[CONTRIBUTION FROM THE DEPARTMENT OF PATHOLOGY AND ONCOLOGY, KANSAS UNIVERSITY MEDICAL SCHOOL]

The Denaturation of Pepsin. II. Hydrogen Ion Equilibria of Native and Denatured Pepsin[1]

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When pepsin is denatured (> *p*H 6) protons are released into the solution and new acid-binding groups may be titrated in the denatured enzyme. The liberation of protons and the inactivation of pepsin proceed at approximately identical rates. The appearance of new titratable groups has been studied by means of potentiometric titrations of native and denatured protein. The *p*H zone of acid release (enzyme instability) was found to decrease with increase in temperature, ionic strength or ethyl alcohol concentration and when small amounts of certain heavy metallic cations were added. The type of weak bond which appears to conform best with all the data is a hydrogen bond formed between a carboxyl group acting as a donor and possibly a second carboxyl group as an acceptor. The evidence that carboxyl groups also function as acceptors should be considered as circumstantial. Since the ionizing groups are carboxyl and not amino, as postulated by Steinhardt, the heat of activation presumably includes only a quite small contribution from the heat of ionization. Therefore, the large values observed for the heat (and entropy) of activation in pepsin denaturation do characterize this type of reaction and generally can be assumed to imply the destruction of a coöperatively hydrogen-bonded network. The cleavage of specific hydrogen bonds would also eliminate a primarily electrostatic mechanism of pepsin denaturation.

Potentiometric titration curves of proteins (and other charged macromolecules) provide information from which may be derived the number and the dissociation constants of the various kinds of ionizable groups that are accessible to titration. In addition, from deviations from predicted behavior, it is possible at times to obtain information about the area adjacent to certain charged groups[3] or of the existence of weak secondary bonds.[4] In the case of bovine serum albumin, alterations in molecular dimensions have been detected from the nature of the titration curve.[5] Various attempts have been made to correlate electrical potentials of linear polyelectrolytes, as determined from titration curves, with molecular configuration.[6]

Crammer and Neuberger,[7] using a spectrophotometric titration method, showed that the ionization constants of the phenolic groups in egg albumin were abnormally high and these groups became accessible only after (or concomitant with) denaturation of the protein. Similar observations on ribonuclease have been reported by Tanford, *et al.*,[4] and by Shugar,[4] Steinhardt and Zaiser,[8] in their extensive investigation of the denaturation of CO- and ferri-hemoglobin, demonstrated by a titrimetric method that 36 acid-binding groups were liberated below *p*H~4.0. The present report provides another instance where acid-binding groups appear concomitantly with the loss in native structure of the protein. Preliminary accounts of some of the results reported presently have appeared.[9]

Materials and Methods

Pepsin was obtained from the Worthington Biochemical Corp. (Freehold, N.J.) as a 2×-recrystallized product. The methods of dissolving pepsin and a description of its physical properties have been reported.[10] Denatured hemoglobin, which was used for pepsin assays, was obtained from the Pentex Corp. (Kankakee, Ill.). All salts were reagent grade. The water used in the titration experiments was freshly distilled from an all-glass unit.

Pepsin assays followed Anson's hemoglobin procedure with some minor modification.[10] The concentration of hydrolysis products was determined by measurement of the optical density (O.D. = $\log I_0/I$) of the trichloroacetic acid supernatant at 280 mμ. Denatured hemoglobin concentrations were adjusted so as to ensure zero order kinetics.

In kinetic experiments, *p*H values were controlled to 0.01 unit by addition of dilute NaOH. A Beckman *p*H meter, model GS, which was equipped with externally shielded general purpose electrodes, continuously monitored the *p*H of the solution.

Standard buffers[11] were used to calibrate the *p*H meter; from *p*H 2.12 to 9.18 the observed values were within 0.02 unit of the standards, and therefore no corrections were applied. The response of the *p*H meter to temperature was checked by titrating histidine and computing its heat of ionization. A value of 7000 cal./mole (in 0.05 *M* NaCl) was found which is in agreement with published values.[12]

Pepsin solutions were titrated by adding NaOH or HCl (~0.10 *M*) from a microburet of 0.20-ml. capacity. Base was always added very slowly to avoid local excess, since native pepsin is very sensitive to hydroxyl ions. In addition, solutions were always stirred continuously during titration (and kinetic) experiments by magnetic stirrers. In the *p*H range of interest, 5 to 9, corrections for the acidity of the solvent were insignificant and have been ignored.

It is not possible to obtain a titration curve of pepsin in the sense that such information can be obtained about other proteins and enzymes, *e.g.*, serum albumin,[5] lysozyme,[13] etc., where *p*H changes are readily reversible over wide ranges. The difficulties stem from two sources: (1) In the neutral *p*H range (> *p*H 6.0) pepsin is rapidly denatured. (2) Throughout most of the *p*H range of pepsin stability slow autolysis can occur, the degree of which appears to correspond to the *p*H–activity curve of pepsin. These autolytic effects are minimal, between *p*H 5 and 6. Pepsin solutions may be stored in this *p*H zone, at low temperatures (~0°) for days without noticeable effects. Attempts to inhibit autolytic activity at *p*H 1.6 by heavy metals have been unsuccessful.[14] In addition, solutions of pepsin generally are

(1) Supported in part by an Institutional Grant from the American Cancer Society and by grants No. C-1974 and RG 4690 of the National Institutes of Health.

(2) National Institutes of Health, Bethesda, Md.

(3) C. Tanford and J. D. Hauenstein, THIS JOURNAL, **78**, 5287 (1956).

(4) C. Tanford, J. D. Hauenstein and D. G. Rands, *ibid.*, **77**, 6409 (1955); D. Shugar, *Biochem. J.*, **52**, 142 (1952).

(5) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

(6) A. Katchalsky, N. Shavit and H. Eisenberg, *J. Polymer Sci.*, **13**, 69 (1954).

(7) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(8) J. Steinhardt and E. Zaiser, *Adv. Protein Chem.*, **10**, 151 (1955).

(9) Third International Congress of Biochemistry, Brussels, Belgium, Aug. 1–6 (1955), 2–36.

(10) H. Edelhoch, THIS JOURNAL, **79**, 6043 (1957).

(11) R. G. Bates, G. D. Pinching and E. R. Smith, *J. Research Natl. Bur. Standards*, **45**, 418 (1950).

(12) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, Chapter 4, Table 6.

(13) C. Tanford and M. L. Wagner, THIS JOURNAL, **76**, 3331 (1954).

(14) Autolysis, here defined in terms of increase in α-amino nitrogen, was measured by the ninhydrin method. When pepsin (~3 mg./ml.) was incubated with the following metals at the indicated concentrations at *p*H 1.6 and 50°, little or no inhibition of formation of α-amino nitrogen was observed: 0.05 *M* Cd^{++}, Fe^{++}, Mn^{++}; 0.005 *M* Cu^{++},

contaminated with appreciable quantities of split products resulting from autolytic activity. Their concentration may be reduced by dialysis, but cannot be completely eliminated by this method. For these reasons it is not possible to obtain a titration curve of pepsin susceptible to the usual methods of analysis. However, data of interest may be obtained by working in restricted *p*H zones or by measuring irreversible effects. In the latter case, interpretation is aided by kinetic data of denaturation rates.

Results

The Liberation of Hydrogen Ions.—When solutions of pepsin were brought to *p*H values near neutrality with dilute NaOH a steady decline or drift in their *p*H was observed. Solutions adjusted to *p*H 6.40 and 6.74 showed a time-dependent decrease in *p*H. The subsequent rate of fall in *p*H, at four time intervals, appears in Fig. 1. The *p*H of pepsin solutions fixed at 6.00 and 7.12 remained unaltered during the same period of time.

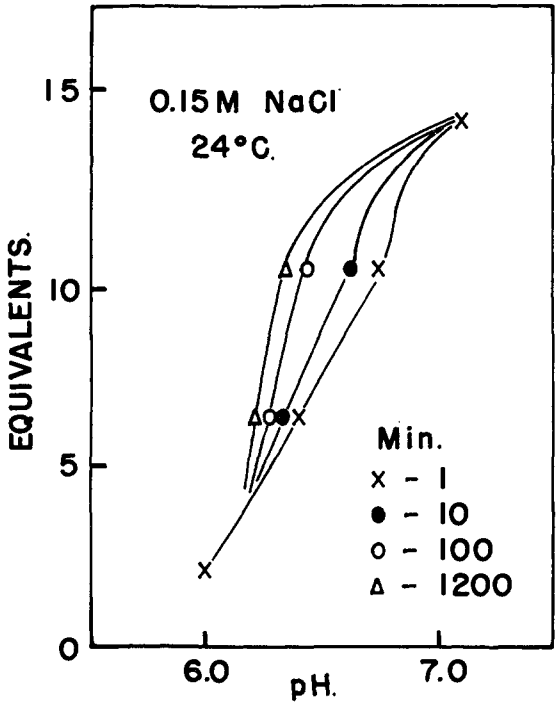

Fig. 1.—*p*H changes in pepsin solutions as a function of time; pepsin concentration = 6.3 mg./ml. (exclusive of 6.3% split products).

It is important to clearly resolve the origin of the *p*H changes shown in Fig. 1 since pepsin titration curves are also not reversible in the region where pepsin shows appreciable autolytic activity. The latter effect precludes the determination of the titration curve below *p*H 4.5. To evaluate the relation between the rates of enzyme inactivation and acid formation, experiments were performed in which both effects were measured simultaneously. In Fig. 2 are reproduced the data of one of these experiments conducted in 0.70 *M* NaCl. The ordinate on the left side of Fig. 2 records the residual peptic activity, in terms of the optical density (at 280 mμ) of the soluble polypeptide products, formed by the action of pepsin on denatured hemoglobin. The line through the points is the theoretical first-order curve of the data. The ordinate on the right is the volume of base (0.0274 *M*) required to maintain the *p*H of the pepsin solution constant at *p*H 6.70.

The amount of H^+ liberated per unit of pepsin activity destroyed decreased as the extent of inactivation increased. The reasons for this decrease are not evident.[15,16] Therefore,

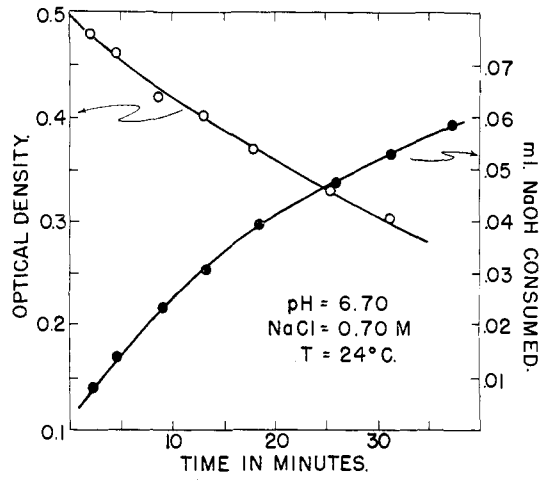

Fig. 2.—Open circles: hemoglobin assay of pepsin activity; line through points is the theoretical first-order curve; filled circles: ml. of NaOH consumed during reaction; solution contained 31.5 mg. of pepsin exclusive of split products (11%).

a first-order plot of the acid liberation data could not be utilized. Consequently, rates of reaction were evaluated by plotting the rate of change in H^+ formation against the time. The data in this plot were then extrapolated to zero time to give initial velocities.

In Fig. 3 is reported the variation with ionic strength of the quantity of acid liberated per constant fraction of pepsin inactivated (0.025 O.D. unit) as determined from the initial rates. This factor is seen to increase below and remain constant above ~ 0.4 *M* NaCl. The increase in acid formation results from the greater difference in electrostatic free energy between native and denatured pepsin solutions at low ionic strengths.

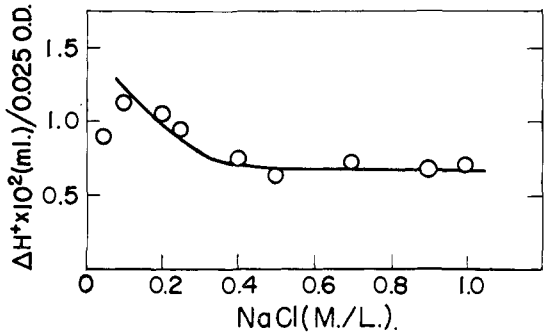

Fig. 3.—Effect of NaCl concentration on acid liberation in pepsin solutions (3.15 mg./ml.); amount of acid liberated per 0.025 O.D. change *at* 0.450 O.D.

The effect of ionic strength on the rate of (1) pepsin inactivation and (2) acid liberation are compared in Fig. 4. As indicated above, below ~0.4 *M* NaCl, the values derived

Hg^{++}. Several metallic ions showed small inhibitory effects at 0.05 *M* Cu^{++}, Zn^{++} and Sn^{++}.

(15) When a solution of 0.55% native pepsin at *p*H 6.70 in 0.10 *M* NaCl was diluted to 0.20% with 0.10 *M* NaCl, the total increase in *p*H was 0.02–0.03 *p*H units. When the same experiment was performed with denatured pepsin, the increase in *p*H was about twice as large. These changes in *p*H on dilution, though tending to reduce the amount of acid liberated, are much too small to account for the observed effects. In 0.01 *M* NaCl, the *p*H increases observed with native and denatured pepsin were twice the values reported above in 0.10 *M* NaCl.

(16) The possibility of peptide bond hydrolysis being in any way responsible for the acid liberated may be ruled out by a consideration of the *pK* values of the α-carboxyl and α-amino groups that would be formed. Typical values of the dissociation constants of these groups are 3.6 (α-carboxyl) and 7.8 (α-amino group). At *p*H 6.7, each α-amino group produced by hydrolysis having a *pK* of 7.8, would liberate only 7.4% of a mole of H^+. Since about 5 or 6 moles of acid are formed per mole of enzyme this hypothesis is clearly untenable. Moreover hydrolytic effects would not be expected to show the pronounced dependence on *p*H which is observed with pepsin.

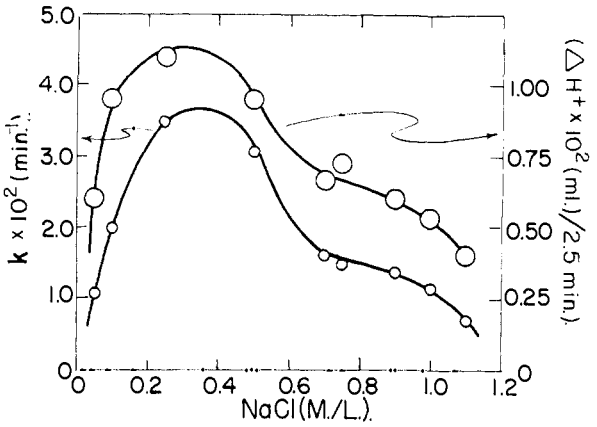

Fig. 4.—Variation of rate of pepsin inactivation with NaCl concentration: small circles, first-order velocity constants (left ordinate) as determined by hemoglobin assay; large circles, initial rates of acid liberation obtained by extrapolating curves of ΔH^+ liberated per 2.5 minutes *vs.* time to zero time.

from acid formation data are enhanced due to electrostatic effects. There appears to be little doubt that the formation of hydrogen ions is intimately associated with the inactivation process.

If pepsin were to produce acid throughout the entire course of its inactivation at the same rate as it does initially, then at high ionic strengths the amount of acid formed was about 7.5 moles per mole of enzyme inactivated. The actual amount released is obviously less than this.

The Alkaline *p*H Limit of Pepsin Stability.—The alkaline limit of pepsin stability was defined by a potentiometric procedure. This method consisted of: (1) the careful addition of a small volume of NaOH (0.053 *M*) to a solution of native pepsin (*p*H ~ 5.4), (2) allowing the solution to stand for five minutes, and (3) neutralization of the base added with an equivalent amount of acid. The *p*H was noted after each change. The final *p*H value was always *less* than the initial value. The difference (Δ *p*H) is plotted in Fig. 5

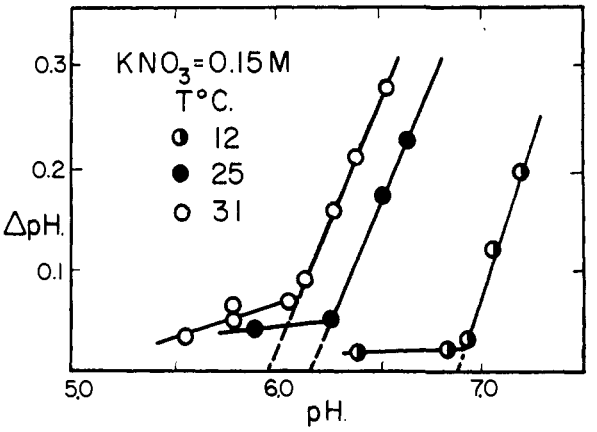

Fig. 5.—Onset of *p*H-instability of pepsin; molarity of acid and base was 0.053; pepsin concentration, 6.0 mg./ml.

against the *p*H of the solution after the addition of base (step 2). A sharp break was observed at a different *p*H value in each curve obtained at three different temperatures. The points acid to the break in the curve (Fig. 5) probably result from a small amount of instantaneous inactivation produced by the alkali.[17] This effect becomes somewhat more pronounced as the *p*H increases and approaches the region of irreversible denaturation, which is indicated by the rapid change observed in Δ *p*H with *p*H (Fig. 5). The *p*H of the onset of irreversibility was considered to be that obtained by extrapolating the alkaline branches to zero value of the ordinate. These values were *p*H 6.00, 6.20 and 6.85 at 31°, 25° and 12°, respectively.

The Titration Characteristics of Native and Denatured Pepsin. I. Effects of Temperature.—The titration curves of solutions of native, denatured, and a mixture of the two forms of pepsin, at 12° and 31°, are shown in Fig. 6. The

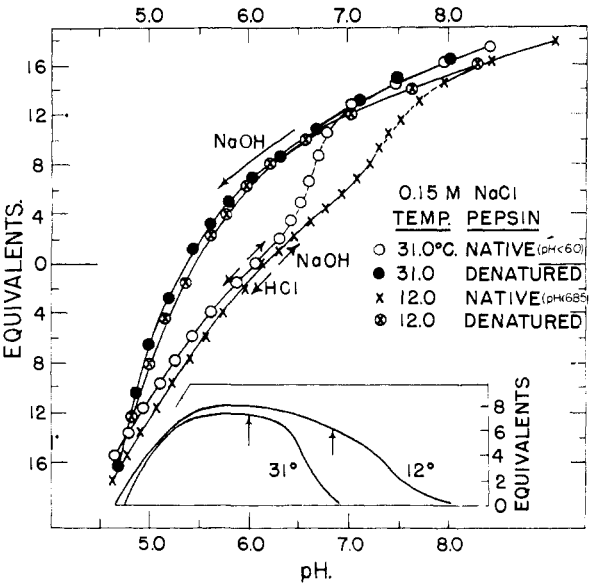

Fig. 6.—The effect of temperature on the titration curves of native and denatured pepsin. Oblique arrows indicate the direction of titration. The broken segments show the *p*H range where acid was liberated at observable rates (the "difference curves" are shown in the inset), pepsin concentration = 4.5 mg./ml.

points encompassed by broken lines represent *p*H values which varied with time and signify the *p*H region of fairly rapid enzyme inactivation. These points were obtained by the arbitrary procedure of waiting until the acid drift in *p*H was less than 0.01 per minute. When this occurred, the titration was resumed by adding more base. The juncture of the forward and reversed curves denotes the *p*H of the complete conversion of the native to the denatured form of pepsin.

The titration curve of denatured pepsin was completely reversible between *p*H 4 and 9. Below *p*H 4 denatured pepsin was not soluble. The titration curve for native pepsin between *p*H 5 and 6 was approximately reversible, in the sense that the reversed curve lies quite close to the forward one. The small differences that were observed (see preceding section) probably result from a small amount of denaturation that occurs when mixing base with native pepsin. Below *p*H 5.0, larger deviations were observed, probably arising from the proteolytic activity (autolysis) of pepsin at more acid *p*H values. For these reasons the titration curve of native pepsin cannot be treated quantitatively. However, the effects of certain environmental variables and reagents were so pronounced and unmistakable that their evaluation, even in a qualitative sense, is quite instructive.

It is evident that the ionization behavior of the titratable groups shown in Fig. 6 is strikingly different in the two forms of pepsin. The pertinent features of the data may be ascertained most readily from a difference curve, obtained by plotting the vertical displacement between the forward

(17) The values of peptic activity found by extrapolating the first-order plots to zero time generally do not account for all the activity present initially. This effect undoubtedly arises from the marked susceptibility of pepsin to alkali inactivation and occurs during the adjustment of the pepsin solution with base to the *p*H value under investigation. Steinhardt[33] has made a careful study of these effects and concluded that an instantaneous inactivation does not occur with pepsin, as postulated by Goulding. The addition of strong base to pepsin solutions results in what appears to be an effect of this kind (A. M. Goulding, H. Wasteneys and H. Borsook, *J. Gen. Physiol.*, **10**, 451 (1926-1927)).

(base addition) and reversed (acid addition) titration curves. These curves measure the combined effects of all the prototropic changes involved in the denaturation process.[18]

It is of significance that the acid segments of the difference curves at 12 and 31° overlap, whereas the alkaline segments are displaced by about 0.70 *p*H unit at their mid-points—considered from the vertical arrow to the alkaline intercept with the abscissa. The vertical arrows in the inset of Fig. 6 indicate the *p*H of the onset of irreversible denaturation as set forth in the preceding section.

The height of the difference curves provides a measure of the amount of acid released by the denaturation process. Both curves (Fig. 6, inset) are reasonably flat over a considerable range of *p*H and account for the formation of between 7 and 8 moles of hydrogen ions. These values are similar in magnitude to those calculated from the kinetic procedures discussed above.

The titration data provide clear evidence of a decrease in the proton affinity of a number of acid binding groups. In the denatured protein these groups are undifferentiated in their titration behavior (*pK*) from the carboxyl groups which were reversibly titrated in the native enzyme. It will be assumed now and discussed more fully later, that these groups are also carboxyl.

The data in Fig. 6 may be analyzed in another way which furnished a somewhat different perspective of the titration curves. If the horizontal differences between the forward and reversed curves are plotted against the ordinate value, the curves portrayed in Fig. 7 are obtained. The ordinate values in Fig. 6 afford a measure of degree of dissociation of hydrogen ions from pepsin (and split products) and hence provide information of the variation in protein charge with *p*H.

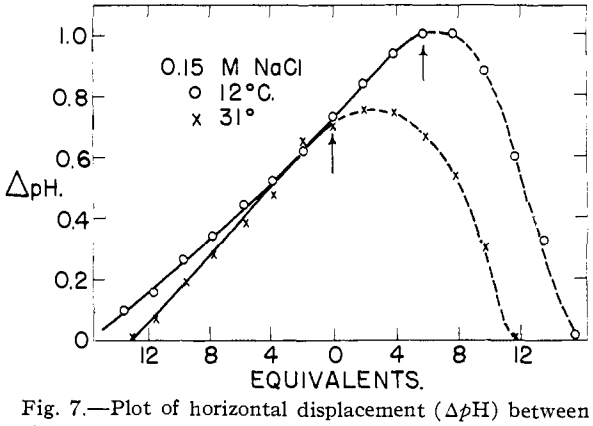

Fig. 7.—Plot of horizontal displacement (ΔpH) between native and denatured pepsin as a function of the net charge—taken from data in Fig. 6. Dashed part of curves indicate region of pepsin instability; vertical arrows obtained from data in Fig. 5.

The Δ *p*H values observed prior to denaturation (vertical arrows in inset, Fig. 6) indicate the magnitude of the *difference* in the electrostatic free energy between native and denatured pepsin. This term is seen to increase almost linearly with charge until the irreversible titration commences. From the larger ΔpH values of the 12° curve, at the onset of denaturation, relative to the curve at 31°, and the shift of the right-hand branch of the curves to larger values of net charge, it would appear that the number of groups dissociated in the native protein prior to denaturation was about 4 to 6 greater at 12° than at 31°.

II. The Effects of Ionic Strength.—The principal influence of neutral salt is to decrease the electric field surrounding the charged pepsin macromolecule. Electrostatic effects modify the ionization behavior of both the reversible and irreversibly titrated carboxyl groups. The experimental titration values should be affected in accord with electrostatic theory though the intrinsic values of the carboxyl dissociation constant should be essentially independent of these effects.[5,19]

The titration curves of pepsin in 0.01 and 0.15 *M* NaCl at 24° are shown in Fig. 8. Zero value of the ordinate indicates the *p*H of the stock solution at the two salt levels. *p*H values of the native curves, acid to the stock solution values, were obtained by titrating with HCl. A back-titration curve from *p*H 4.60 to 5.48 is shown also at 0.15 *M* NaCl by the half-filled circles. This curve lies quite close to the forward curve though it does not coincide exactly with it. Therefore, complete thermodynamic reversibility was not achieved with native pepsin solutions when titrating with either HCl or NaOH.

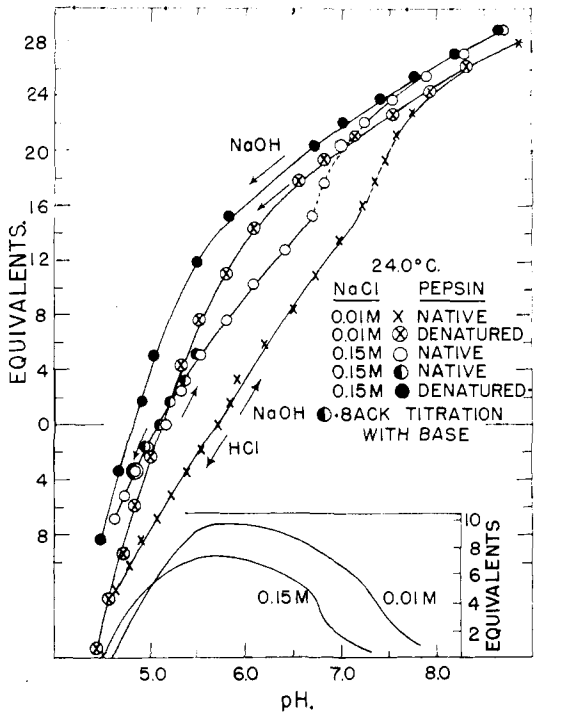

Fig. 8.—The effect of ionic strength (NaCl) on the titration curves of native and denatured pepsin; arrows show direction of titration; region of *p*H instability indicated by broken lines; "difference curves" appear in inset. A back titration curve with base from *p*H 4.60 to 5.64 is shown by half-filled circles (◐); pepsin concentration = 5.0 mg./ml.; split products = 6.3%.

The difference curves are shown in the inset in Fig. 8. Except for the difference in height they resemble the curves in Fig. 6 in that the acid branches overlap while the alkaline branches are displaced from each other. The mid-points of the alkaline segments occur at *p*H 6.65 and 7.25 in 0.15 and 0.01 *M* NaCl, respectively. (In 0.50 *M* NaCl the corresponding point occurred at *p*H 6.30.) In harmony with the kinetic observations reported above more acid was liberated at lower ionic strengths. The maximum in the difference curve at 0.01 *M* NaCl was about 2–3 equivalents larger than at 0.15 *M* NaCl.

An important feature of protein (or any polyampholyte) reactions conducted at constant *p*H, which has at times been neglected, should be emphasized at this point. If we allow that the ordinate is a measure of the degree of dissociation of hydrogen ions from pepsin, then at any *p*H different from that of the isoionic point, the total number of charged groups will not be the same at a given *p*H value at different ionic strengths. Thus, it can be seen from Fig. 8 that at any *p*H between 5.0 and 6.0 the number of charged carboxyl groups and hence the net charge is increased by about 6 as the NaCl concentration changes from 0.01 to 0.15 *M*. This effect will be larger the further displaced the reaction

(18) The contributions of the split products present in pepsin solutions to the titration curves are cancelled in the difference curve since their ionization behavior would be completely reversible. The concentration of split products should remain constant since the autolytic activity of pepsin is very small at these high *p*H values.

(19) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

pH is from the isoionic point of the protein and the greater the disparity in electrolyte concentration. These effects are clearly evident in titration curves performed at several ionic strengths[3,13] and are approximately accounted for by simple electrostatic theory.[20]

III. The Effects of Heavy Metals.—It has been demonstrated that the environmental factors of temperature and ionic strength influence the titration characteristics of pepsin. Under favorable circumstances certain reagents may be shown to produce comparable effects.

When relatively small amounts of certain heavy metal ions (Cu^{++}, Pb^{++}, Cd^{++}) were added to pepsin solutions, the pH range of pepsin instability occurred at more acid values than was observed in the absence of these ions. The effects of metallic ions on pepsin "titration curves" are cogently demonstrated from their difference curves (Fig. 9).

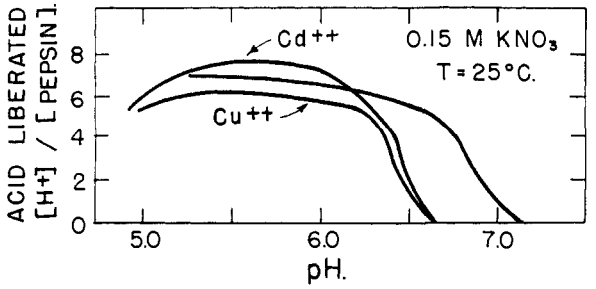

Fig. 9.—Effect of Cu (5.0 moles/mole pepsin) and Cd (8.5 moles/mole pepsin) on the "difference curve" between native and denatured pepsin; unlabeled curve has only KNO_3 and serves as a control; pepsin concentration = 6.6 mg./ml.

A shift in the alkaline wing to more acid values was evident when small amounts of Cu^{++} or Cd^{++} were present.[21] Other metals, such as Mg^{++}, Mn^{++}, Co^{++}, showed no tendency to displace the titration (or difference) curves. Those metals that were effective in causing an acid shift in the irreversible portions of the titration curves proved also to be effective in enhancing the rate of pepsin inactivation. On the other hand, the metals which had little influence on the titration properties were without influence on enzyme inactivation rates. The quantitative effects produced by metallic ions on pepsin inactivation rates are reported in detail in a companion paper.[22]

It is important to note at this time that the effective metals were those which possess a high affinity for carboxylate ions. In fact the order of the efficacy of these metals closely paralleled their affinity constants for carboxylate groups.[22]

IV. The Effects of Ethyl Alcohol.—Difference curves of the hysteresis loop in pepsin titration curves obtained in the presence of ethyl alcohol are shown in Fig. 10.[23] The decrease in pH with alcohol concentration of the right limb of the curves indicates an increase in the acid strength of these groups of $\sim$ 0.25 pH unit per 10% change in ethyl alcohol concentration (v./v.). The decrease in acid strength of the left limb, which also amounts to about 0.25 pH unit for a similar change in ethyl alcohol concentration, conforms approximately to the expected effect of alcohol on the pK values of carboxyl groups in small molecules.[24] In kinetic enzyme inactivation experiments it was necessary to reduce

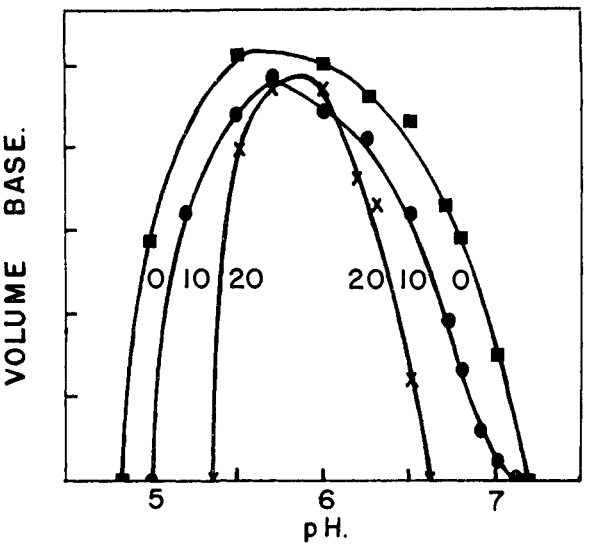

Fig. 10.—Effect of ethyl alcohol on the "difference curves" of pepsin; NaCl = 0.15 M.

the pH by 0.22 unit for each increase of 10% in ethyl alcohol concentration in order to keep the rate constant.[22]

The Effect of Ionic Strength and Temperature on Pepsin Inactivation Rates.—The effect of temperature and ionic strength on pepsin titration curves can be correlated with pepsin inactivation rates. The interrelation between pH, temperature and ionic strength, as determined by the first-order kinetic constants of pepsin inactivation, is depicted in Fig. 11. Velocity constants were found to vary inversely

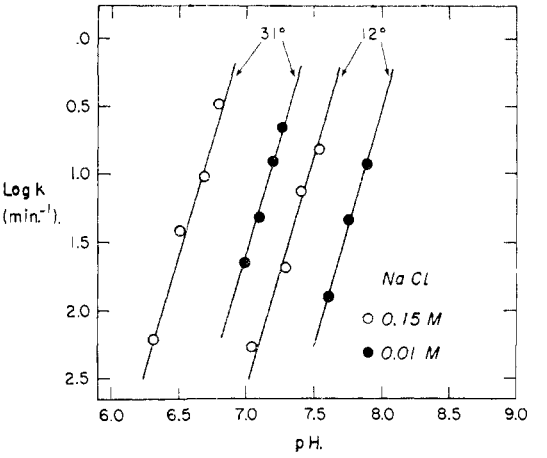

Fig. 11.—pH dependence of the first-order velocity constant (k in reciprocal minutes) of pepsin inactivation on temperature and ionic strength.

with the $\sim$ 3.4 power of the hydrogen ion activity in the pH range investigated. The pH dependence was independent of both NaCl concentration and temperature. Thus in 0.15 M NaCl the two lines in Fig. 11 are parallel to each other but displaced by 0.78 pH unit. In the difference curves of the titration experiments the mid-points of the irreversible segments were displaced by 0.70 pH unit. Considering the qualitative nature of the latter procedure, there does appear to be a significant correspondence between the two methods. These values are also in approximate agreement with pH-stability limits reported earlier (Fig. 5) where a pH difference of 0.85 was noted between these two temperatures (6.00 at 12° and 6.85 at 31°). It can be seen by extrapolating the curves in Fig. 11 to these pH values that these limits correspond to inactivation rates of 0.001 min.$^{-1}$. In 0.01 M NaCl the two lines in Fig. 11 are displaced by 0.70 pH unit. The mid-points of the alkaline

(20) K. Linderstrøm-Lang, *Compt. rend. trav. Lab. Carlsberg*, **15**, No. 7 (1924).

(21) The titration curves with Cu^{++} present were substantially different from the controls due to the combination of Cu^{++} with base. Since this reaction was reversible the difference curve records only the interaction with pepsin.

(22) H. Edelhoch, THIS JOURNAL, **81**, 6648 (1959).

(23) Acid is absorbed in 28.5% ethyl alcohol solutions of pepsin[22]; therefore, at some concentration between 20 and 28.5% the forward and reversed titration curves should coincide and no pH drifts will be observed during inactivation.

(24) The pK values of the carboxyl group of propionic acid in 0, 10 and 20% (w./w.) ethyl alcohol–water mixtures at 25° is 1.336, 0.900 and 0.781 $\times$ 10^{-5}, respectively; see H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," 2nd ed., Reinhold Publ. Corp., New York, N. Y., Table B-10-2, p. 613.

segments of the difference curves were found to be displaced by 0.75 *p*H unit.

From a similar analysis of the effects of ionic strength (Fig. 8) on titration curves obtained at 24° a ΔpH of 0.60 unit was evaluated. At 31° at the corresponding salt levels (0.01 and 0.15 *M* NaCl), the curves in Fig. 12 are 0.50 *p*H unit displaced. Accordingly, there seems to be little doubt that "titration curves," performed in the *p*H region where pepsin undergoes irreversible structural and prototropic changes, do provide significant clues of its kinetic behavior pattern.

The Ultraviolet Absorption of Tyrosine in Acetic Acid–H_2O and Dioxane–H_2O Solutions.—Edsall, Hollingworth and Wetlaufer[25] have observed that acidified solutions of tyrosine show similar differential spectral changes, with reference to the isoelectric state, as occur with insulin[26] and ribonuclease.[27,28] We have found that by changing the dielectric constant of the solvent, as in aqueous solutions of acetic acid or dioxane, notable increases in tyrosine absorption occurred. In acetic acid–H_2O solutions the differential spectral peaks, which occurred at about 278 and 285.5 mμ, increased linearly with the content of acetic acid to 70% (v./v.), where the peaks were about 0.04 and 0.10 O.D. unit above the values of a 0.56 m*M* solution of isoelectric tyrosine in water, which served as the reference solution. Above this acetic acid–H_2O ratio, the optical density of the differential spectral peaks increased even more rapidly.

In dioxane–water solutions, the differential peaks were shifted to slightly higher values (about 1 mμ for each peak). A linear increase in O.D. of the difference peaks was observed from 0 to 80% (v./v.) dioxane. In the 80% solution of dioxane the O.D. values at the two peaks were 0.23 and 0.38 unit greater than that of a 0.56 m*M* solution of aqueous tyrosine.

The Ultraviolet Absorption of Native and Denatured Pepsin.—Perlmann[29] reported a shift in the ultraviolet absorption spectrum peak, from 2780 to 2760 Å., when pepsin was denatured in 8 *M* urea at *p*H 5.3. Similar modifications in the spectrum of insulin[26] and ribonuclease[27,28] have been interpreted in terms of the rupture of hydrogen-bonded tyrosyl hydroxyl groups.

When pepsin was denatured either by raising its *p*H from 5.8 to 7.2 in 0.10 $\Gamma/2$ phosphate buffer or by briefly heating the *p*H 5.2 solution at $\sim$ 70°, a diminution in its spectrum below 295 mμ was observed as shown in Fig. 12. The peaks at 279 and 286 mμ are due to tyrosine while the peak at 292 mμ presumably arises from the tryptophan residues. The O.D. values were half as great in solutions containing half as much pepsin. When we consider the significant changes that can be induced in tyrosine absorption by solvent effects, it would seem premature to assign a specific structural character to the tyrosine hydroxyl groups in pepsin at this time. The folding of the peptide chains has been shown to be drastically altered when pepsin is denatured.[10] This process undoubtedly modifies the environment of the hydrophobic residues from a relatively lipophilic to a predominantly aqueous one, which may also account for the observed spectral changes.

Discussion

Anomalies in the titration characteristics of pepsin have been noted earlier by Conn, *et al.*[30] The rapid advances that have been made in recent years in our concepts of protein structure,[31] and especially of intramolecular hydrogen bonding situations, have permitted the identification of the major irreversible effects encountered in pepsin titration curves, in terms of specific hydrogen-bond donor groups.

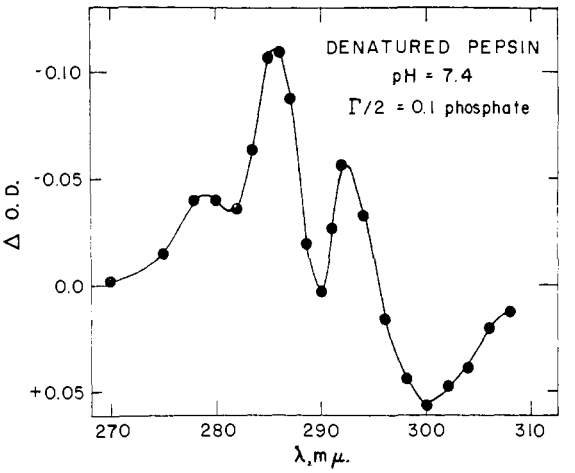

Fig. 12.—Ultraviolet difference spectra of denatured pepsin at *p*H 7.4, 0.1 $\Gamma/2$ phosphate. The control cuvette (1.00-cm. path) contained a sample of native pepsin at *p*H 5.8, 0.1 $\Gamma/2$ phosphate. The pepsin concentration was 1.72 mg./ml.

Steinhardt and Zaiser[8] have provided recently a very valuable review of the hydrogen ion equilibria in native and denatured proteins. They list 4 basic criteria which must be fulfilled when prototropic groups become accessible in the conversion of a native protein to a new modification. As has been shown, pepsin is an unqualified member of this class of proteins.

The chemical bond whose properties, though certainly not understood *in extenso*, appear to conform in outline to essentially all the behavior characteristics observed with native pepsin when subjected to a wide variety of experimental conditions, is a hydrogen bond between a carboxyl group which acts as the proton donor and, as yet, an unspecified acceptor group. Various possible acceptor groups will be considered subsequently. It is of interest to note that Fraenkel-Conrat has observed the appearance of new titratable groups in tobacco mosaic virus when it is denatured by sodium dodecyl sulfate.[32] These groups titrate between *p*H 3.5 and 7.0 and tentatively have been assumed to be carboxyl groups which are hydrogen-bonded in the intact virus. Moreover, Fraenkel-Conrat has found that Pb^{++} ions can substitute for the hydrogen-bonded protons.

Of the data we possess of pepsin denaturation, the large temperature coefficients[33] and the action of urea and guanidine[34] indicate the involvement of hydrogen bonds. The large *p*H dependence of inactivation rates, the variation of the order of inactivation in urea and guanidine solutions with respect to *p*H and the effects of certain metallic cations specifically point to the participation of carboxyl groups in the hydrogen bonds. The detailed effects of urea, guanidine and heavy metals

(25) J. T. Edsall, B. R. Hollingworth and D. B. Wetlaufer, Abstracts of Papers, Amer. Chem. Soc., Sept., 1957, New York, p. 32c.

(26) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga, *Biochim. et Biophys. Acta*, **19**, 581 (1956).

(27) H. A. Scheraga, *ibid.*, **23**, 196 (1957).

(28) W. F. Harrington and J. A. Schellman, *Comp. rend. trav. Lab. Carlsberg, Ser. Chim.*, **30**, 167 (1957).

(29) G. Perlmann, *Arch. Biochem. Biophys.*, **65**, 210 (1957).

(30) J. B. Conn, D. C. Gregg, G. B. Kistiakowsky and R. M. Roberts, THIS JOURNAL, **63**, 2080 (1941).

(31) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci. U. S.*, **37**, 205 (1951); L. Pauling and R. B. Corey, *ibid.*, **37**, 235-283 (1951).

(32) H. Fraenkel-Conrat, *Fed. Proc.*, **16**, 810 (1957); also personal communication.

(33) J. Steinhardt, *Kgl. Danske Videnskab. Selskab. Mat. fys. Medd.*, **14**, No. 11 (1937).

(34) J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. Chim.*, **29**, 223, 230 (1955).

are presented in the companion paper.[22] The pertinent aspects of this work, insofar as it provides evidence for the existence of carboxyl hydrogen bonds, will now be summarized.

If urea and guanidine affected only the intrahelical hydrogen bonds it would seem unlikely that the concentration of urea or guanidine needed to produce a given rate of denaturation would be dependent on the *p*H. However, from *p*H 6.2 to 6.5 the concentration sufficing to give a rate constant of $k = 0.01$ (min.$^{-1}$) varies approximately with the square of the hydrogen ion activity; this dependence on acidity is closer to first order from *p*H 5.7 to 6.0.

The marked effects of certain metals in enhancing inactivation rates serves further to incriminate carboxyl groups as the hydrogen donors. The order of the relative effectiveness of Cu^{++}, Pb^{++} and Cd^{++} closely parallels their affinity for carboxylate ions. Furthermore, these metal ion effects are only evident in the ionization range of the carboxyl hydrogen-bonded groups thereby indicating that they function competitively by virtue of their affinity for carboxylate ions.

The contribution of the α-helical peptide groups to pepsin stability is not evident from any of our measurements. If the increase in levorotation that occurs on denaturation reflects the disorganization of intramolecular hydrogen-bonded α-helices, as proposed by Yang and Doty,[35] then we may conclude from the equivalence in rates of denaturation as observed by optical rotation and acid liberation[36] that the α-helical structure (secondary) is lost at the same rate as the side chain carboxyl hydrogen bonds. The secondary structure therefore possesses, at best, marginal stability which must be supplemented by other intramolecular bonds to provide the energy necessary to stabilize the native configuration.[37] It is of particular interest to note that pepsin undergoes by far the smallest optical rotatory change on denaturation when compared with a group of ten enzymes and proteins, as listed by Schellman and Schellman.[38] In addition to carboxyl hydrogen-bonded groups, hydrophobic forces undoubtedly contribute significantly to the stability of the active enzyme.[22]

Number of Hydrogen-Bonded Groups.—The *total number* of protons liberated, and consequently acid groups ionized, may be estimated from the "difference curves" when measured at high supporting electrolyte concentrations. The height of these curves decreases with ionic strength and reaches a limiting value of 5 to 6 in 1 *M* NaCl. The various complexities involved in kinetic measurements and the uncertainties encountered in the titration experiments prohibit the evaluation of a more definitive value of the number of carboxyl-linked hydrogen bonds

The Nature of the Acceptor Groups.—The hypothesis originally proposed by Steinhardt,[33] which is still quite tenable, is that the unusually large *p*H dependence of inactivation rates suggests an ionization process involving one type of functional group as part of the activation mechanism. These groups now appear to be carboxyl which are stabilized in the active enzyme by hydrogen bonding.[39] The *p*K values of these carboxyl groups will depend, to a certain extent, on the nature of their acceptor groups. Laskowski and Scheraga[19] (and Loeb and Scheraga[40]) have analyzed various hydrogen-bonding combinations and shown that the *pK* of the donor group may be independent of, increase or decrease with *p*H depending on the nature of the acceptor group(s). When the *pK* decreases with increase in *p*H, the dissociation curve will become steeper than in the non-hydrogen bonded case. In addition, the *p*H range of ionization (apparent *pK*) will increase as the energy of the hydrogen bond or the extent of coöperation between hydrogen-bonded groups increases. If the rate of inactivation is a function of the degree of ionization of the hydrogen-bonded groups, then this type of coördination could furnish an explanation of the marked *p*H dependence of pepsin denaturation rates. The only simple hydrogen-bonding situation that would exhibit this unusual variation in ionization with *p*H would also require the acceptor group to form a coöperative bond and possess a dissociable group with a *pK* quite close to that of the carboxyl donor group. This mechanism immediately rules out positively charged nitrogen groups, which cannot function as proton acceptors[41]; it would also preclude the participation of all non-ionizable acceptors and leave only a second carboxyl group as suitable since other possible ionizable groups (phenolic hydroxyl, etc.) do not have appropriate *pK* values. The carboxyl–carboxyl groups could form either an homologous double bond or a coöperative system if more than two groups were coupled. Though not constituting what we might refer to as further proof of this structure it is of importance to note that the unusual distribution of charged groups in pepsin is quite compatible with the bonding situation just discussed. There are only seven free basic groups in pepsin (1 α-amino, 2 histidine, 2 lysine and 2 arginine) compared with about 38 free carboxyl (1 α-carboxyl). It is perhaps not without significance to this study to note that carboxyl-carboxyl hydrogen bonds (in acetic acid) are energetically about the strongest known.[42]

(35) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(36) H. Edelhoch, *ibid.*, **76**, 2644 (1956).

(37) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. Lab Carlsberg, Ser. Chim.*, **30**, 21 (1956).

(38) C. Schellman and J. A. Schellman, *ibid.*, **30**, 463 (1958).

(39) Since the isoelectric point of pepsin is below *p*H 1 there will be an appreciable electrostatic contribution to the ionization of all charged groups. The magnitude of the decrease in the acidity of the reversibly titrated carboxyl groups due to electrostatic effects is shown in Fig. 8. This decrease can amount to almost 1 *p*H unit. If the onset of the irreversible sections of the titration curves may be taken as a rough measure of the free energy of the hydrogen-bonded carboxyl groups (*p*H 6.0 and 6.85 at 12 and 31°, respectively) then the difference between these values and the non-bonded carboxyl groups is within the range that might be expected for hydrogen bonding effects, as determined by Laskowski and Scheraga.[19] Since the next nearest ionizing group is imidazole with a *pK* value about 2 units above that of carboxyl, this and more basic groups may be ruled out as possible hydrogen bond donor groups observed in the titration experiments.

(40) G. I. Loeb and H. A. Scheraga, *J. Phys. Chem.*, **60**, 1633 (1956).

(41) Uncharged nitrogen groups are also precluded as possible acceptors since they must be protonated in mildly acidic solutions where pepsin is quite stable. Moreover, a carboxyl-uncharged imino (or amino) bond would not liberate protons when cleaved in the neutral *p*H range.

(42) F. W. Johnson and L. K. Nash, THIS JOURNAL, **72**, 547 (1950).

Comparison with Steinhardt's Results.—Any report on pepsin inactivation rates would be incomplete without recognition of the classical investigation of Steinhardt twenty years ago.[33] The data obtained in the present study are essentially in accord with his results.[43] However, various aspects of Steinhardt's interpretation of the experimental results need some modification.

Steinhardt interpreted the change in pH dependence in terms of a series of acid dissociations of 5 critical groups in which the observed rate depended solely on the concentration of the ionic form of the last group to be ionized. The shape of the resultant rate–pH curve then depicts a gradual reduction in slope as the concentration of the terminal ionized species becomes appreciable. Steinhardt deduced a pK value of 6.76 for the ionization constant of the five critical groups and suggested that they might be the five free amino groups of cystine. Moreover, since the effect of temperature on reaction rate was principally to shift the rate–pH curve horizontally without change in slope, Steinhardt considered the ΔpK of this displacement to include the heat of ionization of these five basic groups. Accordingly, each dissociation was assigned a ΔH of 9040 calories. In this way, a $\Delta H^{\mp}$ value of 18.3 was obtained for the activation process which would then be comparable in magnitude to that observed for small molecule reactions. This hypothesis is open to question since the critical groups are now identified as carboxyl. In addition, later amino acid analysis show only 3 cystine residues, all of which form cross-links in the polypeptide chain.[44]

Other Theories of Pepsin Denaturation.—McLarin and Lewis[45] and also Kauzmann[46] have developed theoretical expressions relating intramolecular electrostatic effects to protein stability. Schellman and Linderstrøm-Lang have calculated a free energy change of 15.7 kcal./mole in favor of the unfolded (and completely extended) form of clupein relative to an α-helical structure.[34] There is not much doubt that electrical effects can considerably modify the equilibrium between native and unfolded or expanded forms of proteins, as is evident in the behavior of serum albumin.[5,47]

The rather large increase in velocity with electrolyte concentration observed by Steinhardt was interpreted on the basis of a primary salt effect (Brönsted theory). However, if we conceive of pepsin denaturation as resulting from an imbalance of intramolecular forces then the effects of ionic strength must be looked upon in another way. One force tending to promote denaturation is electrostatic in origin. Pepsin possesses an appreciable net negative charge in the neutral pH range which undoubtedly leads to intramolecular strain.[45,46] If pepsin denaturation resulted principally from these electrostatic forces, then we would expect an increase in electrolyte concentration to reduce these intramolecular repulsive forces and consequently the rate of denaturation to fall. This of course is not what is observed. However, increase in ionic strength also serves to increase the net charge on the protein when measurements are made at constant pH (see Fig. 8). It is this factor which appears to be principally responsible for the large increase in rate that is observed below $\sim 0.4\ \Gamma/2$.

It is of interest to note that pepsin attains a larger net negative charge prior to denaturation at 12° than at 31° (Figs. 6 and 7). If electrostatic repulsive forces were solely responsible for pepsin denaturation, then the rate would be expected to be greater at 12° than at 33°. It would appear therefore that the rupture of a few carboxyl hydrogen bonds is the primary event leading to inactivation. It should also be mentioned that there is no physical evidence, either from sedimentation velocity or viscosity, suggestive of the occurrence of other macroscopic forms of pepsin which may have been induced by intramolecular repulsive forces.

Relationship of Stability and Activity.—It is of interest to note briefly the conclusions of Herriott[48] concerning the active sites of pepsin. From data relating the degree of inactivation with the number of bound reagent molecules (sulfur mustard, ketene and iodine) Herriott has implicated both the carboxyl and tyrosyl groups as the sites of peptic activity. In fact, a maximum number of 5 and 7–8 have been assigned to each group, respectively. The close similarity of these data with respect to the carboxyl groups with that derived from denaturation studies is rather striking. However, it would appear to be premature at this time to advance the hypothesis that the enzymic stability and activity are dependent on the identical groups in the native molecule until the molecular structure of the various derivatives has been very closely examined.

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(43) An apparent discrepancy between Steinhardt's data and ours is in the pH dependence of inactivation rates. His inverse fifth power dependence on hydrogen ion activity was obtained from data in the velocity range of log k (min.$^{-1}$) of ~ -4.5 to -2. As Steinhardt states,[33] "At the highest velocities, the proportionality of velocity to the inverse fifth power of the hydrogen-ion concentration no longer prevails; further increase in pH produce a relatively smaller increase in velocity." In fact, in the legend to his Fig. 5, from a limited number of experimental points, he states that a slope of three expresses the data. It appears, therefore, that the pH dependence varies from a factor of five at very low rates (low pH values) to 3.4 at the rates investigated presently.

(44) Green and Neurath, in "The Proteins," ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., Vol. II, Part B, p. 1087, on other grounds have indicated the unliklihood of the five critical groups being amino groups, as postulated by Steinhardt.[33]

(45) A. D. McLarin and C. Lewis, *J. Polymer Sci.*, **5**, 379 (1950).

(46) W. Kauzmann in "Mechanism of Enzyme Action," ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954.

(47) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, THIS JOURNAL, **77**, 6421 (1955); C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(48) R. M. Herriott, *J. Cell. Comp. Physiol.*, **47**, Suppl. 1, 239 (1956).