by the fact that glycolamide has a greater compressibility[34] and diffusion rate[4,5] than glycine in water. On the other hand, the behavior of the alkali metal halides is contrary; for example, the more compressible aqueous solutions of lithium chloride show a lower diffusion rate than those of sodium chloride.[35,36] The results of this investigation indicate a higher limiting diffusion coefficient D_0 of 93.92×10^{-7} cm.^{2}/sec. for the most polar isomer, beta-alanine, in water at 25.00° than the corresponding value of 91.46×10^{-7} cm.^{2}/sec. for alpha-alanine,[8] a difference of over 2.5%. On the other hand the value of D_0 for lactamide,[30] the least polar isomer, is $99.11(10)^{-7}$ cm.^{2}/sec.

(35) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publ. Corp., New York, N. Y., 1950 pp. 180, 181.

(36) J. W. Williams and L. C. Cady, *Chem. Revs.*, **14**, 171 (1934).

Furthermore, the work of Mason, Kampmeyer and Robinson[14] shows that solutions of beta-alanine in water exhibit lower relative viscosities and lower activation energies for viscous flow than do solutions of the alpha-isomer. These comparisons of the properties of the alanines lead to the conjecture that a macroscopic physical measurement such as electrostriction, compressibility and heat capacity, indicating closeness of nearest-neighbor molecules in a liquid, is not necessarily a precise measure of the effective size of the kinetically independent particles in a flow process.

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[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY OF HARVARD UNIVERSITY, AND THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA]

Degradation of Poly-α,L-glutamic Acid. I. Degradation of High Molecular Weight PGA by Papain

BY WILMER G. MILLER[1]

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Long chain poly-α,L-glutamic acid under suitable conditions is shown to be an excellent substrate for papain. The maximum velocity (V_m) is extremely *p*H dependent and in the region of the helix-coil transition changes with *p*H faster than the change in hydrogen ion concentration. Chromatographic analysis of the products of degradation shows that the enzyme attacks bonds randomly except for discrimination near the ends of the chain. The interpretation of the data seems most consistent with the assumption that enzymic hydrolysis occurs almost exclusively at helix-coil junctions in the polypeptide. The importance of electrostatic charge in influencing enzymic attack could not be determined explicitly.

Introduction

The degradation of proteins by proteolytic enzymes has been investigated many times. The configurational state of the protein often plays an important role but interpretation is generally handicapped by a lack of knowledge of the conformation of the protein. The increasing availability of high molecular weight synthetic polypeptides of known conformation permits a study of the effect of conformation on degradation. Waley and Watson[2] made an extensive study of the hydrolysis by trypsin of poly-L-lysine in the random coil conformation. Other[3] less extensive studies of the hydrolysis of synthetic polypeptides have been made but there appears to be no investigation of the effect of changing conformation.

The present study was undertaken in an attempt to determine the effect of conformation, in particular the effect of going from an α-helix to a random coil. Although the simple synthetic polypeptides can hardly be called synthetic proteins, they nevertheless should serve as useful model compounds. The enzyme papain seemed attractive to study since the kinetics of its action on small substrates has been thoroughly studied[4] and since it had been reported[5] to attack poly- α-L-glutamic acid (PGA). The water-soluble PGA has been shown to exist in solution both as an α-helix and as a random coil[6] and the helix-coil transition occurs at a *p*H where the activity of papain is nearly maximum. Unfortunately the use of PGA is complicated by the fact that an ionization of the polypeptide accompanies the helix-coil transition.

Experimental

Poly-α,L-glutamic acid was purchased from Pilot Chemicals, Inc. The procedure used for synthesis is such that the PGA has an unblocked C-terminal carboxyl, as far as is known.[7] The PGA was received as the sodium salt monohydrate. Titration of the carboxyls gave a residue weight of 170, the expected result for the sodium salt monohydrate. PGA with a weight average molecular weight (MW_w) of 58000 in the acid form as determined by viscosity measurements[8] was used during most of the kinetic runs. In all, four preparations were used with weight average weights of 50000–60000. Papain (crystalline 2X) was obtained from Nutritional Biochemical Corporation and was used without further recrystallization. Other chemicals were of analytical grade.

Unless stated, all measurements were made at 25°, controlled to ±0.02° for viscosity-kinetic runs and to ±0.5° for runs lasting several days or longer. Optical rotations were measured with a Rudolph model 200 high precision polarimeter, using a mercury lamp as the light source and a 40 cm. cell. Concentrations were 0.2–1.0% and the ro-

(1) Public Health Service Postdoctoral Fellow 1958–1959. Present address: State University of Iowa, Iowa City, Iowa.

(2) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(3) M. Sela and E. Katchalski, *Adv. Protein Chem.*, **14**, 391 (1959).

(4) See J. R. Kimmel and E. L. Smith, *Adv. Enzymology*, **19**, 267 (1957), for an excellent review.

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

(6) P. Doty, A. Wada, J. Yang and E. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

(7) Mark Hyman, Jr., Pilot Chemicals, Inc., private communication.

(8) We are indebted to Drs. Paul Doty and A. Wada for making available the calibration of the molecular weight–viscosity scale for L-PGA (A. Wada and P. Doty, to be published).

tations were calculated for the sodium salt monohydrate. The *p*H was measured with a Radiometer TTl and Beckman G and H2 *p*H meters. Since the rate is so sensitive to *p*H, it is unfortunate that one instrument could not be used for all measurements. Viscosities were measured with a thermostatted Ubbelhode type viscometer with solvent efflux times of two and four minutes. The kinetic energy correction was estimated using solvents of known viscosity and found to be negligible under the experimental conditions.

The activator–buffer system of Smith, *et al.*,[4] was followed as closely as possible. Unless stated, all measurements were made in a system containing 0.2 *M* NaCl, 1 m*M* ethylenediaminetetraacetate (EDTA), 5 m*M* cysteine and 10 m*M* citrate. The rate of hydrolysis was markedly dependent upon the cysteine concentration but independent of EDTA and citrate concentrations. Citrate was used in order to have a good buffer over the *p*H range of most interest.

Papain activity was assayed by the procedure of Kimmel and Smith[9] using α-benzoyl-L-argininamide as the substrate. Absolute protein concentration was determined by dry weight (110°). At 39° the papain had a specific activity of 0.6, which is somewhat lower than commonly reported.[4] The enzyme had been stored for several months before the absolute concentration was determined, and the lower specific activity undoubtedly reflects the presence of some inactive enzyme. It was difficult to determine activities to better than ± 10%. For routine kinetic studies a stock solution of enzyme was stored at 4° as a solution composed of enzyme, 1 m*M* EDTA, 5 m*M* cysteine and 20 m*M* citrate, *p*H 5.5. When the stock solution showed considerable loss of activity, fresh solutions were prepared. By storing the enzyme in the activator–buffer system, the enzyme used in kinetic runs could thus be added as the fully activated enzyme.

Viscosity–kinetic measurements were made with a thermostatted, closed reaction cell equipped for magnetic stirring and having openings for introduction of electrodes, microburet, nitrogen line and the tip of the viscometer. A stream of nitrogen was passed over the solution on all runs above *p*H 5 and occasionally on runs below 5. To measure rate as a function of PGA concentration at a given *p*H, activator-buffer solution of twice the desired concentrations was diluted and adjusted to give the desired *p*H and concentrations. PGA was added to another aliquot of the concentrated buffer solution and the solution diluted to the same *p*H and buffer concentrations. The solutions were filtered through a coarse porosity sintered-glass filter funnel. Aliquots of the two solutions were pipetted into the reaction cell to give both the desired PGA concentration and a total volume of 6 ml. All measurements at a given *p*H were made using aliquots of the same stock solutions, otherwise the data were very erratic due to the extreme *p*H sensitivity of the rate. After determining the viscosity of a solution, 0.2 ml. of stock enzyme solution was added and the viscosity taken periodically. The enzyme concentration was kept low enough such that the change in viscosity during the time of measurement was small compared to the total change in viscosity during a run. Nevertheless a correction was made for the fact that the measured viscosity did not correspond to the viscosity at the time of starting the measurement but was an average over the time of efflux. The enzyme activity was assayed during each set of runs. Substrate concentrations ranged from 1 to 20 g./l. and enzyme concentrations from 1 mg./l. to 2 g./l. (5×10^{-8}–10^{-4} *M*). In general, the substrate concentration was varied by a factor of 5–10 and wherever possible bridged the Michaelis constant. Rate constants above *p*H 6 are maximum estimated values as the change in viscosity over a period of hours at very high enzyme concentrations was of questionable significance. Below *p*H 4 the Michaelis constant is so small that it was not possible to get meaningful viscosity–time curves at low enough substrate concentrations to permit evaluation of the Michaelis constant.

Solutions to be chromatographed were adjusted to *p*H 7 and diluted to less than 0.02 *M* in NaCl. The solutions were quantitatively transferred onto a diethylaminoethyl cellulose (Brown Company) column in the chloride form and water was passed through the column until no NaCl or cysteine appeared in the eluant. A 1 × 13 cm. column was used for series A, B and D and a 2 × 30 cm. column for series C and E. The polypeptides were eluted with a linear NaCl gradient (0.4 m*M*/ml.). The flow rate was 0.5–1.0 ml./min./cm.^{2} for the 1 cm. column and 0.2–0.5 for the 2 cm. column. Fractions of 7.7 and 16 ml. were collected from the 1 and 2 cm. columns, respectively, and aliquots were analyzed for amino nitrogen with ninhydrin.[10] Glutamic acid was used as a standard for the ninhydrin analyses. Saturated NaCl solution or 0.1 *N* NaOH was run through the column and analyzed for peptide at the end of a run to make certain all polypeptide was eluted.

The identity of the peaks was established in three ways. The early well-resolved peaks became progressively closer with increasing salt concentration. With increasing degradation the earlier peaks increased at the expense of the later peaks but no new peaks appeared, thus suggesting that the position of the peak was proportional to the number of residues (net charge) in the polypeptide. Next an aliquot of a peak was titrated for carboxyls with HCl and another aliquot was either titrated for amino groups by titration in alcohol with NaOH[11] or assayed with ninhydrin. From the ratio of carboxyls to amino groups the number of residues per molecule can be calculated directly. Rather dilute solutions were used for the titrations and the results were good only to ± 1 residue. For the final identification we were fortunate in obtaining a sample (MI-294) of fractionated PGA from Dr. E. R. Blout[12] which showed a strong infrared absorption band at 1625 cm.$^{-1}$ and very little absorption around 1650 cm.$^{-1}$. From this it had been concluded that the polymer had an average degree of polymerization (DP_n) below that which stabilizes the α-helical conformation.[12] When this PGA was chromatographed, seven peaks appeared; the first six were strong and the seventh was barely discernible. A solution containing another aliquot of this fractionated PGA, 0.4 mg. of glutamic acid and small amounts of what titration had shown to be 3-mer and 8-mer was then chromatographed. When normalized to the same amount of fractionated PGA and compared to the first column, the first and third peaks were much larger than in the first column and an eighth peak appeared properly spaced after the seventh peak. From this it was concluded that the peaks were being properly identified.

Results

Chromatography.—The yield of resolved peptides for a series of degradations over widely varying times and for various values of enzyme concentration, substrate concentration and *p*H is given in Table I. The initial amount of PGA was known (except for C-6 and C-7 which were normalized) and values have been converted to micromoles per gram of initial PGA. In some instances changes in concentration of several hundred to one thousand could be observed as the degradation proceeded. Quantities of less than 5 μmoles are subject to considerable error, in some instances possibly as much as a factor of 2. On chromatograms that gave completely resolved peaks, a material balance on the amount of peptide recovered showed 80–105% recovery. As there was no trend in degree of degradation with per cent. recovery, it is concluded that the ninhydrin assays for amino nitrogen were independent of chain length and that the per cent. recovery is an indication of the error of analysis. Equal aliquots were reproducible to ± 2% but widely varying amounts of peptide showed much larger differences, with larger quantities of peptide generally showing relatively lower yields. When the salt gradient breaks through the column it removes a trace of cysteine not removed in the wash-

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

(10) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(11) W. Grassmann and W. Heyde, *Z. physiol. Chem.*, **183**, 32 (1929).

(12) We are deeply indebted to Dr. Blout for providing this sample and outlining its properties and history.

TABLE I

YIELDS OF SMALL PEPTIDES UNDER VARIOUS CONDITIONS OF DEGRADATION

Run no.	pH	Reaction time (min.)	e_0t (g. min./l.)	Max.[a] e_0/PGA (g./g.)	Hydrolysis, %	μmoles of x-mer per gram of PGA										
						1	2	3	4	5	6	7	8	9	10	11
A-1	4.85	78	0.363	0.0003		?	1.3	2.0	2.2	3.0	3.3	3.4	2.7	2.2	..	..
A-2		169	0.785	.0003		?	1.0	6.5	8.4	10.2	10.0	9.4	10.0	6.8	6.7	8.4
A-3		587	2.73	.0003		2 (?)	4.2	31.7	40.0	50.0	48.5	38.4	36.7	25.0	18.4	21.7
A-4		793	6.38	.0012	16	1 (?)	16.7	96.8	125	139	115	93.5	58.4	48.4	25.0	18.4
A-5		2588	176	.0091	25	2.5	100	437	396	304	164	46.7	10.7	2.2	1	0
A-6		7200	8200	.19	46	375	1155	711	7							
B-1	4.85	<2[b]	<0.8	.1		?	4.0	1.6	2.4	2.6	2.8	2.2	1.7	.	..	..
C-1	4.7	1290	600	.11	35	132	537	958	410	46.7						
C-2		2880	1340	.11	38	210	670	1010	267							
C-3		4200	2650	.24	40	265	820	1090	164							
C-4		5500	4700	.39	45	533	910	1020	60							
C-5		8500	9450	.39	47	573	1080	1020	22							
C-6		30400[c]	44000	.39	43	191	1380	1050								
C-7		1.3×10^{5}[c]	4×10^{5}	1.0	43	102	1480	950								
D-1	5.7	2350	772	0.04		2.5	10.5	31.4	30.9	38.5	31.1	28.0	24.0	22.0	17.3	13.2
D-2		4250	2672	.13		3.8	28.7	57.2	54.8	66.6	55.7	43.5	39.3	30.8	22.1	18.4
E-1	3.65	15	1.65	.1		5	130	212	136	81	43.7	19.8	12.6	8.6	5	<1

[a] Maximum ratio of enzyme to substrate during reaction. Approximate concentrations of PGA were 17, 7, 4, 10, 1 g./l. for series A through E, respectively. [b] Enzyme added as solid suspension; time of activation unknown. [c] Substrate concentration unknown and values are normalized to give 1 g. of PGA.

ing step and the washing procedure used in the first few columns made this peak overlap the monomer peak. A question mark appears when it was doubtful whether the peak was cysteine or monomer.

Five complete chromatograms are shown in Fig. 1. The spacing of the peaks is sensitive to the salt gradient, and the slight variations in spacing are due to slightly differing gradients. The areas under the peaks are proportional to moles of peptide and can obviously be converted to moles of peptide by application of the appropriate known factors. For the present discussion, the important observations to be made from Table I and Fig. 1 are that there is always much less monomer than dimer, dimer than trimer (except C-6 and C-7) and above trimer the concentration of x-mer is, within experimental error, slightly greater than the concentration of $(x + 1)$-mer. This is qualitatively what is expected for random breakage of peptide bonds with the two end bonds being less reactive.

Evaluation of Rate Constants.—In Fig. 2, $1/\eta_{red}$ (c/η_{sp} with concentration calculated as g./100 ml. solution) is plotted against time for a series of substrate concentrations at pH 5.05. This is typical of curves obtained at all pH values. To convert reduced viscosity to intrinsic viscosity, information on the concentration dependence of three molecular weights ranging from 30000 to 60000 was investigated at two pH values, one where the conformation was predominantly helix and the other predominantly random coil. The Huggins expression,[13] $\eta_{sp}/c = [\eta] + k[\eta]^2c$, relating reduced viscosity to concentration was adequately followed in the concentration range of interest and the constant in the Huggins expression was within error the same for each molecular weight. Assuming this to be true over the pH range of interest, the Huggins constant, which varied slightly over the pH range investigated, can be determined at a given pH from the concentration dependence of the viscosity of the initial polypeptide, and the intrinsic viscosity as a function of time may then be calculated. A knowledge[8] of the exponent in the modified Standinger equation permits the conversion of intrinsic viscosity to a quantity proportional to the molecular weight (MW_w). The plots of $1/\eta_{red}$, $1/[\eta]$ and $1/MW_w$ against time are shown in Fig. 3 for a single run. There was a slight tendency for $1/MW_w$ to bend downward in some instances, particularly by the time MW_w had dropped by a factor of two or three. This procedure for converting reduced viscosity to MW_w will be strictly valid only if the molecular weight distribution during the degradation differs little from the distribution in the original PGA.

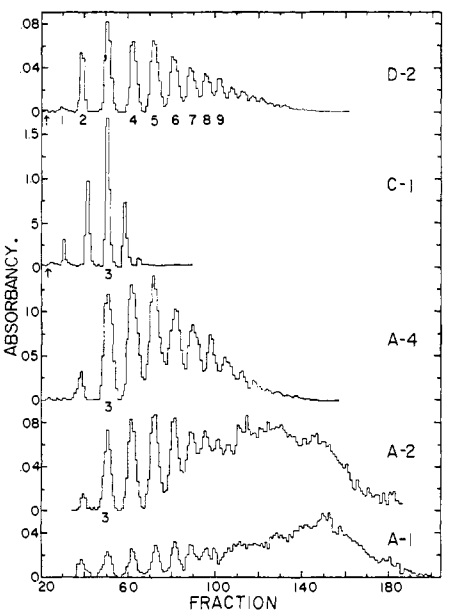

Fig. 1.—Chromatograms during various degrees of degradation; conditions given in Table I. Quantity of material chromatographed was variable, therefore quantities of peptide from different columns cannot be compared directly. Arrows indicate point where NaCl first breaks through column; numbers under peaks refer to length of peptide chain.

(13) M. L. Huggins, THIS JOURNAL, **64**, 2716 (1942).

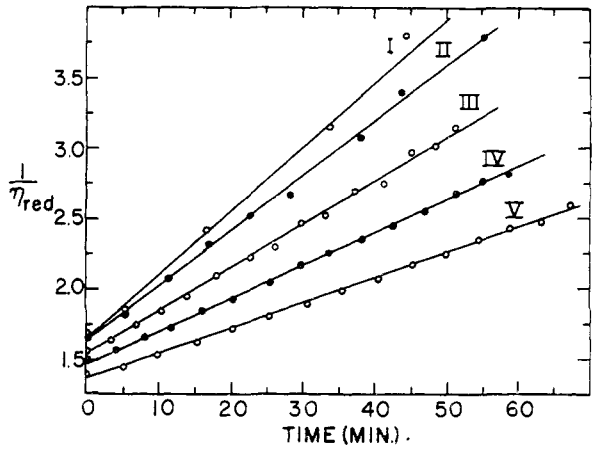

Fig. 2.—Typical plots of $1/\eta_{red}$ against time. Enzyme concentration = 1.0×10^{-7} *M*, *p*H 5.05; substrate concentrations (g./l.) were 1.6, 3.3, 6.6, 9.9 and 16.4 for I through V, respectively.

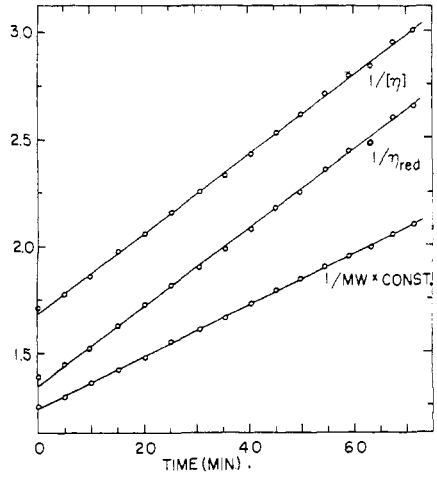

Fig. 3.—Typical plots of $1/\eta_{red}$, $1/[\eta]$ and $1/MW_w$ against time. Occasionally plots of $1/MW_w$ show slight downward curvature.

Poly - γ - benzyl - L - glutamate, the precursor of PGA, has a rather narrow weight distribution[14] but debenzylation with HBr to give PGA breaks a few peptide bonds[15] which theoretically[16] as well as experimentally[17] widens the weight distribution considerably. Thus the initial PGA should have very nearly a random or Gaussian weight distribution. Theoretically,[16] a plot of $1/MW_w$ against degree of depolymerization will be linear for random degradation of an initially random distribution and in general will not be linear for most other types of idealized degradation.[18] For random degradation of a random distribution, MW_w will drop to one half of its initial value when one bond has been broken per initial number average molecule. Therefore, a knowledge of the initial MW_n permits the calculation of the rate of breaking bonds in absolute units. Since the chromatograms as well as the plots of molecular weight against time indicate random degradation, and since the original PGA has a random distribution, the procedure used to convert reduced viscosity to molecular weight is completely justified.

(14) R. D. Lundberg and P. Doty, THIS JOURNAL, **79**, 3961 (1957).
(15) M. Idelson and E. R. Blout, *ibid.*, **80**, 4631 (1958).
(16) A. Charlesby, *Proc. Roy. Soc.* (*London*), **A224**, 120 (1954).
(17) C. E. Hall and P. Doty, THIS JOURNAL, **80**, 1269 (1958).
(18) R. Simha, *J. Applied Physics*, **12**, 569 (1941).

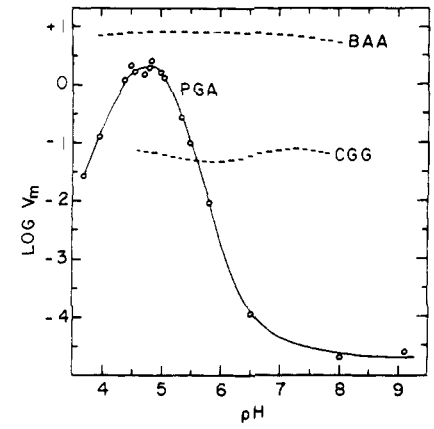

Fig. 4.—Plot of log V_m as a function of *p*H. For PGA, values above *p*H 6 are *estimated maximum values*. Curves for α-benzoyl-L-argininamide (BAA) and carbobenzoxyglycylglycine (CGG) were obtained from data of Smith, *et al.*[21,22]

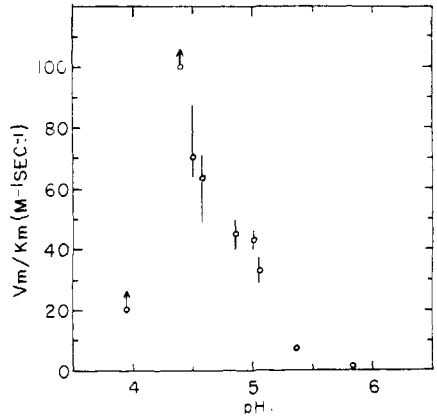

Fig. 5.—Plot of V_m/K_m against *p*H. Maximum and minimum values indicated; minimum values only (♂) are given in two cases.

The rate of breaking bonds was calculated from plots similar to Fig. 3 by measuring the time necessary for the molecular weight to drop to $^1/_2$ its initial value, which corresponds to breaking less than 0.5% of the total bonds. At a given *p*H, a plot of reciprocal rate against reciprocal substrate concentration permits the evaluation of the two constants (V_m and K_m) in the Michaelis–Menten rate law. As might be expected, the degradation follows the Michaelis–Menten rate law within experimental error. In Fig. 4 is a plot of log V_m against *p*H, and Fig. 5 a plot of V_m/K_m as a function of *p*H. Since the rate is dependent on the concentration of bonds and not on the concentration of macromolecule, the Michaelis constants were calculated in terms of the concentration of glutamate residues, which at high degrees of polymerization is nearly equal to the concentration of peptide bonds. The rate was first order in enzyme concentration and if there was any trend away from this, it was towards a higher power. The maximum velocity, calculated from the smooth

TABLE II

WEIGHT DISTRIBUTION AS A FUNCTION OF EXTENT OF DEGRADATION

Chain length	Weight fraction A-1 Exp.	A-1 Calcd.	A-2 Exp.	A-2 Calcd.	A-3 Exp.	A-3 Calcd.	A-4 Exp.	A-4 Calcd.
1	?	0.0005	?	0.0023	0.0004	0.016	0.0002	0.072
2	0.0005	.0009	0.0003	.0032	.001	.027	.006	.103
3	.0010	.0014	.0033	.0047	.016	.036	.049	.116
4	.0015	.0018	.0057	.0060	.027	.042	.085	.113
5	.0026	.0022	.0087	.0072	.043	.046	.118	.104
6	.0034	.0025	.0102	.0083	.045	.048	.117	.091
7	.0044	.0029	.0111	.0093	.050	.049	.111	.077
8	.0036	.0032	.0136	.0101	.050	.049	.080	.064
9	.0035	.0036	.0105	.0109	.038	.048	.074	.053
10	....	....	.0114	.0116	.031	.047	.043	.043
11	....	....	.0156	.0124	.041	.045	.034	.035
% Degradation		1.75		3.75		12.5	16	26.8
DP_n		45.5		23.8	8–9	7.8	6.0	3.7

curve in Fig. 4, is plotted in Fig. 6 together with optical rotation and titration curves taken in the same activator-buffer system as the rate measurements. This shows V_m in relation to the helix-coil transition and to the charge on the polypeptide.

Discussion

Type of Degradation.—Predominantly cage or one-by-one type mechanisms can be ruled out as they would give unreacted material, completely degraded products and no intermediate weight polymers; this is clearly not the case. Equal reactivity of molecules would give a different distribution of products and different molecular weight–time curves from those observed.[18] The molecular weight–time curves quantitatively indicate random breakage of bonds but cover the breaking of a negligible fraction of the total bonds. The chromatograms, however, cover the breaking of significant fractions of total bonds, and it is of interest to look at these quantitatively.

If the average molecular weight is large, the weight distribution expected for a random distribution is conveniently calculated from the normalized expression

$$w_x = \frac{x}{(DP_n)^2} e^{-x/DP_n}$$

where w_x is the weight fraction of the peptide containing x residues. For a small average molecular weight, it is better to use the statistical expressions of Montroll and Simha.[19] The calculated and observed values for four degradations at pH 4.85 are shown in Table II. When the molecular weight could not be determined, the integrated Michaelis–Menten equation,[20] $V_m e_0 t = (P) - K_m \ln [1 - (P)/(S)_0]$, was used to calculate the percentage of bonds broken for each value of $e_0 t$. Knowledge of the number of bonds broken permits calculation of w_x. The rate of splitting off monomer and dimer is always less than that expected for random degradation. It is significant that in A-3, where the calculated DP_n is 7.8 compared to the initial DP_n of about 225 and where peptides are analyzed on both sides of the distribution peak, the calculated and observed values agree reasonably well. This indicates that bond reactivity is approximately the same in relatively short chains, except for the end bonds. The end effect would be expected to be unimportant until a significant fraction of the remaining bonds were end bonds, at which time the rate would be drastically reduced and deviations from calculations would be expected. This is seen to be the case both in Table I and Table II.

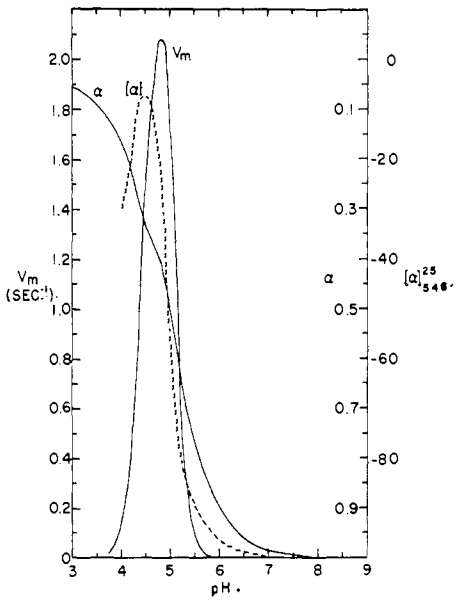

Fig. 6.—Plots of V_m, specific rotation ($[\alpha]^{25}_{546}$) and degree of dissociation (α) in same activator–buffer–solvent system as function of pH; V_m was obtained from the smooth curve through the data in Fig. 4.

In addition to the extensive data at pH values where the rate of bond breakage is maximum, Table I includes data on both sides of the maximum. At the lowest pH the configuration is predominantly α-helix and at the highest, predominantly random coil. There is no obvious change in molecular weight distribution over the entire pH range investigated, thus indicating that the type of degradation is independent of pH.

Before leaving a discussion of the type of degradation, the back reaction must be considered. Synthesis is thermodynamically unfavorable, and the simple back reaction will merely decrease the net rate of hydrolysis. There is a more subtle back reaction, however, whereby a bond is broken and

(19) E. Montroll and R. Simha, *J. Chem. Phys.*, **8**, 721 (1940).
(20) R. A. Alberty, *Adv. Enzymology*, **17**, 1 (1956).

one fragment dissociated, leaving behind an enzyme fragment compound (an acyl enzyme compound, for example). In the back reaction of the enzyme-fragment compound, a peptide bond may be formed between the fragment and any PGA molecule in the medium. This type of back reaction can be called a transpeptidation reaction as it involves no net hydrolysis. If the enzyme-fragment compound is formed randomly and the transpeptidation reaction is either much slower or else occurs randomly, the weight distribution will be random and the interpretation as random bond scission still valid. If formation of the enzyme-fragment compound is non-random and slow while the transpeptidation is random and fast, the weight distribution will still appear essentially random and the interpretation as random bond scission is erroneous. The net hydrolysis of peptide bonds at optimum *p*H is so large (see next section), however, that for transpeptidation to be faster seems unlikely. Furthermore, the weight distribution is random for hydrolysis over all values of *p*H, even when the net rate of hydrolysis is very small. Unless the rate of hydrolysis and of transpeptidation fortuitously have the same *p*H dependence, transpeptidation is not likely to be obscuring the interpretation of the hydrolysis.

Rate of Degradation.—The rate of degradation depends upon *p*H, chain length and position in the chain. As is shown in Figs. 4 and 6, V_m is extremely dependent on *p*H and on the alkaline side of the maximum decreases to less than 0.0001 of the maximum value in 1.5 *p*H units. The rate changes faster than the hydrogen ion concentration, a phenomenon which has never been observed in the extensive studies of Smith and co-workers on the hydrolysis of small synthetic substrates.[4,21,22] The maximum rate of 2.5 sec.$^{-1}$ is within a factor of 2 or 3 of the maximum of α-benzoyl-L-argininamide at 25°, one of the best small substrates for papain.[21] The specific activity of our enzyme was approximately one half or less of that routinely reported by Smith, and, if the data are adjusted to their specific activity, the V_m is virtually the same as that for α-benzoyl-L-argininamide. The Michaelis constant has a value of 0.050 ± 0.015 (calculated as molar concentration of residues) from *p*H 6 to *p*H 4.5 with no significant trend over this region. Below *p*H 4.5, K_m decreases and no meaningful value could be determined. The value of K_m falls within the same range as those for hydrolysis of small esters, amides and peptides.[21,22] The *p*H dependence of V_m/K_m is normally a bell-shaped curve for papain substrates with apparent pK's of about 4 and 8.[4] The *p*H dependence is clearly different for PGA, although unfortunately values of V_m/K_m could not be determined below *p*H 4.5. Studies with small substrates have shown that the best substrates are positively charged, with negatively charged substrates generally being hydrolyzed at much slower rates.[22,23] The hydrolysis of PGA is quite in contrast as it is an excellent substrate when the carboxyls are 40–60% dissociated.

As was discussed in an earlier section, the breaking of peptide bonds is random down to relatively short chain lengths except for discrimination in the two end bonds. The preceding paragraph applies, then, to all bonds within the polypeptide chain except the end bonds. The rate of breaking the end bonds in a long polypeptide chain may be estimated from chromatograms of samples having a relatively large DP_n. The average rate of splitting off a dimer is 5–10 times less than breaking an interior bond, and the rate of breaking the end bond appears to be at least 100 times less than an interior bond. There is also a systematic trend in the data which indicates that the third and fourth bonds from the end may be a few per cent. less reactive.

Although it is difficult to make a calculation, there appears to be a marked difference in the rate of breaking off a monomer or dimer in a long polypeptide and in a very short chain. In the C series in Table I, where all bonds are within two units of an end, the rate of splitting 4-mer seems to be extremely slow and it is not certain that trimer is split at all. Ignoring any spurious effects in C-6 and C-7, the concentration of trimer is virtually the same at the end of three months at very high enzyme concentration as it was at the end of the first day (C-1). At this enzyme concentration, the viscosity-kinetic measurements would be completed in about one second. At this *p*H, dimer, trimer and tetramer are extremely poor substrates, while bonds are split in the long chain polypeptide at a rate comparable to the best of the small synthetic substrates. This is a very striking example of the effect of neighboring groups on reactivity.

Mechanism of Degradation.—The most important question to answer is the relative importance of conformation *versus* charge. Unfortunately it is also the most difficult to answer. In Fig. 6, the optical rotation and rate of hydrolysis change with *p*H in a strikingly similar manner on the alkaline side of the maximum. The optical rotation is a function of the conformation[6] and if certain conditions are met[24] linear interpolation between extremes in rotation can be used as the content of α-helix. The rate of hydrolysis therefore approximately parallels the helix content, and the rate is meaningfully unmeasurable at *p*H values where there is essentially no α-helix. Since the helix-coil transition is very sharp,[6,25] the extremely rapid change in rate with *p*H becomes reasonable if only helical regions are attacked. This is somewhat puzzling, however, as the peptide bond in an α-helix is less accessible to attack than a peptide bond in a random coil.[26] If charge as well as configuration is important, attack at helical regions becomes more tenable as the degree of ionization in a helical region is somewhat less than in a random coil region.[25,27] If the helical regions are attacked

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much more readily because of a more favorable charge distribution, V_m (% helix) would show a very strong pH dependence as the charge in the helical regions changes with pH. Experimentally V_m changes faster with pH than does the specific rotation but apparently not to a marked degree. The extreme slowness of breaking bonds in peptides of 5 residues or less compared to the rate of breaking long chains at the same pH is also favorable to helical attack only, as several investigators have shown the existence of a critical chain length below which the α-helix does not exist.[28-30] In PGA this critical chain length is around six.[28] The 3 end residues on either end of an α-helix can have only one hydrogen bond per residue and are less stable than the rest of the α-helix. The difference in reactivity of the end bonds in a long chain would be expected, then, as ends are less likely to be helical. At pH values where the conformation is neither all helix nor all random coil, helical regions will be found at various points along the chain, which is consistent with the randomness of the degradation.

It is difficult to explain the rapid decrease in rate on the acid side of the maximum by assuming attack at helically regions, with or without a charge effect. The charge continually decreases and the helical content increases as the pH is lowered, thus necessitating further explanation of the rate behavior. One possibility is that the peptide bond becomes inaccessible due to tightening up of the helix as the charge is diminished. It has been suggested that pairs of uncharged side-chain carboxyls intramolecularly hydrogen bond,[31] this could easily block the attack on a peptide bond by a large enzyme molecule. An alternate explanation may be that the attack occurs only at the junction of helix-coil regions. Rationally this is reasonable as the fraction of bonds involved in junctions will be zero for the pure helix, rise to a maximum value when the configuration is a mixture and fall back to zero for completely random coil. Theoretically[25] the maximum will occur at the midpoint of the helix-coil transition and this is close to what is found experimentally, as seen in Fig. 6. The pH dependence will depend upon the parameters used in making a theoretical calculation and can easily be made as sharp as the V_m-pH curve using reasonable parameters. The previous arguments for the attack on helical regions directly will also apply to attack at junctions. One also avoids the unpleasant situation of a favored attack on a less accessible bond.

Attack at helix-coil junctions is not a well-defined term as several residues will be involved in a junction. Attack at junctions may mean, however, that bond scission is greatly enhanced if the bond is sterically accessible and if the residues near the bond are held at a relatively restricted angle. From this viewpoint one would expect both peptides below the critical helix length and segments of random coil in general to be hydrolyzed but at a very reduced rate. The degradation of the small monodisperse peptides near the critical chain length will be of particular interest and will be the subject of a future publication.

To summarize the over-all view of the mechanism, the data indicate that although there may be an effect of charge, there is also an effect of conformation. A consistent and seemingly reasonable mechanism is enzyme attack on peptide bonds at helix-coil junctions. The mechanism is so tentative and the differences so large that a discussion of the significance of this investigation in relation to the degradation of natural-occurring proteins seems inadvisable at this time.

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