[CONTRIBUTION FROM THE DEPARTMENT OF PATHOLOGY AND ONCOLOGY, KANSAS UNIVERSITY MEDICAL SCHOOL, AND THE INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

The Denaturation of Pepsin. III. The Effects of Various Protein Denaturants on the Kinetics of Pepsin Inactivation[1]

BY HAROLD EDELHOCH[2]

RECEIVED MARCH 7, 1958

The effects of guanidine and urea, metallic cations, ethyl alcohol and trimethyldodecylammonium chloride, and several combinations of reagents on the enzyme stability of pepsin have been evaluated by means of kinetic inactivation experiments. First-order velocity constants were obtained in all experiments. In all instances a range of *p*H was investigated. It was adduced that *p*H, urea (and analogs) and specific metallic cations modified the stability of pepsin by their effect on carboxyl (donor) hydrogen bonds. The latter bonds are assumed to be responsible for the strong *p*H dependence observed for pepsin inactivation. Ethyl alcohol and trimethyldodecylammonium chloride, however, principally perturb the intramolecular hydrophobic interactions and consequently lead to decreased stability of the carboxyl hydrogen bonds. An independent contribution by the α-helical hydrogen bonds to pepsin stability has not been found. Pepsin may be inactivated either by rupture of its carboxyl hydrogen bonds or by loss of its hydrophobic bonding. The latter mechanism is favored in high concentrations of alcohol while the former predominates in aqueous medium or dilute alcohol solutions. When pepsin was denatured in water–ethyl alcohol solutions containing guanidine or $Pb(NO_3)_2$, the velocity constants indicated that each reagent exerted an independent effect. Hence, it appears likely that ethyl alcohol acts on different bonds from those attacked by guanidine and $Pb(NO_3)_2$.

In the first report of this series[3] the molecular properties of solutions of native and alkali-denatured pepsin were characterized by various methods. In a second communication,[4] the influence of temperature, ionic strength, ethyl alcohol and certain metal cations on the titration curves of native and denatured pepsin was examined. The titration data could best be interpreted in terms of the existence of 5 or 6 carboxyl (COOH–donor) hydrogen bonds. The purpose of the present investigation was to obtain kinetic evidence of the mode of interaction of a variety of so-called denaturing reagents with pepsin, paying particular attention to the carboxyl hydrogen-bonded groups. To this end the kinetics of pepsin inactivation was studied over as broad a *p*H range as seemed of interest with all the denaturants.

Methods and Materials

All rate measurements were obtained by enzymatic assay of pepsin activity by the hemoglobin substrate method.[3] No buffers were used. Since acid was liberated in most experiments, the *p*H of the reaction was maintained constant by the continuous manual addition of dilute base to the temperature-controlled vessel which contained shielded glass and calomel electrodes of the Beckman GS *p*H meter. Normally the *p*H could be regulated to about ± 0.01 *p*H unit during a run. Since the rate of pepsin inactivation varies with the 3.4th power of the *p*H,[4] a change 0.01 *p*H unit leads to an uncertainty in rate constant of about 10%. The velocity constant data were obtained from the slopes of first-order plots which allowed a variation of about 20% or less. Occasionally experimental points obtained at the latter stages of inactivation were found to lie above the line connecting the earlier points. If the initial part of the reaction conformed to a first-order plot the later points were ignored. Good data were somewhat more difficult to obtain when detergents were present.

In pepsin solutions containing 28.5% (v./v.) or more of ethyl alcohol, acid was absorbed (*p*H tended to increase) during inactivation. In these experiments it was necessary to add acid to keep the *p*H constant. The rate of acid uptake corresponded to the rate of enzyme inactivation. The change from acid release to acid binding at about 25% ethyl alcohol can be rationalized if we assume that the principal component[3] of denatured pepsin contains fewer basic groups than the native enzyme. It has been observed that the titration curve of the denatured enzyme always intersects that of the native enzyme at about *p*H 4.5 in aqueous media.[4] In alcohol the intersection of the two curves occurs at higher *p*H values. At the same time the alkaline junction point decreases in *p*H due to the lower stability of the enzyme in alcohol solution. Between 19 and 28.5 ethyl alcohol, the hysteresis loop must disappear and the position of the titration curves reverse.

In solutions of Arquad-12 and in many of the metal ion experiments as well as at higher concentrations of guanidine and ethyl alcohol (>28.5%) pepsin solutions became increasingly turbid as inactivation progressed. However, the appearance of turbidity or even flocculation had no effect on the course of the reaction which continued to follow first order kinetics.

All experiments were conducted at 28.0° unless otherwise specified.

Pepsin Concentration.—The concentration of active pepsin was generally close to 0.2%. Only in the experiments where heavy metal cations were added were the pepsin solutions dialyzed before use. These were dialyzed for two days or more and served to reduce the concentration of split products from 15–20%, to less than 10%. In experiments with other additives or denaturants dialysis was not performed.

Ionic Strength.—The ionic strength of the pepsin solutions was primarily controlled by 0.15 *M* KNO_3 which was present in all experiments. The contribution of most of the reagents to the over-all ionic strength should be small except in the case of guanidine. In all our experiments in guanidine solution it is the guanidinium ion that is present and referred to—even though not stated explicitly. Since the rate of pepsin inactivation is not affected appreciably at ionic strengths above ~0.6,[4] when guanidine is present at this level or higher it should act principally as a denaturing reagent. At lower levels of guanidine there will be an independent effect due to its contribution to the ionic strength of the solution. This should be small, however, since all experiments have been conducted in 0.15 *M* KNO_3 and the marked effects of ionic strength occur at much lower values.

Sources of Materials.—2×-Crystallized pepsin was obtained from the Worthington Biochemical Corp. (Freehold, N.J.). Guanidine hydrochloride, methylurea and 1,3-dimethylurea purchased from Eastman Kodak Co. Urea and thiourea were Mallinckrodt products. Trimethyl dodecylammonium chloride (Arquad-12) was purchased from Armour and Co. The metallic salts were reagent grade products. All materials were used without further purification. All solutions were prepared with glass-distilled water.

The *p*H of Water-Organic Solvent Mixtures.—The *p*H values recorded with ethyl alcohol–water solutions were those obtained with the Beckman *p*H meter using a glass–calomel cell. The *p*H meter was standardized with an aqueous *p*H 7.00 buffer. To relate the *p*H values of the glass–calomel cell to hydrogen electrode values we have obtained

(1) Supported in part by an Institutional Grant from the American Cancer Society and by Grant No. G-2175 from the National Science Foundation.

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

(3) H. Edelhoch, THIS JOURNAL, **79**, 6100 (1957).

(4) H. Edelhoch, *ibid.*, **80**, 6640 (1958).

the pK values of acetic acid in dioxane–water solvent mixtures with the Beckman pH meter. The pK values of these solutions have been determined by Harned and colleagues[5] with hydrogen silver–silver chloride cells.

When the pH meter reading was plotted against log $\alpha/1-\alpha$ (where α is the degree of ionization) in accord with the Henderson–Hasselbalch equation ($p\mathrm{H} = pK + \log \alpha/1-\alpha$) straight lines were obtained in all solutions. Moreover the slopes had the theoretical value of one in all cases except at 60 and 70% (v./v.) dioxane. In the latter solutions a slightly larger slope was observed (~1.05). It has been shown by Harned and Fallon[6] that pK and mole fraction (N_2) of dioxane are linearly related. The data for acetic acid are reproduced in Fig. 1 along with the results which we have

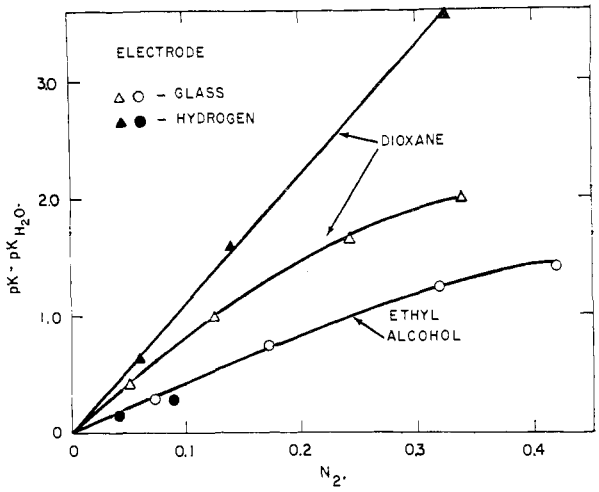

Fig. 1.—The variation of apparent pK of acetic acid with mole fraction (N_2) dioxane and ethyl alcohol as determined by the glass–calomel cell of the Beckman pH meter, model GS; acetic acid concentration ≅ 0.08 M; KNO_3 ≅ 0.2 M; hydrogen electrode data taken from Harned and Owen[5]: ▲, △ = dioxane; ⊙, ● = ethyl alcohol; T = 28°.

obtained with the Beckman model GS pH meter—equipped with externally shielded electrodes. The discrepancy between the two curves in dioxane solutions increases with the mole fraction of dioxane and becomes in excess of one pK unit in 60% dioxane (N_2 ≅ 0.24). However, in 40% dioxane (N_2 ≅ 0.12) the difference in pK values is ~0.4 unit and decreases with the concentration of dioxane.

Hydrogen electrode data are not reported by Harned and Owen[5] for acetic acid in ethyl alcohol–water solutions. However, data of Patterson and Felsing[7] are quoted for propionic acid in 10 and 20% (w./w.) ethyl alcohol solutions. These data are shown also in Fig. 1. For some reason the pK values do not vary linearly with mole fraction of ethyl alcohol as reported by Harned and Fallon[6] for acetic acid in dioxane–water solutions. pK values obtained for acetic acid in ethyl alcohol–water solutions with the glass–calomel cell are reported also in Fig. 1. All plots of pH *vs.* log $\alpha/1-\alpha$ were linear; only the 70% (v./v.) ethyl alcohol solutions had a slope slightly greater than unity. The value of $pK - pK_{H_2O}$ taken from the data of Patterson and Felsing[7] (for propionic acid) in 10% ethyl alcohol falls on the line obtained with acetic acid by the Beckman pH meter. At 20% the agreement is poor with the hydrogen electrode value, in this case smaller than the glass–calomel value.

If the dielectric constant of the solvent is the principal factor responsible for the failure of the glass–calomel cell to reproduce the hydrogen electrode results, then we would expect the ethyl alcohol–water system to show better agreement than dioxane–water solutions.

For the purposes of the present investigation we have assumed therefore that the pH meter values vary properly with ionization of acetic acid in all ethyl alcohol solutions. However, the pK values determined from the pH meter readings are only reasonably satisfactory below 40% ethyl alcohol (v./v.). At higher concentrations deviations between pH meter and hydrogen electrode values are quite large and meter values become rather empirical and of very limited utility for correlative purposes.

Results

Inactivation by Single Reagents: Urea Analogs.—Pepsin shows a marked response to urea and guanidine; this susceptibility is undoubtedly related to its pH sensitivity. When pepsin inactivation occurs at measurable rates at room temperatures ($p\mathrm{H} > 6$) quite small amounts of denaturant suffice to stimulate the rate. However, considerably larger amounts are needed to produce observable rates when otherwise the rate would not be noticeable ($p\mathrm{H} < 6$). At low, pH values these reagents are practically without influence. Kauzmann and Simpson[8] could not observe any effect of 10 M urea at pH 4.3 (at 30°) on the optical rotation of native pepsin.

The dependence of the first-order velocity constant of pepsin inactivation on guanidine concentration, between pH 5.70 and 6.50, is illustrated in Fig. 2. A linear relation was

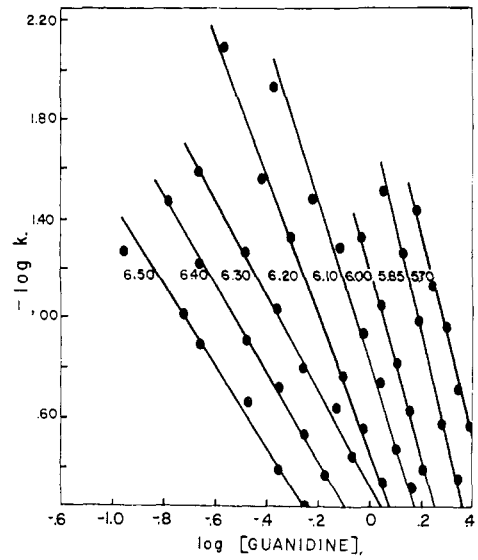

Fig. 2.—Effect of pH on the log–log curves of pepsin inactivation rates *vs.* guanidine concentration; KNO_3 = 0.15 M, T = 28.0°.

observed between the logarithms of the first-order velocity constants (k) and the molarity of guanidine. The slopes of the lines, usually considered as representing the order of the reaction, decreased smoothly from 4.45 to 1.70 with increasing pH. A plot of the slopes *vs.* pH is presented in Fig. 3.

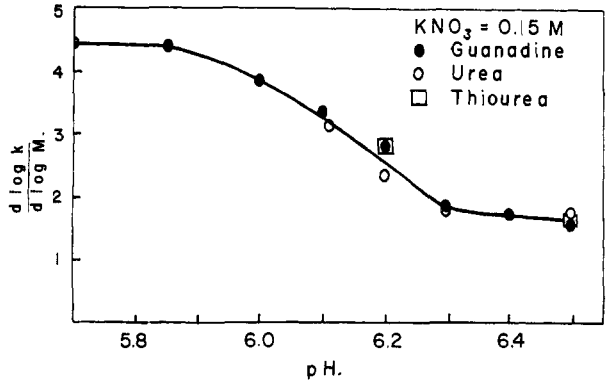

Fig. 3.—Effect of pH on the slopes (*i.e.*, order of reaction) of the log–log plots of pepsin inactivation rates.

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

(6) H. S. Harned and L. D. Fallon, THIS JOURNAL, **61**, 2377 (1939); see also ref. 5, p. 509.

(7) A. Patterson and W. A. Felsing, *ibid.*, **64**, 1480 (1942).

(8) W. Kauzmann and R. B. Simpson, *ibid.*, **75**, 5154 (1953).

The curve becomes rather flat at either end and probably represents the limits of variation in order with *p*H for inactivation by guanidine.

A similar log-log plot of the velocity constants of inactivation against urea concentration appears in Fig. 4[9] while the

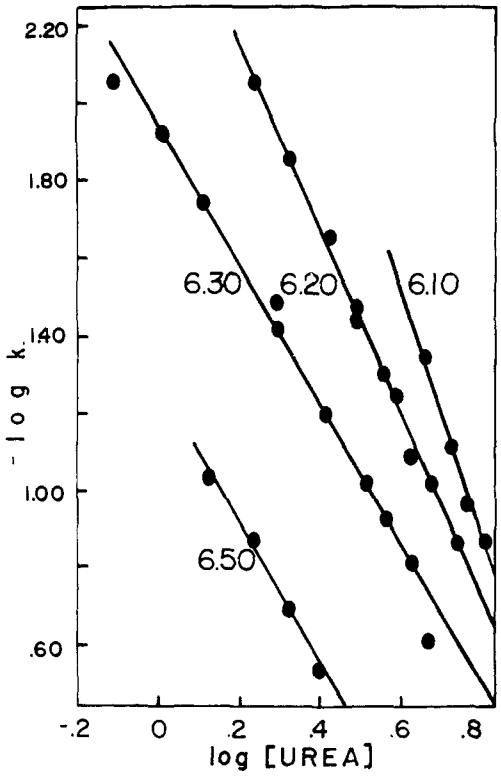

Fig. 4.—Effect of *p*H on the double logarithmic plot of pepsin inactivation rates *vs.* urea molarity; KNO_3 = 0.15 *M*, *T* = 28.0°.

slopes are recorded also in Fig. 3. The points fall on essentially the same curve as was obtained with guanidine though the data are not as extensive. The slopes observed with guanidine and urea at any *p*H are quite similar; however, the quantities of each reagent required to produce a given rate of inactivation at a particular *p*H are significantly greater for urea. About 7.5 times as much urea as guanidine was needed to produce an equivalent effect on rate within the *p*H interval 6.1 to 6.5. With ovalbumin, Kauzmann and colleagues[10] found guanidinium chloride to be about 3 times as effective as urea. The difference in relative effectiveness of these two reagents may possibly be related to the different types of residue hydrogen bonding proposed for each protein. Harrington[11] and Crammer and Neuberger[12] have presented evidence for the existence of carboxylate–tyrosyl b nds in ovalbumin. The *p*H-stability range of pepsin precludes a carboxylate bond while conforming to the predicted behavior of a carboxyl bond.[4]

Since pepsin inactivation proceeds at measurable rates above *p*H 6.0 at 25°, it furnishes a convenient system to compare the effects of other urea derivatives whose solubility is too low to be tested against the more stable proteins. Thiourea proved to be almost as efficient as guanidine in enhancing the denaturation rate of pepsin.[13] The slopes of the log-log plot are in close agreement with the results obtained with guanidine and urea at the two *p*H values investigated (see Fig. 3). It is interesting to note that oxygen, which is more electronegative than either nitrogen or sulfur and presumably capable of forming the strongest hydrogen bonds of the three elements, is the weakest reagent when compared in the form of analogs of urea. It is probable therefore that urea, being the smallest of the three molecules, has the poorest fit with the labile hydrogen bonds of pepsin.

Experiments with methylurea at *p*H 6.4, between 0.70 and 1.70 *M*, gave results which indicated that it had about the same potency as urea in denaturing pepsin. The slope however was 1.3 which is somewhat less than found with urea. With 1,3-dimethylurea at *p*H 6.5 a linear plot was obtained to 2.7 *M*, though the slope was only 0.5, which is significantly less than observed with the other urea analogs.

Metallic Cations.—In the studies of Gurd and colleagues[14] and Tanford[14] on the binding of metal cations to serum albumin they have presented binding data which serve to relate the reactive sites with specific functional groups of the protein. Thus Zn^{++} is bound preferentially to free imidazole groups while Pb^{++} binds predominantly to the carboxylate anion. Cu^{++}, however, reacts initially with sulfhydryl groups[15] and then with other functional groups.[16] When the binding of a metal is a simple association, the intrinsic affinity constant for the metal–protein system is in quite good agreement with that of the metal–residue affinity constants.[14,17]

There are many examples where rates of reaction have been shown to depend on the equilibrium properties of one of the reactant molecules, *i.e.*, acid–base catalysis, *m*- and *p*-substitution effects, etc.[18] In the field of protein chemistry the rate of reaction of the mercuric dimer of serum albumin with halide ions has been shown to be in approximate accord with the affinity constants of the corresponding mercuric halides.[19]

The reaction of pepsin with metals may be expressed in terms of the functional group involved. The equation which s in best accord with all the data is

$$\text{COOH—Acc} + M^{++} \longrightarrow \text{COOM}^{+} + H^{+} + \text{Acc}$$

where COOH—Acc indicates a carboxyl group which is hydrogen bonded to an unspecified acceptor group. In the course of this transition native pepsin is converted to the denatured form where *all* the carboxyl groups are available for binding with metal ions. The data shown in Fig. 5 have been expressed in terms of the relative rate of inactivation (k/k_0), where k_0 refers to the rate in the absence of added metal ions, thereby accounting for the effects of *p*H.

The relationship between rate and equilibrium constants has been formulated in a very general way[18] as

$$k_i = GK_i^x$$

or

$$\log k_i = \log G + x \log K_i \qquad (1)$$

where $K_i = [\text{COOM}^{+}]/[\text{COO}^{-}]\,[M^{++}]$ for the present system and k_i is the rate of pepsin inactivation. Before examining the reaction in terms of the foregoing scheme several features of the data shown in Fig. 5 should be discussed first.

Of particular interest are the very low levels of Cu^{++} and Pb^{++} which produce a significant acceleration of inactivation rates. One or two moles of metal is sufficient to produce a noticeable effect on the rate. Their effectiveness is even more striking when we consider that there are about 6 to 7 times as many free carboxyl groups which are also available for binding as are present in the hydrogen-bonded form. Both the Cu^{++} and Pb^{++} curves show a maximum in rate at higher levels of metal indicating a mild stabilizing effect.

(9) Kinetic data obtained at *p*H 6.50 in urea solutions of less than 1 *M* and at *p*H 6.60 at still lower concentrations of urea, appeared not to fit a first-order plot as well as the other data and consequently have not been reported.

(10) R. B. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5139 (1953); J. Schellman, R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5152 (1953).

(11) W. F. Harrington, *Biochim. et Biophys. Acta*, **18**, 450 (1955).

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

(13) H. Edelhoch, *Biochim. et Biophys. Acta*, **22**, 401 (1956).

(14) F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952); F. R. N. Gurd and G. R. Murray, Jr., *ibid.*, **76**, 187 (1954); see also, C. Tanford, *ibid.*, **74**, 211 (1952).

(15) I. M. Klotz, J. M. Urquhart, T. A. Klotz and J. Ayers, *ibid.*, **77**, 1919 (1955).

(16) I. M. Klotz and H. G. Curme, *ibid.*, **70**, 939 (1948); I. M. Klotz, J. L. Faller and J. M. Urquhart, *J. Phys. Colloid Chem.*, **55**, 101 (1951).

(17) F. R. N. Gurd and P. E. Wilcox, *Adv. Protein Chem.*, **11**, 311 (1956).

(18) See, for example, L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940. Chapt. III.

(19) H. Edelhoch, E. Katchalski, R. H. Maybury, W. L. Hughes, Jr., and J. T. Edsall, THIS JOURNAL, **75**, 5058 (1953).

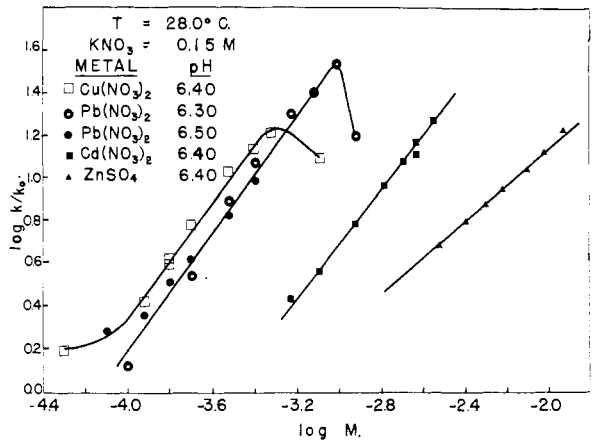

Fig. 5.—The effect of small amounts of several bivalent metallic cations on the rates of pepsin inactivation; k_0 is the first-order velocity constant of inactivation observed in the absence of added bivalent ions.

With all four metallic ions shown in Fig. 5, log k/k_0 varied linearly with the log of the molarity of metal, at intermediate concentrations of metal, for a factor of about 10 in rate. As k/k_0 approached one the Cu^{++} and Pb^{++} curves departed from linearity and asymptotically approached the rate observed in the absence of metal. The slopes of the linear regions were equal to 1.30 for Cu^{++}, Pb^{++} and Cd^{++}; the Zn^{++} curve had a smaller slope which was equal to 0.85. It is not known whether the anion of the Zn^{++} salt (sulfate), which was different from the other anions (nitrate), had any influence on the slope. If the similarity in slope implies a parallelism in mechanism, the effectiveness of the metals showing the same slope may be compared with the properties of each metal.

Since the slopes are constant the rates may be compared at any arbitrary metal concentration with the equilibrium constants between the metals and the functional groups of pepsin. Values of the constants of equation 1 appear in Table I where K_1 is for carboxylate–metal ion association and

TABLE I

EFFECTS OF METALLIC CATIONS ON THE RATE OF PEPSIN INACTIVATION

	log k/k_0[a]	log K[b]		$-\log G$
Cu^{++}	1.42	2.16		0.74
Pb^{++}	1.29	2.0		.71
Cd^{++}	0.42	1.3		.88
			Av.	0.78
Zn^{++}	0.12	1.03		

[a] Determined at log $M = -3.2$. [b] Table of F. R. N. Gurd and P. E. Wilcox, ref. 17.

x is taken as equal to one. The approximate constancy of G attests to the applicability of equation 1. According to this relation a metal ion with a value of log K of less than 0.74 should have no effect at a molar concentration of $10^{-3.2}$ or less. Zn^{++} has slightly larger value of log K[17] and does show appreciable stimulation but at higher concentrations than predicted. Of the other metallic cations tested which show little or no enhancement in inactivation rate at comparable concentrations (Hg^{++}, Ag^{++}, Mn^{++}, Co^{++}, Mg^{++}, Ni^{++}, Fe^{++}) in water or in 28.5% ethyl alcohol, the first association constants in water (with the exception of Hg^{++}) are considerably smaller than Zn^{++} or are not available.[17] A similar analysis using the affinity constants of metal–imidazole or metal–amine (i.e., ammonia) groups does not lead to a constant value of G in eq. 1. The possibility of the amino groups serving as sites can be eliminated also on quite different grounds. Herriott and Northrop[20] were able to show that acetylation of all the primary amino groups in pepsin did not significantly affect either its pH–stability or pH–activity characteristics.

(20) R. M. Herriott and J. H. Northrup, *J. Gen. Physiol.*, **18**, 35 (1934).

There are a number of complicating factors which tend to limit the validity of the quantitative correlation established by the data in Table I. In the first place the K_1 values quoted in Table I were not obtained at constant ionic strength but varied from 0 (Cu^{++}) to 3 M (Cd^{++}).[17] Secondly, we have used the total concentration of metal in the abscissa in Fig. 5 rather than that of the free metal concentration, since the amount of metal bound to the non-bonded carboxyl groups at each concentration was not known. In addition, the split-products present in dialyzed pepsin preparations (4–10%) also tend to reduce the concentration of active metal by complex formation. All three factors make the quantitative correlation less certain; nevertheless, there is little doubt of the general significance of the data in its present form.

In Fig. 6 the effect of Pb^{++} in promoting the rate of inactivation of pepsin is seen to be essentially independent of pH. The rate varies by $10^{1.7}$ between pH 6.1 and 6.6 due to pH alone. Since both metal and hydroxyl ions are believed to act on the carboxyl hydrogen bonds, the failure to observe interaction effects between these two reactants would make suspect the thesis that a single type of bond in pepsin is the site of their activity. The data can be rationalized, however, if the two ions are acting on different parts of the carboxyl hydrogen bond, *i.e.*, the metal for the potential carboxylate ion and the hydroxyl for the dissociable proton.

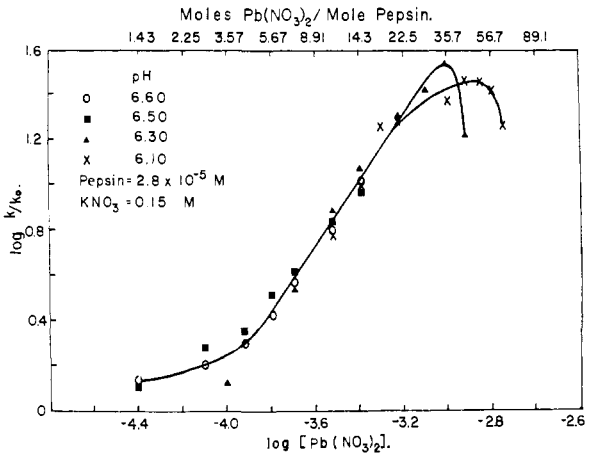

Fig. 6.—The influence of $Pb(NO_3)_2$ on the rate of pepsin inactivation as a function of pH between 6.10 and 6.60; k_0 is the first-order velocity constant obtained in the absence of $Pb(NO_3)_2$: T = 28.0°.

With Cu^{++}, Cd^{++} and Zn^{++} the effects of pH are not quite as consistent as the Pb^{++} data in showing independence of interaction though they permit no other simple interpretation considering the pronounced effect of pH *per se*. The slopes of all four metals are essentially constant with variation in pH, though all the data do not fall on a single curve as observed with Pb^{++}. The curves obtained with Cu^{++} at pH 6.2 and 6.6 are shifted by about 0.12 log unit to higher metal concentrations relative to the pH 6.4 curve (Fig. 5). Both curves also exhibit a maximum in rate, though at pH 6.2 it occurs at log $k/k_0 = 1.2$, while at pH 6.6 it occurs at log $k/k_0 = 0.85$.

The curve found with Cd^{++} at pH 6.2 is displaced by about 0.20 log unit to higher metal concentrations when compared with the pH 6.4 curve. At pH 6.5 and 6.6 the data fall on the curve reported for pH 6.4 (Fig. 5) except at low metal concentrations where they lie above the (extrapolated) curve and asymptotically approach the rate observed in the absence of metal. The Zn^{++} data at pH 6.6 lie on a curve about 0.10 log unit to the right of the curve observed at pH 6.4 (Fig. 5).

Ethyl Alcohol.—The influence of ethyl alcohol on the pH–rate curves of pepsin is shown in Fig. 7. Below about 35% ethyl alcohol the pH dependency of log k remains constant at ~3.4, while above 35%, the slopes decline. At 70.5% the rate of inactivation was too rapid to measure at any pH value as far down as a meter reading of 1.5. It seems likely that the slope approaches zero at 70.5% where the inactivation process becomes essentially independent of pH.

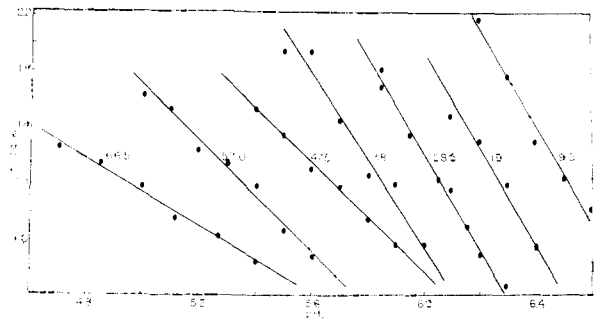

Fig. 7.—The influence of pH on the rate of pepsin inactivation as a function of ethyl alcohol concentration between 9.5 and 66.5% (v./v.); KNO_3 = 0.15 M, T = 28.0°.

Between 9.5 and 38% ethyl alcohol the curves relating log k and pH (Fig. 7) are displaced about 0.21 unit in the acid direction with each increase of 9.5% in alcohol concentration. This shift is interpreted to indicate a decrease in the stability of the carboxyl hydrogen bonds since it has been demonstrated[4] that the titration range ($\sim pK$ values) of the carboxyl hydrogen-bonded groups decreased by close to this amount (while that of the free carboxyl groups increased) with a corresponding increase in alcohol concentration. The behavior of the free groups was in the same direction as observed with small molecules.

The decrease in the stability of the hydrogen-bonded groups suggests a dependency or interaction of these bonds with the hydrophobic bonds in pepsin. The stability of the latter interactions will of course depend on the composition of the solvent. The reaction path may be visualized in terms of the breakage of carboxyl hydrogen bonds at low alcohol concentrations and on the dissolution of the hydrophobic bonding situation at high alcohol levels. At intermediate levels (~35 to 70%) both mechanisms are probably operative. It appears consequently that the stability of the carboxyl hydrogen bonds is linked directly to the intramolecular micellar regions formed by lipophilic amino acid residues.

A very rough estimate of the energy of the hydrophobic interactions may be surmised if we relate the slope or order (of the inactivation in ethyl alcohol) to the number of bonds broken. Restricting the usable data to below 38% ethyl alcohol, a reasonably good linear relation was found between log k and log ethyl alcohol concentration which had a slope of 4.5 (Fig. 8). If we assign an energy of 1.2 kcal. for the CH_2 group,[21] then each molecule will supply an interaction energy of 2.4 kcal. Therefore, as an approximation, the total energy provided by 4.5 molecules is about 11 kcal. It is probable that at least this much cohesive energy is lost when the active enzyme is denatured by alcohol since the driving force of the reaction probably originates in the large gain in entropy that occurs when the compact native structure is unfolded into a randomly coiled polyelectrolyte. The 11 kcal. principally serves to reduce the kinetic barrier inhibiting denaturation.

Detergents.—The anionic detergent sodium dodecyl sulfate, at a concentration of 0.0067 M, was essentially without effect on the rate of pepsin inactivation in the pH range 6.3 to 6.7 in 0.15 M KNO_3 at 28°. On the other hand, the cationic detergent Arquad-12 (trimethyldodecylammonium chloride) produced a marked stimulation in denaturation rates. Since pepsin has an appreciable negative charge in the pH range of denaturation, electrostatic interactions with detergent must play a significant role. It is known from the work of Putnam and Neurath[22] that ionic detergents will precipitate proteins only when the protein carries a charge of opposite sign to that of the detergent and adequate amounts of detergent are present. At lower levels of detergent, statistical binding usually occurs which appears to be reversible.[23,24]

The amounts of Arquad-12 sufficing to augment the rate of inactivation at pH 6.4, where about 25 to 30 of the carboxyl groups of pepsin are neutralized, are below the stoichiometric equivalence of these groups. At lower pH values, between 5.8 to 6.2 (Fig. 9), the molar ratio (detergent/protein carboxylate groups) is closer to unity. Detergents are known to comprise one of the most effective class of reagents in their ability to denature proteins. Pepsin conforms to this general behavior though the effects of specific metallic cations are comparable.

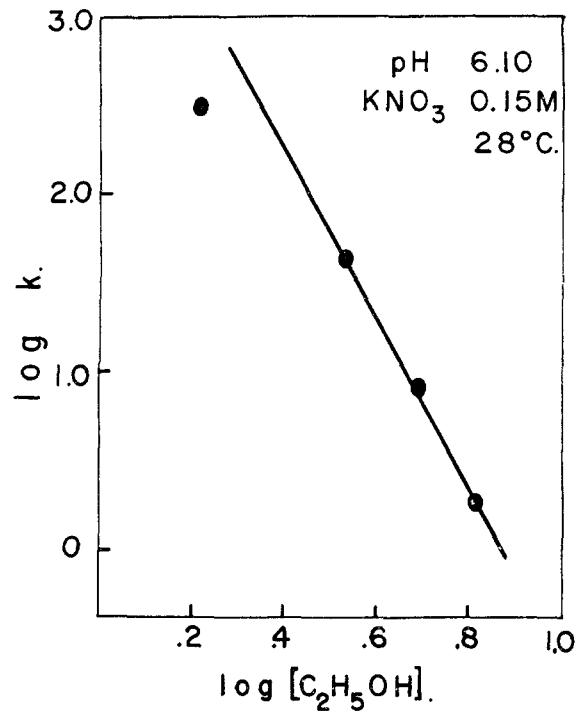

Fig. 8.—Double logarithmic plot of rate constant *vs.* molarity of ethyl alcohol; data obtained from curves between 9.5 and 38% ethyl alcohol concentration reported in Fig. 7.

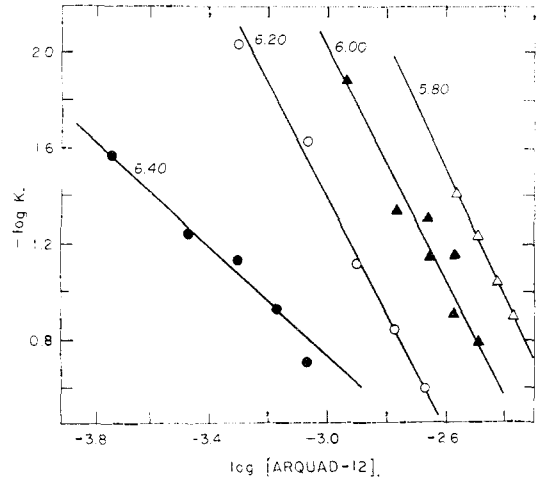

Fig. 9.—The effect of pH on the log-log plot of pepsin inactivation rates *vs.* trimethyldodecylammonium chloride (Arquad-12) concentration; KNO_3 = 0.15 M, T = 28.0°.

From pH 5.80 to 6.20 the slopes of the log-log plot (Fig. 9) are approximately constant and equal to 2.6 (±0.1). At pH 6.4 it is about half this value. If we consider only the data where the slope is constant, which would imply a common mechanism, the interrelations of the other variables may be assessed. At a constant concentration of detergent the rate of inactivation varies inversely with the 2.8 power of the hydrogen ion concentration, which is somewhat less than that found in the absence of detergent. Thus the pH and

(21) P. Debye, *Ann. N. Y. Acad. Sci.*, **51**, 575 (1949).
(22) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944).
(23) J. D. Teresi and J. M. Luck, *J. Biol. Chem.*, **174**, 653 (1948).
(24) F. Karush and M. Sonenberg, THIS JOURNAL, **71**, 1369 (1949).

detergent are not quite independent variables in their interaction with pepsin—as was found between *p*H and Pb^{++}.

The significance of the 2.6 value of the slope is difficult to interpret. If Arquad-12 affects the same bonds as does ethyl alcohol then it may be instructive to compare their slopes with their structures. The slope observed with ethyl alcohol was 4.6. The hydrocarbon chain of Arquad-12, however, contains 6 times as many CH_2 groups. It therefore appears that an appreciable part of the chain is unreactive in breaking hydrophobic bonds. However, it is well known that the binding of detergent molecules increases with chain length.[23,24] It seems therefore that only part of the chain is effective in displacing intramolecular hydrophobic bonds though a long chain is needed for efficient binding to the surface. The small sizes (and hydrophobic bond-forming capacity) of most of the amino acid residues, except perhaps for the aromatic amino acids, would tend to support this supposition.[25]

Inactivation by Two Reagents: Guanidine and Ethyl Alcohol.—The influence of guanidine on the rate of inactivation of pepsin in solutions containing 38% ethyl alcohol is shown in Fig. 10. It is evident from Fig. 6 that measurable rates are obtained only between *p*H 5.6 and 6.0 in 38% alcohol. On the other hand, the *p*H must be above 6.0 to observe inactivation in solutions containing less than 1 *M* guanidine. The fact that rate data can be secured at *p*H 5.3 in solutions containing less than 1 *M* guanidine supports the hypothesis that these two reagents interact with different types of bonds in pepsin and each contributes independently to the reduction in stability. The slopes of the log–log plot also reflect the combined attack of the two reagents. In 38% alcohol a *p*H dependence near 3 was observed. The slopes of the data shown in Fig. 10 decline from 1.85 at *p*H 5.30 to 0.90 at *p*H 5.70. The variation in slope with *p*H conforms to the results found in the absence of ethyl alcohol though their magnitudes are considerably less. The smallest slope observed in guanidine solutions was 1.70.

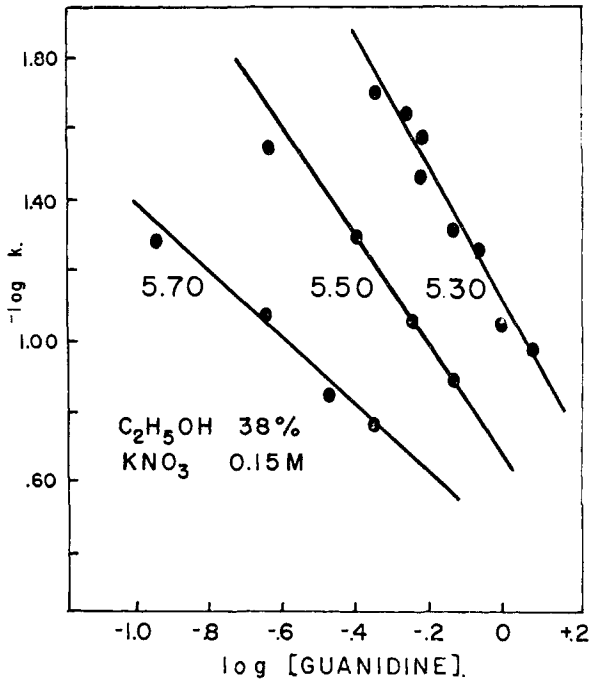

Fig. 10.—The effect of *p*H on the log–log plot of pepsin inactivation rates (in 38% ethyl alcohol) *vs.* molarity of guanidine; $T = 28°$.

Lead and Ethyl Alcohol.—The effects of Pb^{++} on the first-order velocity constants in 28.5% ethyl alcohol are illustrated in Fig. 11. The values of log k_0 at *p*H 5.5 and 5.7 were too low to measure and were obtained by extrapolation from data at higher *p*H values (Fig. 7). The results show a relatively greater enhancement in rate at the lower *p*H values. In the absence of ethyl alcohol the effects of Pb^{++} were essentially independent of *p*H. Though the curves in Fig. 11 are not linear, it is readily evident that the rates increase much faster with Pb^{++} than was observed in the absence of ethyl alcohol. It is apparent also that the effects produced by Pb^{++} are considerably influenced by ethyl alcohol.

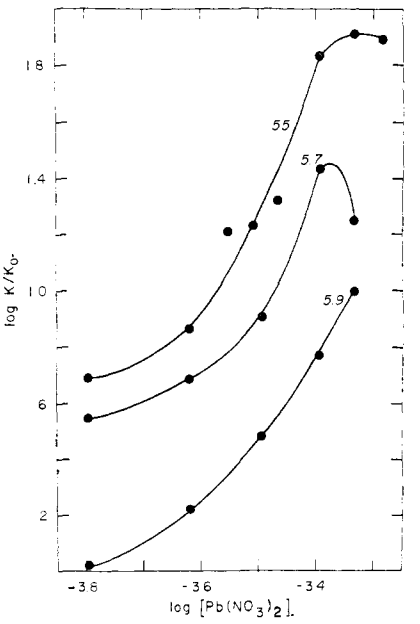

Fig. 11.—The effect of *p*H on the log–log plot of relative pepsin inactivation rates (in 28% ethyl alcohol) *vs.* $Pb(NO_3)_2$ concentration; k_0 is the first-order velocity constant in the absence of $Pb(NO_3)_2$; $KNO_3 = 0.15\ M$, $T = 28.0°$.

Discussion

The various types of weak bonds which have been postulated to play a role in the stabilization of the native form of pepsin are the same kind which have been proposed and described in most proteins.[26,27] Pepsin has been selected for the present investigation since we possess fairly detailed knowledge of one group of bonds (5 or 6 carboxyl hydrogen bonds) whose integrity is vital to its stability and whose disappearance can be followed kinetically from the *p*H dependence of its inactivation rate.

Of the known weak bonds present in native pepsin (carboxyl hydrogen bonds, α-helical peptide hydrogen bonds, hydrophobic bonds) which are lost on denaturation, none appear to be sufficient by themselves to preserve the structure of the native enzyme. Pepsin furnishes a particularly favorable case to evaluate the interdependence of its weak bonds since denaturation above *p*H~6 is always complete and unencumbered by transition states in the sense that upon reversal of the conditions producing denaturation, no reversal of any molecular change has been found to occur.[3] In addition, the kinetics of denaturation as determined by changes in the macromolecular properties and

(25) See, for instance, an estimate of the hydrophobic character of the amino acid residues as treated by D. F. Waugh, *Adv. Protein Chem.*, **9**, 325 (1954).

(26) For further discussion of these bonds see Chapter by W. Kauzmann in "Mechanism of Enzyme Action," ed. W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954; see also ref. 25, 30.

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

optical rotation of pepsin solutions are identical to those obtained by enzyme assay or formation of acid-insoluble protein.[28] Before discussing the contribution of the various types of hydrogen bonds in pepsin to its stability, it would seem necessary to discuss their characteristics as evidenced in other pertinent situations.

Harrington and Schellman[29] have presented evidence, based on the structure of oxidized ribonuclease, that a linear polypeptide will not form an α-helix in an aqueous medium. In a solvent of low water activity, however, such as concentrated LiBr, the random-coil configuration of this molecule readily assumes the folding of an α-helix.[30] These results are not too surprising since the free energy of formation of a peptide-like hydrogen bond in water, as exemplified by urea dimerization, is quite unfavorable, *i.e.*, $\Delta F^\circ \approx 1990$ cal.[27] A similar situation pertains to the carboxyl–carboxyl hydrogen bonds. Acetic acid is completely dimerized in the absence of water[31] where the energy of a single —C=O—H—O— bond is 6,910 cal./mole. In water, however, the association is quite feeble.[32] The interaction of tyrosine with acetate,[33] guanidinium and ammonium ions[34] has been shown to be very small in aqueous media. The proved stability of α-helical configurations, such as α-glutamic acid polypeptide,[35] is therefore possibly due to other factors, such as lateral hydrogen bonding of side chain carboxyl groups. Hence the energy of either peptide or carboxyl–carboxyl hydrogen bonds is inadequate to stabilize the α-helical structure, though when supporting each other the latter is favored. The inherent weakness of the carboxyl–carboxyl bond, even when located on a polymer chain, is evident in the structure of (natural) γ-glutamic acid polypeptide, since no evidence of hydrogen bonding could be inferred from its molecular behavior in water.[36]

There are two important factors which can affect the stability of groups capable of forming hydrogen bonds. These may be classified as: (a) solvent effects, which principally influence the heats of hydrogen bond formation, and (b) steric factors, which have a greater influence on the entropy of reaction. Solvent interactions have been demonstrated by Doty, *et al.*,[37] to play a crucial role in the random-coil↔α-helix transformation of α-benzylglutamyl polypeptide in non-aqueous solvents. Steric factors have been evident for some time in the ability of salicylic acid[38,39] to form a strong intramolecular hydrogen bond while phenol and acetate show only very minor interaction.[33] The intramolecular formation of hydrogen bonds are favored since no loss in translational (or rotational) entropy is involved as would occur in intermolecular association.[41] Thus entropic factors can obviously play an important role in determining whether a particular type of hydrogen bond will be stable under specific environmental conditions.

None of the hydrogen bonding possibilities discussed above is therefore sufficiently stable, when divorced from coöperating interactions, to account for the compact, ordered structure of the native enzyme. Neither will the hydrophobic interactions suffice if we may use as a model the stability properties of detergent micelles.[42] These structures are held together solely by hydrophobic forces and represent the simplest example of this kind of bonding in a macromolecule. All detergent micelles, however, show critical phenomena at a specific concentration of detergent. Below this critical concentration the micelles dissociate and only monomer remains. Since micelles like, *e.g.*, Na lauryl sulfate, presumably offer a much greater degree of intramolecular cohesive interaction than a "random mixture" of non-polar residues and are relatively unstable, it seems hardly probable that a protein can be stabilized solely by hydrophobic forces.

Our principal concern in the present study is the effect of various denaturing agents on the stability of pepsin and the manner in which the carboxyl hydrogen bonds are affected. The participation of the latter type of bonds can be followed from the *p*H-dependence of inactivation rates. If the various reagents employed to denature pepsin attack a single type of intramolecular linkage, then any deviation from the log k–*p*H relation found in the absence of denaturant, will be interpreted as indicating interaction between the bonds attacked by the reagent and the carboxyl hydrogen bonds. In other words, the internal structure of pepsin is envisioned as composed of an "integrated" system of weak bonds; the stability properties of any group of bonds may therefore be dependent on the preservation of any other group. The ability of the solvent to modify the α-helical content of a protein and hence its structure has been demonstrated.[30,43]

The decrease in the order of reaction of pepsin inactivation that occurs between *p*H 5.8 and 6.3 in guanidine (urea and thiourea) solutions would appear to be related to the degree of activation of carboxyl hydrogen-bonded groups. In the ab-

(28) H. Edelhoch, THIS JOURNAL, **78**, 2644 (1956).

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

(30) W. F. Harrington and J. A. Schellman, *ibid.*, **30**, 167 (1957).

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

(32) A. Katchalski, H. Eisenberg and S. Lifson, *ibid.*, **73**, 5889 (1951).

(33) D. Wetlaufer, *Comp. rend. Lab. Carlsberg, Ser. Chim.*, **30**, 135 (1956).

(34) C. Tanford, THIS JOURNAL, **76**, 945 (1954).

(35) P. Doty, *Proc. Natl. Acad. Sci.*, **42**, 791 (1956); P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

(36) H. Edelhoch and J. B. Bateman, THIS JOURNAL, **79**, 6093 (1957).

(37) P. Doty, J. H. Bradbury and A. M. Holtzer, *ibid.*, **78**, 947 (1956).

(38) C. T. Abichandani and S. K. K. Jatkar, *J. Indian Inst. Sci.*, **21A**, 417 (1938).

(39) Another interesting example of the effect of hydrogen bonding on the dissociation constant is that of O-carboxybenzenephosphonic acid.[40] The pK of the secondary phosphono group is increased by 2.28 units from that of the *p*-isomer. In addition the pK value of the carboxyl group is reduced indicating that the carboxylate ion forms a more stable hydrogen bond than the uncharged carboxyl group. In 4-NO_2-O-hydroxybenzenephosphonic acid the pK of the hydroxyl group is increased by at least 4 units while that of the secondary phosphoryl is decreased by 1.02 units.

(40) H. H. Jaffé, L. D. Freedman and G. O. Doak, THIS JOURNAL, **76**, 1548 (1954).

(41) H. H. Jaffé, *ibid.*, **79**, 2373 (1957).

(42) M. E. L. McBain and E. Hutchinson, "Solubilization," Academic Press, Inc., New York, N. Y., 1955, see Chapt. III.

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

sence of denaturant the activation energy of these bonded groups declines by about 50 kcal. between *p*H 6.0 and 6.7.[44] Or in terms of the theory developed by Simpson and Kauzmann[8] this decrease in order should reflect the reduction in the difference $(\bar{n}' - \bar{n})$ in the average number of guanidine (or urea) molecules bound to pepsin in the activated $(\bar{n}')$ and native $(\bar{n})$ states, respectively. This mechanism implies that there occurs a lessening in the number of binding sites (*i.e.*, carboxyl hydrogen-bonded groups) in the activated complex as the *p*H is increased.

It is of importance to note that the *p*H dependence of inactivation is about 3.6 in 1 *M* and 3.0 in 1.6 *M* guanidine.[45] Since these values are in approximate agreement with the value found in water, *i.e.*, 3.4, it is likely that the effects of guanidine and hydroxyl ions are independent and presumably involve the same sites or groups in pepsin.

The order of the inactivation reaction with respect to Pb^{++} is independent of *p*H (Fig. 6) and smaller (~1.3) than observed with guanidine at any *p*H. This contrast in behavior of the two reactants would tend to preclude a common site on pepsin as the reactive center. It is, of course, possible that the metal ions react directly with only some of the bonds susceptible to attack by guanidine since the latter are probably capable of forming weak bonds with many types of hydrogen bond forming groups. Furthermore, the influence of very small amounts of metallic ions would be expected to be rather specific. It is also possible that steric or electrostatic factors prevent Pb^{++} from reacting with all the carboxyl hydrogen bonds. This appears to be somewhat unlikely since guanidine is positively charged also and of appreciable size. However, the much higher order observed in Pb^{++}–alcohol mixtures (~4–5) may be rationalized if the alcohol induces an intramolecular rearrangement which makes all the carboxyl hydrogen bonds accessible to Pb^{++}.

Since ethyl alcohol and urea affect the dielectric constant of water in opposite ways but both produce an increase in denaturation rates, it is unlikely that the dielectric properties of the solvent *per se* appreciably affect the stability of pepsin. Moreover, at *p*H 4.3 pepsin is quite stable in 10 *M* urea at 30°[10] and in <70 ethyl alcohol at 28°. It is necessary, therefore, to seek rather specific interactions in order to explain the effects of these reagents.

It has been demonstrated potentiometrically that the *p*H range of liberation of protons from the carboxyl hydrogen bonds is lower in ethyl alcohol than in water.[4] This result clearly indicates a lessening of the strength of these bonds. The *free* carboxyl groups, however, titrate at more alkaline *p*H values in alcohol in accord with their behavior in small molecules. Since the effects of alcohol on the carboxyl hydrogen bonds cannot be explained on the basis of a small molecule model we are compelled therefore to look for other groups as the site of ethyl alcohol activity. These are presumably the hydrophobic amino-acid side chains which prefer a lipophilic to an aqueous environment. Thus as the intramolecular hydrophobic interactions are progressively weakened with increase in alcohol content, the stability of the carboxyl hydrogen bonds is directly affected. (Ordinarily the strength of hydrogen bonds would be expected to increase in media of reduced water activity.) This is evident in the progressive shift to lower *p*H values seen in the dependence of rate on *p*H (Fig. 7), which occurs without change in slope up to about 35% alcohol and is considered to represent a common reaction mechanism based on breakage of carboxyl hydrogen bonds.

The stability of carboxyl hydrogen bonds in pepsin thus appears to be directly dependent on the extent of intramolecular hydrophobic bonding. This is not altogether surprising since the carboxyl hydrogen bonds postulated to exist in pepsin are not stable enough to exist on their own in aqueous media. They must therefore gain free energy by other means. The hydrophobic interactions may indirectly supply this free energy either by providing a medium of low dielectric constant for the hydrogen bonded groups or by bringing the hydrogen bonding groups into close juxtaposition with each other so that there is very little entropy loss encountered in bond formation.

At higher alcohol concentrations the hydrophobic bonds in pepsin are sufficiently weakened so that inactivation occurs along another route not directly dependent on the rupture of carboxyl hydrogen bonds in the same manner as occurred at alcohol concentrations below 35%.

The behavior of pepsin toward Arquad-12 can be interpreted by the interaction of the detergent with the same bonds in pepsin that are affected by ethyl alcohol. The greater efficacy of detergents as compared with smaller chain uncharged molecules is undoubtedly related to the larger extent of binding of the detergent. Pepsin has been shown to bind Mg^{++},[46] Ca^{++}[47] and even K^{+}.[48] The binding of Arquad-12, which must precede denaturation, undoubtedly is enhanced by its long hydrocarbon chain.

KANSAS CITY, KANS.
BETHESDA, MD.

(14) In preparation, H. Edelhoch.

(45) At a lower concentration of guanidine the *p*H dependence of log *k* is not linear. This results from the large variation in order that occurs. At lower *p*H values and hence larger quantities of reagent, the *p*H dependence is about the same as in the absence of guanidine. We have assumed therefore that the mechanism of inactivation remains essentially the same throughout.

(46) C. W. Carr and K. R. Woods, *Arch. Biochem. Biophys.*, **55**, 1 (1955).

(47) C. W. Carr, *ibid.*, **46**, 424 (1953).

(48) C. W. Carr, *ibid.*, **62**, 476 (1956).