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Degradation of Synthetic Polypeptides. II. Degradation of Poly-α,L-glutamic Acid by Proteolytic Enzymes in 0.20 *M* Sodium Chloride[1]

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The initial action of carboxypeptidase A and B, α-, β-, γ-, and Δ-chymotrypsin, elastase, ficin, leucine aminopeptidase, papain, pepsin, subtilisin, and trypsin on high molecular weight poly-α,L-glutamic acid in 0.20 *M* NaCl at 25° has been investigated. The endopeptidases chymotrypsin, elastase, ficin, papain, and subtilisin exhibit the same pH dependence of the rate of hydrolysis. Using one-dimensional Ising models with nearest-neighbor interactions, various models to explain the large pH dependence were considered. One model, that of enzymic attack of peptide bonds with adjacent side chains uncharged in random coil regions of the polymer, stood out over all others in predicting the pH behavior observed experimentally. Assuming this mechanism to be the correct one, the first-order rate constants for hydrolyzing a glutamyl–glutamyl bond with adjacent side chains uncharged in a random coil section of polyglutamate were 0.01, 3.0, 250, 40, and 40 sec.$^{-1}$, respectively. The pH dependence of the pepsin-hydrolyzed reaction was different from the other endopeptidases. Of the exopeptidases only carboxypeptidase B showed catalytic activity.

Introduction

The enzymic hydrolysis of high molecular weight poly-α,L-glutamic acid (PGA) has been the subject of several studies in the past few years.[2-6] Most previous work was concerned with the end products of exhaustive hydrolysis and whether or not a particular proteolytic enzyme would hydrolyze PGA. Except for hydrolysis by papain,[4] very little detailed work has been published on the initial rate of hydrolysis of long chain molecules and the pH and conformation effects on the initial rate.

This paper reports a systematic study of proteolytic enzyme action on PGA over a reasonably wide range of pH in an attempt to establish enzyme specificity, initial rates of hydrolysis, and mechanism of action. The enzymes used were those which could be obtained commercially in a high state of purity. All four available forms of chymotrypsin were investigated to see if the rate was responsive to modifications of the enzyme.

High molecular weight PGA undergoes a helix to random coil transition in 0.2 *M* NaCl around pH 5,[7] with most of the transition occurring between 4.5 and 5.5. Below 4.5 the polymer is only slightly soluble. At pH 7 over 99% of the side-chain carboxyls are ionized and there is little change in physical parameters with increasing pH. Consequently, the pH range of easy accessibility and most interest is 4–7.

Experimental

Poly-α,L-glutamic acid was purchased from Pilot Chemicals, Inc. Its synthesis and the method of determining the molecular weight was the same as that used in the PGA–papain study.[4] Polymer having a weight average molecular weight of 92,000 (as sodium salt) was used for most of the investigation.

The enzymes were obtained from Worthington Biochemical Corp. unless otherwise noted. The source and manufacturer's grade were as follows: trypsin (bovine pancreas), crystallized three times and lyophilized; α-chymotrypsin (bovine pancreas), crystallized three times; β-chymotrypsin, crystallized three times (Nutritional Biochemical Corp.); γ-chymotrypsin, crystallized two times and lyophilized; Δ-chymotrypsin, lyophilized; elastase (pancreatic), crystallized two times; ficin (fig latex), crystallized two times; papain (papaya latex), crystallized two times; subtilisin (*B. subtilis*), crystalline; pepsin (swine stomach mucosa), crystallized two times; leucine aminopeptidase (hog kidney), lyophilized; carboxypeptidase A, carboxypeptidase A-DFP(bovine pancrease), crystallized; carboxypeptidase B (hog pancrease), frozen solution from column elution. All enzymes were used as received without further purification.

Enzyme solutions were prepared immediately before use (generally less than 30 min.). Enzyme concentration was determined by dry weight. When an enzyme was received as a suspension, dry weight was checked against the manufacturer's stated concentration. Enzyme concentration ranged from 1.0×10^{-10} to 4×10^{-4} mole/l., depending on the catalytic ability under the conditions being investigated. Molar concentrations were calculated from the dry weight and the molecular weight of the enzyme. They do not necessarily reflect the specific activity of the enzyme. When there was a loss of activity due to several months storage, the reported concentrations were corrected for the loss in activity.

All solutions were 0.20 *M* in NaCl. When ficin or papain was the catalyst, the solutions were also 5 m*M* in cysteine. When leucine aminopeptidase was the catalyst, the solutions were 2 m*M* in $MnCl_2$ and 5 m*M* in $MgCl_2$. Substrate concentrations

(1) Presented in part at the 145th National Meeting of the American Chemical Society, New York, N. Y., September 8–13, 1963. This investigation was supported in part by Public Health Service Research Grant GM-08409.

(2) M. Green and M. Stahmann, *J. Biol. Chem.*, **197**, 771 (1952).

(3) E. Simons, G. Fasman, and E. Blout, *ibid.*, **236**, PC64 (1961).

(4) W. G. Miller, *J. Am. Chem. Soc.*, **83**, 259 (1961).

(5) E. Katchalski, Y. Levin, H. Neumann, E. Riesel, and N. Sharon, *Bull. Res. Council Israel*, **10A**, 159 (1961).

(6) H. Neumann, N. Sharon, and E. Katchalski, *Nature*, **195**, 1002 (1962).

(7) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958).

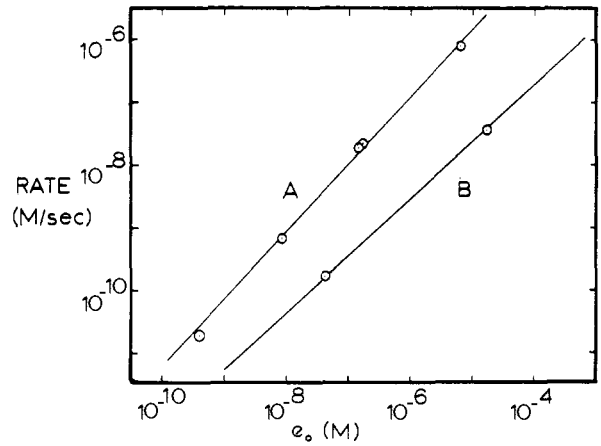

Fig. 1.—Dependence of reaction rate on enzyme concentration: A, elastase, pH 5.0, substrate concentration 7.5 g./l.; B, subtilisin, pH 6.10, substrate concentration 6.7 g./l.

were generally in the range 6–8 g./l. A wider range was used when estimating Michaelis constants.

All kinetic measurements were made at 25.0 ± 0.03° in Ostwald viscometers. Up to eight kinetic runs were made simultaneously. In a typical run 0.50 ml. of freshly prepared enzyme solution was added to 5.0 ml. of substrate solution. Five milliliters of the reacting solution were pipetted into a viscometer and flow times taken periodically. Corrections to the viscosity due to a contribution from the enzyme were necessary in the few cases where the enzyme concentration was greater than 0.5 mg./ml. Rates were calculated from the viscosity measurements by a method described previously.[4] In order to determine rate of bond cleavage in long-chain molecules, rates were calculated from data corresponding to cleavage of less than three bonds per molecule, equivalent to less than 1% of the total peptide bonds. Where not limited by solubility, enzyme concentration was adjusted so that the rate was 0.1–10 μmoles/l./min. Rates less then 2×10^{-10} M min.$^{-1}$ gave viscosity changes that were less than the experimental uncertainty and were arbitrarily assigned a value of zero.

Addition of the enzyme to the substrate solution, even in buffered solutions, generally resulted in a change in pH. This was not recognized in the PGA–papain study, and the values reported there refer to the pH of the substrate solution before addition of enzyme. As hydrolysis proceeds the pH drops slowly. The pH change due to hydrolysis was not perceptible, however, until several per cent of the total peptide bonds were hydrolyzed. Since most of the systems studied showed a marked dependence of the rate on pH, it was found absolutely necessary to determine the pH after the addition of enzyme. As rates were determined during the first per cent of reaction, it was most expedient to measure the pH immediately after determining the rate rather than after addition of enzyme but before the rate determination. In approximately half of the rate studies 10 mM PO_4 was used to buffer the system. Any effect of the buffer on the rate was less than the precision of the rate measurements.

Chromatography analyses of partially degraded polypeptide on diethylaminoethyl cellulose were carried out using methods described previously,[4] except that a Technicon AutoAnalyzer was used for peptide detection.

Results

Rate Law.—All endopeptidases yielded data which could be analyzed in the framework of the Michaelis–Menten mechanism. The rate was expected to be first order in enzyme concentration. It was necessary to confirm this as the enzyme concentration was varied over a wide range to keep the rate measurable. The data in Fig. 1, in the case of elastase covering a 17,000-fold change in enzyme concentration, show first-order behavior and are typical of the endopeptidases over the entire pH region studied.

When the reaction was followed over a period of several hours or when pH regions of known enzyme instability were being investigated, the rate dropped off faster than expected, indicating a loss of enzyme activity. As the loss in enzyme activity is likely a first-order process, increasing the enzyme concentration will not eliminate the drop-off. When such cases were encountered, the rate extrapolated to zero reaction time was taken as the initial rate.

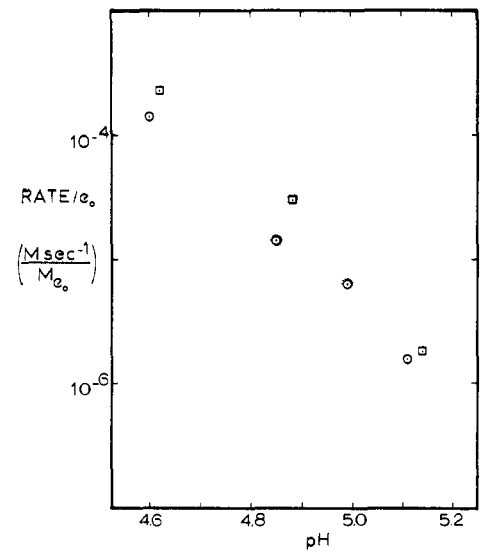

Fig. 2.—Hydrolysis of PGA by pepsin: (○), substrate to enzyme concentration 2 g./l.; (□), substrate to enzyme concentration 10 g./l.

Exopeptidases.—Leucine aminopeptidase, concentration 2×10^{-6} M, caused no detectable viscosity change in a 7 g./l. PGA solution in 50 hr. reaction time over the pH range 5.0–7.7. Chromatograms showed no monomer peak. Carboxypeptidase A (DFP treated to remove endopeptidases) gave similar results.

Carboxypeptidase B, concentration 1.3×10^{-6} M, gave easily detectable reactions in 7 g./l. PGA solutions at pH 4.6, 4.9, and 5.2. Chromatograms showed only monomer was split from the polymer. The specific viscosity dropped to one-half its initial value in 1–2 hr. Absolute reaction rates are not easily calculated from viscosity measurements when monomer is the only hydrolysis product. The rate of reaction did not appear to be strongly pH dependent in the limited pH range studied.

Endopeptidases.—Trypsin, concentration 100–200 μM, in the pH range 5.0–8.6 gave either no detectable reaction or an insoluble complex, as was expected.[2,8] The other endopeptidases, the chymotrypsins, elastase, ficin, papain, pepsin, and subtilisin, showed catalytic activity and the rate was greatly dependent on the pH. The pH dependence of the rate gave a curve whose shape seemed the same for each of the active endopeptidases except pepsin. The data for pepsin are shown in Fig. 2. If indeed the other enzymes exhibit the same pH-rate curve, multiplying each rate for a particular enzyme system by a scaling factor would make the curves superimpose. Scaled data for the chymotrypsins, elastase, ficin, papain,[4] and subtilisin are presented in Fig. 3.

Many of the points around pH 5 are averages over several kinetic determinations. The scaling factors are listed in Table I along with the rate at the pH optimum,

(8) E. Delbert and M. Stahmann, *Nature*, **176**, 1028 (1955).

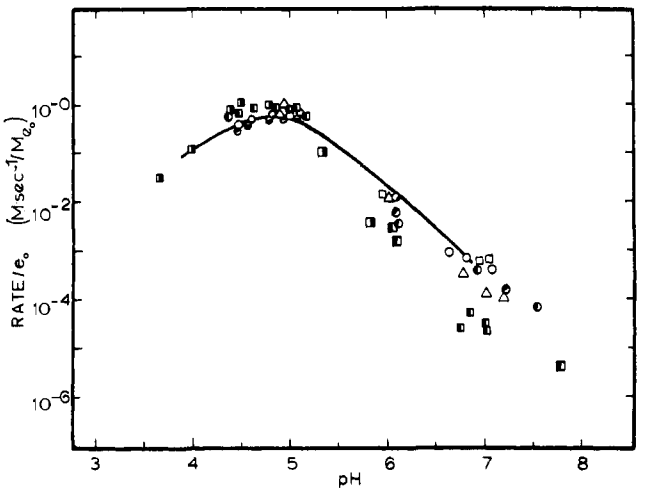

Fig. 3.—Hydrolysis of PGA by α (○), β (◒), γ (◓), Δ (◐) chymotrypsin, elastase (□), ficin (△), papain[4] (□■), and subtilisin (■□). Rates have been multiplied by scaling factors given in Table I. Solid line is theoretical curve calculated for model G assuming $w/kT = 2$.

the isoelectric point, and the pH optimum for hydrolysis of small molecular weight substrates.

TABLE I
RATE, SCALING FACTOR, ISOELECTRIC POINT, AND pH OPTIMUM

Enzyme	Rate/e_0 at pH optimum, M sec.$^{-1}/M_{e_0}$	Scaling factor	Isoelectric point	pH optimum for small substrates
α-Chymotrypsin	2.1×10^{-4}	4000	8[a]	7–8[a]
β-Chymotrypsin	0.9×10^{-4}	9000		
γ-Chymotrypsin	1.6×10^{-4}	6300		
Δ-Chymotrypsin	5×10^{-4}	2000		
Elastase	0.13	7	8.5[b]	~8[b]
Ficin	8	0.14		6–7[c]
Papain	1.2	0.9	8.7[d]	5–7[d]
Subtilisin	1.0	1.0	9.4[a]	8–10[a]

[a] "The Enzymes," Vol. 4, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press, Inc., New York, N. Y., 1960, p. 103 ff. [b] D. Hall and J. Czerkawski, *Biochem. J.*, **73**, 356 (1959). [c] B. R. Hammond and H. Gutfreund, *ibid.*, **72**, 349 (1959). [d] Ref. 11.

Rate data are reported in the units moles of bonds broken per liter per second divided by the molar enzyme concentration (M sec.$^{-1}/M_{e_0}$) rather than in sec.$^{-1}$, as the data do not represent maximum rates (V_m) under the condition of substrate-saturated enzyme. Estimates of the Michaelis constants (K_m) were made. The results are shown in Fig. 4. It is difficult to obtain very precise values for the Michaelis constants, especially when the rates are low. In most cases the substrate concentrations used in obtaining the data in Fig. 3 are approximately equal to the Michaelis constants. In general, then, the maximum rates can be no more than a factor of two larger, which is only slightly larger than the experimental error in the lower pH range and less than the experimental error at pH 7 and higher. In several instances the reaction was followed until most of the polymer was of low molecular weight. The rate dropped sharply as this condition was reached. Chromatograms showed on a qualitative basis that bond cleavage was essentially random with discrimination against cleavage near the end of a chain. These results are similar to a more quantitative and detailed study of the hydrolysis by papain.[4] Although a detailed systematic study was not made, it appears that this may be a general result for endopeptidases.

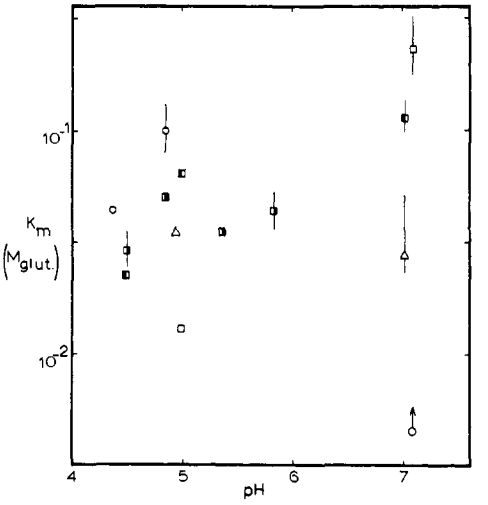

Fig. 4.—Estimated Michaelis constants as a function of pH. (○), chymotrypsin; (□), elastase; (△), ficin; (□■), papain; (■□), subtilisin.

Discussion

Comparison to Previously Published Results.—Subtilisin, elastase, and leucine aminopeptidase have not been investigated previously as catalysts for hydrolysis of PGA. Chymotrypsin has been reported as not being a catalyst.[2] Considering the very low rate and the less sensitive α-amino nitrogen analysis used by Green and Stahmann, it is not surprising that catalysis was undetected. Although chymotrypsin can generally be obtained in a very high state of purity, it is possible that an impurity is the catalyst in our preparations. Chymotrypsins purchased from different batch numbers showed the same specific activity (±5%). Polylysine was hydrolyzed much more rapidly than polyglutamate.[9] It would furthermore seem that different modifications would show considerably different activities if an impurity was responsible. The rates of thermal hydrolysis are less than 1% of the observed rates. Green and Stahmann also report PGA is hydrolyzed by carboxypeptidase, which presumably was the exopeptidase from bovine pancrease or carboxypeptidase A. We have been unable to observe this reaction even though the reaction was tried several times, using several different samples of enzyme and polypeptide.

The hydrolysis by pepsin as investigated by Simons, *et al.*,[3] was carried out at 37.9°. At pH 4.18 and a substrate concentration of 0.023 M glutamyl residues they observed a rate of 3.9×10^{-9} M min.$^{-1}$/mg. of pepsin/ml. Under similar conditions, except a temperature of 25°, we observe a rate 30 times larger. This discrepancy can be rationalized if one assumes the pH dependence of the rate is at least partially controlled by the polypeptide conformation or state of ionization. The helix–coil transition is shifted to a lower pH as the temperature is increased and may shift the pH–rate curve towards a lower pH. The study of Katchalski, *et al.*,[5] was made at 37° and a very low ionic strength. The polypeptide conformation and titration curve is quite sensitive to ionic strength and renders any intercomparison meaningless.

Specificity for High Molecular Weight PGA.—The scaling factors in Table I provide relative values of the intrinsic ability of an enzyme to catalyze the

(9) W. G. Miller, *J. Am. Chem. Soc.*, **86**, 3918 (1964).

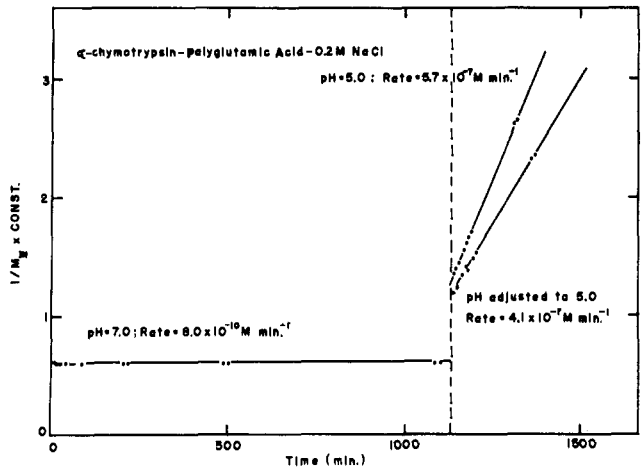

Fig. 5.—Stability of chymotrypsin in the presence of polyglutamate.

hydrolysis of PGA. The curve in Fig. 3 is a factor which must be intrinsic to the substrate. The observed rate is, then, the product of a factor intrinsic to the substrate and a factor intrinsic to the enzyme.

The specificity toward PGA varies widely. The variation in the scaling factors for the various modifications of chymotrypsin is greater than the experimental error. Since the scaling factors fell in roughly the same order for the chymotrypsins acting on polysine,[9] the variation is most likely a result of differing specific activities of the enzymes rather than a difference in intrinsic ability to hydrolyze PGA.

It is of interest to compare the optimum rates to the rates of peptide bond cleavage in small substrates. The extensive work of Bergmann and co-workers[10] is reported as per cent hydrolysis after a specified time of reaction, so the data are not in a form that can be compared directly. Some data on the hydrolysis of dipeptides were found in a form which could be compared.[11-13] The rates were much lower than those observed with PGA. More extensive data exist on the hydrolysis of amide and ester bonds in amino acid derivatives. The higher rates in Table I are in the same range as those observed for good ester and amide substrates.

The pH Dependence of the Rate Catalyzed by the Chymotrypsins, Elastase, Ficin, Papain, and Subtilisin.—As PGA is known to inactivate trypsin,[8] it is remotely possible that the large pH dependence of the rate is a result of a similar process. In an attempt to answer this α-chymotrypsin was allowed to hydrolyze PGA at pH 7.0 for 20 hr. The reacting solution was suddenly changed to pH 5.0 by addition of HCl, as shown in Fig. 5. The rate increased 500-fold and was within experimental error equal to the rate in a freshly prepared chymotrypsin–PGA solution at pH 5.0. Thus, if there is inactivation by substrate, it is fully reversible with the activity regained within seconds or less. Inactivation seems an unlikely explanation of the pH dependence of the rate.

In gross detail the pH dependence of the rate of hydrolysis is the same for each of these enzymes. This suggests that the pH dependence is controlled by a

(10) M. Bergmann and J. S. Fruton, *Advan. Enzymol.*, **1**, 63 (1941).
(11) J. R. Kimmel and E. L. Smith, *ibid.*, **19**, 267 (1957).
(12) E. L. Smith, V. J. Chavre, and M. J. Parker, *J. Biol. Chem.*, **230**, 283 (1958).
(13) E. J. Casey and K. J. Laidler, *J. Am. Chem. Soc.*, **72**, 2159 (1950).

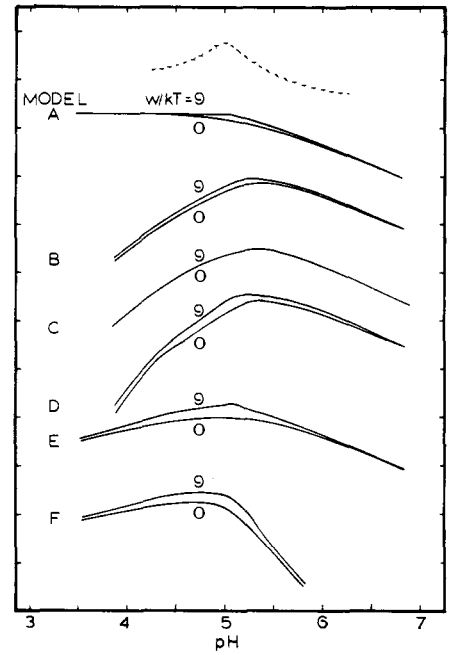

Fig. 6.—Model calculations for pH dependence of rate. Dashed curve is concentration of helix–random coil junctions.[17] On ordinate scale each division is one power of 10.

factor common to each enzyme–substrate system. There seems to be no correlation with the pH dependence of these enzymes acting on small substrates. It should be pointed out, however, that most small peptide pH studies have been on amide and ester rather than peptide bond hydrolysis. Any property of the substrate is obviously a feature common to each system. Various properties of the substrate which might possibly affect the pH dependence of the rate will now be considered.

A. Electrostatic Effects.—The pH region investigated was well below the isoelectric point of each enzyme, hence each enzyme carried a net positive charge. The apparent pK of the side-chain carboxyls in PGA in 0.2 *M* NaCl is about 5.0. The electrostatic potential in the vicinity of a peptide bond increases considerably as the side chains are titrated and might affect the catalysis. The electrostatic free energy of a polyelectrolyte can be estimated.[14] The manner in which electrostatic interaction would affect the reaction rate is not clear. For small substrates Hammes and Alberty[15] assume the entire effect can be described in changes in formation of the enzyme–substrate complex and obtain qualitative agreement with experiment. The experimental facts which we must explain are a 2- to 3-fold increase in rate in going from 25 to 45% dissociation, a decrease by a factor of two in going from 45 to 65%, a 7- to 10-fold decrease in going on to 85%, and a further 10- to 15-fold decrease in going to 95% dissociation. The largest changes in rate occur during the last few per cent dissociation, which is just the region where the electrostatic potential is changing very little. Assuming a linear relationship between the electrostatic free energy and the log of the rate, an effect is predicted which is not comparable to that observed. An additional assumption that only random coil regions of the polymer will be hydrolyzed allows a maximum

(14) S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions," Academic Press, Inc., New York, N. Y., 1961, p. 283 ff.
(15) G. Hammes and R. A. Alberty, *J. Phys. Chem.*, **63**, 274 (1959).

but will not alter the curve at high degrees of dissociation. Furthermore, the detailed charge distribution around the "active site" must not be important, otherwise superposition of the pH–rate curves would not be observed.

The more sophisticated pH dependence of enzyme reactions as given by the Kirkwood charge fluctuation theory[16] is equally unable to explain the data. A charge density that is physically unreasonable is needed to produce the observed effect.

B. Helix–Coil Junctions.—In discussing the hydrolysis by papain we suggested[4] that "a consistent and seemingly reasonable mechanism is enzyme attack on peptide bonds at helix–random coil junctions." A maximum around pH 5 is then expected and the fall-off on either side is a function of two adjustable parameters. Zimm and Rice,[17] evaluating these parameters from the titration curve of PGA, predict the concentration of junctions as a function of pH as shown by the dashed line in Fig. 6. The fall-off near the maximum will be a sensitive function of the two parameters while the slope further out will be rather insensitive to these parameters. If the theory of Zimm and Rice is qualitatively correct in predicting the concentration of junctions, comparison with experimental data gives rather poor agreement.

C. Conformation and Specific Charge State of Side Chains Adjacent to a Peptide Bond.—A number of enzymes catalyze a reaction involving a substrate in one charge state, while showing little or no catalysis if the charge state is changed. The pH dependence of the rate may be quantitatively predicted by assuming specific charge states of the molecule are necessary for catalysis. It is of interest to consider models in which the charge state of the side chains adjacent to a peptide bond are specified. Models which would predict a maximum in the pH dependence of the rate were considered, their description being given in Table II.

TABLE II

MODELS FOR HYDROLYSIS OF PGA ASSUMING SPECIFIC CHARGE STATE AND CONFORMATIONAL RESTRICTIONS

Model	Charge state of the two side chains adjacent to bond	Conformational restriction for catalysis	Mathematical description of model (F)[a]
A	Up to 1 charge	None	$f_{00} + f_{01}$
B	Up to 1 charge	Random coil	$(f_{00} + f_{01})f_{r.c.}$
C	1 specific uncharged[b]	Random coil	$(f_{00} + {}^1/_2 f_{01})f_{r.c.}$
D	1 charge only	Random coil	$f_{01}f_{r.c.}$
E	1 charge only	None	f_{01}
F	1 charge only	Helix	$f_{01}(1 - f_{r.c.})$
G	No charge	Random coil	$f_{00}f_{r.c.}$

[a] $F_A = f_{00} + f_{01}$; $F_B = (f_{00} + f_{01})f_{r.c.}$; etc. [b] One specific uncharged—the "amine side" (or the "carboxyl side") of the peptide bond has no charge. The charge state of the other side chain has no effect.

Models taking into account a specification of charge state farther away than the adjacent side chains were not considered, as they could be classified more appropriately as electrostatic effects.

The statistical quantities to be calculated are the number of pairs of adjacent side chains per bond that are unionized (f_{00}), the number of pairs per bond with one ionized (f_{01}), and the number of pairs per bond with both ionized (f_{11}), each as a function of the degree of dissociation, α. These quantities are not all independent as the number of side chains must be conserved and are related, neglecting end effects, by

$$f_{01} + 2f_{11} = 2\alpha$$
$$f_{01} + 2f_{00} = 2(1 - \alpha)$$

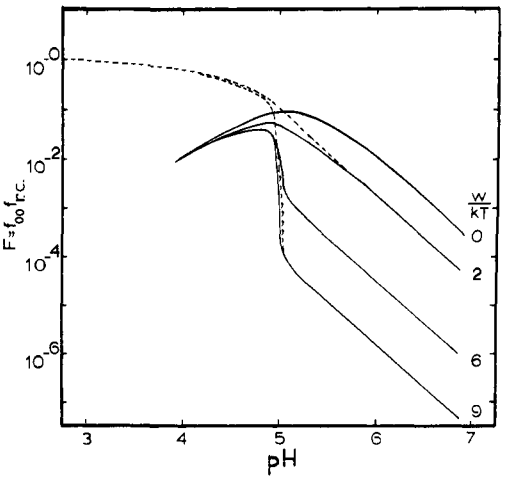

Fig. 7.—Model G calculations for pH dependence of the rate. Dashed lines correspond to removing the restriction on conformation.

The charged side chains will interact and distort the distribution from a random one. In order to have a readily manageable mathematical description we will use a one-dimensional Ising model assuming nearest-neighbor interactions only. Following the standard approach to the Ising model, its partition function is written down and approximated by its maximum term.[18] As a result the relationship

$$f_{01} = 4\alpha(1 - \alpha)/(1 + \beta)$$

is obtained where

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

and w is the pairwise interaction energy for nearest-neighbor interaction. Assuming values of w/kT, f_{00} and f_{01} can be calculated as a function of α. The degree of dissociation may be obtained from the titration curve.[4] The fraction of the residues that are in the coil conformation, $f_{r.c.}$, may be estimated from the theoretical calculation of Zimm and Rice,[17] optical rotation or hypochromicity measurements. Only model G around pH 5 and at large values of w/kT and model F will be sensitive to the precise form of the helix–coil transition. Results from calculations assuming w/kT = 0, 2, 6, and 9 are shown in Fig. 6 and 7, using hypochromicity at 202 mμ to estimate the fraction of residues in the random coil conformation. For models which are insensitive to w/kT, the calcuations for 0 and 9 are shown. Comparison with Fig. 3 shows that models F and G are the only models which even approximately fit. Model F fits rather poorly and seems intuitively unreasonable from what is known about enzymic hydrolysis of proteins. Two other models involving hydrolysis in helical sections, namely helix with no charge and helix with up to one charge, predict no maximum and show a fall-off in rate above pH 5 similar to model F. Model G seems reasonable and describes the data

(16) J. G. Kirkwood, *Discussions Faraday Soc.*, **20**, 78 (1955).
(17) B. H. Zimm and S. A. Rice, *Mol. Phys.*, **3**, 391 (1960).
(18) T. L. Hill, "An Introduction to Statistical Thermodynamics," Addison-Wesley Publishing Co., Inc., Reading, Mass., 1960, p. 235 ff.

very well if $w/kT = 2$, as can be seen in Fig. 3 where it is superimposed on the rate data.

Discussion of Model G.—The side-chain carboxyls in the random coil are about 10 Å. apart and an interaction energy of $2kT$ is quite reasonable. The titration curve of a helical region is different from that of a random coil region whereas we used the average titration curve in making the calculations. As soon as the helix–coil transition is complete, we will have no error. Error from this source, therefore, will contribute only below pH 5. Another source of error is higher order interactions. This cannot be easily corrected without going to machine calculations. At high degrees of ionization it can be approximated by assuming that the corrected value for the pairwise nearest-neighbor interaction energy is somewhat less than the value for w necessary to fit the data.

If the experimental data are corrected to maximum velocities, the shape of the curve will be changed very little, at least in the pH range of 4–7. Due to ionizable groups involved in the catalytic site, the activity of the enzymes will change with pH. However, from pH 5 to 7 the correction factor for papain, ficin, and chymotrypsin will amount to little more than the experimental error. The pH dependence of subtilisin and elastase is not known well enough to allow a similar estimate.

Assuming the model correctly explains the dominant features in the pH dependence of the rate, dividing the rate at any pH by the corresponding value of F_G will give the rate at which the enzyme hydrolyzes a bond with adjacent side chains uncharged in a random coil region. These rates, corrected to V_m through use of the Michaelis constants and expressed as first-order rate constants, are given in Table III.

Changing w should produce a large effect on the rate above pH 5. The interaction energy can be altered by changing the ionic strength and should provide a good check on the validity of this interpretation of the mechanism.

The pH Dependence of the Rate Catalyzed by Pepsin.—The data for the pepsin-catalyzed reaction are scanty and are included mostly to point out the difference between it and the other endopeptidases. Pepsin is a poor catalyst and at pH 4.6 the rate is the same as that for chymotrypsin. Polyglutamate is difficult to work with below pH 4.5 unless one goes to much lower concentrations, where the rates become difficult to measure. A further complication results from the poor catalytic ability. Enzyme concentrations comparable to the substrate concentration must be used in order to obtain measurable rates, thus making the steady-state assumption questionable. This apparently leads to the observed results of reduced rates at a given pH becoming dependent on the substrate to enzyme ratio. Bull and Currie[19] report pepsin has an ionizable group with a pH of 2.1 involved in the catalytic site. Above pH 3, then, the log of the enzyme activity should vary linearly with pH. The slope of the curve in Fig. 2 is much greater than this. It has been suggested that pepsin acts on the helical form of polyglutamate.[3] Our data support this but cover too narrow a pH range to make possible knowledgeable statements.

TABLE III

RATE CONSTANT FOR HYDROLYSIS OF A PEPTIDE BOND WITH ADJACENT SIDE CHAINS UNCHARGED IN A RANDOM COIL REGION OF PGA, ASSUMING MODEL G

Enzyme	Rate constant, sec.$^{-1}$
α-Chymotrypsin	0.01
Elastase	3.0
Ficin	250
Papain	40
Subtilisin	40

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Degradation of Synthetic Polypeptides. III. Degradation of Poly-α,L-lysine by Proteolytic Enzymes in 0.20 *M* Sodium Chloride[1]

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The initial action of carboxypeptidase A and B, α-, β-, γ-, and Δ-chymotrypsin, elastase, ficin, leucine aminopeptidase, papain, pepsin, subtilisin, and trypsin on high molecular weight poly-α,L-lysine in 0.20 *M* NaCl at 25° has been investigated. The endopeptidases chymotrypsin, elastase, ficin, papain, and subtilisin, after correcting to constant enzymic activity, exhibit the same pH dependence of the rate of hydrolysis. Analogous to the action of these enzymes on polyglutamic acid, the pH dependence is quantitatively predicted assuming enzymic attack of lysyl–lysyl bonds with adjacent side chains uncharged in random coil regions of the molecule. Trypsin is shown to be insensitive to the specific charge state of the polylysine side chains. Below pH 9 the rate is quantitatively accounted for, assuming trypsin has an ionizable group with a pK of 6.6 associated with its catalytic activity. Of the exopeptidases, carboxypeptidase B and leucine aminopeptidase showed catalytic activity.

Introduction

The hydrolysis of poly-α,L-lysine by tryspin has been extensively studied by Waley and Watson at pH 7.6.[2] Several less quantitative studies have been carried out,[3–5] mostly concerning hydrolysis products, though pH

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