqueous solvent. In a more recent study Sluyterman (1967) finds substantial reduction in the rate of hydrolysis of benzoylglycine ethyl ester and of benzoylarginine ethyl ester by papain upon addition of methanol. The applicability of these results to the hydrolysis of the peptide bond is unknown. If one does apply them, the correction would be at most a factor of ten. Thus the experimental effect is still considerably greater than the calculated effect.

Recently it has been proposed (Schechter and Berger, 1967; Brubacher and Bender, 1967) that proteolytic enzymes may be similar to lysozyme in that they may contain a groove into which the substrate must fit. This hypothesis might account for the inability of the enzymes studied here to hydrolyze the helix, and also explain the inability of our proposed model to take better account of the rate in alcohol–water solutions. Using the experimental free energy of Hermans (1966b) for PGA in 40% ethanol, σ equal to 0.003, and eq 7, the average length of a random coil sequence at low pH is calculated to be only 2.5 residues/molecule for a chain of 512 peptide bonds. Such a short sequence of random coil residues may not fit properly for hydrolysis to occur, and a more elaborate theoretical model would be required.

Returning to the dependence of the rate on ionic strength, the calculated effect is seen to be in only semiquantitative agreement with the experimental data. As with the mixed solvent results, several correction factors can be considered. The factor f_{ec} has been ignored in the calculations and is a function of the pH, the pK's of ionizable groups in the enzyme, and the ionic strength. Studies on small substrates (*e.g.*, Stockell and Smith, 1957) show only a small ionic strength dependence. Considering all of these factors, f_{ec} should vary by no more than a factor of two or three in the pH range investigated. Corrections of this magnitude would bring the calculated and experimental results at low pH into nearly quantitative agreement.

The hydrolysis of diastereoisomers of small peptides studied by Schechter and Berger (1967) is pertinent to our work. They find that the active site of papain recognizes up to seven amino acid residues. Our working model assumes both charge and conformational restrictions, but we have already seen that under conditions of very high helical content the model is not adequate. Turning to conditions where the polymer is predominantly random coil the rate is controlled by the charge state of the side chains. Although our experimental rates of less than 10^{-3} sec^{-1} were obtained at such high enzyme to substrate ratios that they may be questioned, a careful inspection indicates the experimental rate has a greater pH dependence than the calculated one. Introduction of further charge restrictions such as requiring a triplet of random coil residues to be uncharged will give a much larger calculated pH dependence. This restriction predicts too large a pH dependence but, considering the imprecision of our data, cannot be completely ruled out. It is also conceivable that charge restrictions two or more units removed from the peptide bond to be hydrolyzed may not be an all or none restriction. Detailed analysis of lysine–glutamate copolymer hydrolysis may be a better means of deciding the extent of the charge dependence.

We have made little comment on the ionic strength dependence of the hydrolysis of polylysine. The effect of ionic strength on the physical properties of polylysine has been studied less thoroughly than the polyglutamate system. Applequist and Doty (1962) found the helix–coil transition shifted toward higher pH as the ionic strength is lowered, whereas the titration curve shifted in the opposite direction. Only a small shift in the rate–pH curve was observed in going from 0.2 to 0.04 M NaCl. The opposing shifts observed by Applequist and Doty would tend to make $f_{oo}f_{rc}$ rather insensitive to ionic strength, in line with the observed effect on the reaction rate.

A comparison of different methods of calculating $f_{oo}f_{rc}$, applied to PGA in 0.2 M NaCl, is shown in Figure 7. Parameters were assigned such that the total electrostatic interaction in the fully charged random coil was the same in each case. When optical data are used to determine f_{rc} and the maximum helical content is assigned in agreement with Ising model calculations, the three methods are seen to be in rather good agreement. Electrostatic interactions are long range. However, at moderate and higher ionic strengths truncation of these interactions into a first neighbor dependence works surprisingly well, as was noted by Zimm and Rice (1960). Inasmuch as the matrix method will have to be used in making detailed calculations for hydrolysis of copolymers, it was important to compare calculations based on this method with those based on other methods.

Acknowledgment

We wish to thank Dr. C. Goebel for the use of a fourth neighbor charge computer program.

References

Applequist, J., and Doty, P. (1962), *in* Polyamino Acids, Polypeptides and Proteins, Stahmann, M., Ed., Madison, Wis., University of Wisconsin Press, p 161 ff.

Brubacher, L., and Bender, M. (1967), *Biochem. Biophys. Res. Commun. 27*, 176.

Cassim, J. Y., and Taylor, E. W. (1965), *Biophys. J. 5*, 573.

Flory, P. J., and Miller, W. G. (1966), *J. Mol. Biol. 15*, 284.

Hermans, Jr., J. (1966a), *J. Phys. Chem. 70*, 510.

Hermans, Jr., J. (1966b), *J. Am. Chem. Soc. 88*, 2418.

Hermans, Jr., J. (1966c), *J. Am. Chem. Soc. 88*, 5719.

Iizuka, E., and Yang, J. T. (1965), *Biochemistry 4*, 1249.

Kimmel, J. R., and Smith, E. L. (1954), *J. Biol. Chem. 207*, 515.

Kono, N., and Ikegami, A. (1966), *Biopolymers 4*, 823.
Lake, A. W., and Lowe, G. (1966), *Biochem. J. 101*, 402.
Miller, W. G. (1961), *J. Am. Chem. Soc. 83*, 259.
Miller, W. G. (1964a), *J. Am. Chem. Soc. 86*, 3913.
Miller, W. G. (1964b), *J. Am. Chem. Soc. 86*, 3918.
Miller, W. G., and Nylund, R. E. (1965), *J. Am. Chem. Soc. 87*, 3542.
Nagasawa, M., and Holtzer, A. (1964), *J. Am. Chem. Soc. 86*, 538.
Rifkind, J., and Appelquist, J. (1964), *J. Am. Chem. Soc. 86*, 4207.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27*, 157.
Sluyterman, L. A. (1967), *Biochim. Biophys. Acta 139*, 418.
Snipp, R. L., Miller, W. G., and Nylund, R. E. (1965), *J. Am. Chem. Soc. 87*, 3547.
Stockell, A., and Smith, E. L. (1957), *J. Biol. Chem. 227*, 1.
Zimm, B. H., and Bragg, J. K. (1959), *J. Chem. Phys. 31*, 526.
Zimm, B. H., and Rice, S. A. (1960), *Mol. Phys. 3*, 391.

On the Determination of Molecular Weight of Proteins and Protein Subunits in the Presence of 6 M Guanidine Hydrochloride*

A. Ullmann, M. E. Goldberg, D. Perrin, and J. Monod

ABSTRACT: For six proteins of well-established molecular structure, the apparent molecular weights have been measured in 6 M guanidine hydrochloride. It is shown that this apparent value is proportional to the true molecular weight of the protein subunits. The proportionality constant can be computed and may be used for the determination of molecular weights of polypeptide chains in 6 M guanidine hydrochloride.

Guanidine hydrochloride is now widely used as a denaturing and solubilizing agent for proteins, because of its property of abolishing noncovalent inter- and intramolecular interactions of polypeptide chains. Therefore it should be a valuable tool for determining the molecular weight of the subunits (protomers) of oligomeric proteins. However because of uncertainties regarding the partial specific volume and the degrees of hydration and solvation of proteins in the presence of high concentrations of guanidine hydrochloride, centrifugation experiments have not so far allowed molecular weight determinations in the presence of guanidine hydrochloride. However, to the extent that in the presence of guanidine hydrochloride the secondary, tertiary, and quaternary structures of proteins are completely abolished, one would presume that the relevant physical properties mentioned above should depend only on the amino acid composition of the polypeptide chain, regardless of its actual sequence. Then, for most natural polypeptides, the amino acid composition of which do not differ widely, the partial specific volume, degrees of hydration and solvation in guanidine should be practically identical. Therefore and to the extent that this assumption is correct, the apparent molecular weight of a polypeptide chain in guanidine hydrochloride should be proportional to its molecular weight.

In order to test this assumption, six proteins, whose molecular weights and subunit structures seemed well established, have been studied. Their apparent molecular weights have been determined, using the Archibald method, in the presence of 6 M guanidine hydrochloride. The experiments which we report in the present paper appear to justify the above assumptions allowing a direct determination of the molecular weight of proteins in 6 M guanidine hydrochloride solutions.

Materials and Methods

Protein Sources. All proteins used during this work were highly purified or crystalline preparations. Lysozyme, rabbit muscle lactic dehydrogenase, *Escherichia coli* alkaline phosphatase, beef liver glutamic dehydrogenase, and bovine serum albumin were commercial preparations. Rabbit muscle phosphorylase *b* was prepared according to Fischer and Krebs (1958) and the twice-recrystallized preparation was freed from 5′-AMP[1] by passing it through a Norit A column. β-Galactosidase from *E. coli* was prepared according to Perrin (1965) and was a twice-crystallized preparation.

Guanidine Treatment. The guanidine hydrochloride

* From the Service de Biochimie Cellulaire, Institut Pasteur Paris, France. *Received July 14, 1967.* This work was been aided by grants from the National Institutes of Health, the "Délégation Générale à la Recherche Scientifique et Technique," Euratom, and the "Centre National de la Recherche Scientifique."

[1] Abbreviation used: 5′-AMP, adenosine 5′-monophosphate.