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CATALYZED HYDROLYSIS OF AMIDE AND PEPTIDE BONDS IN PROTEINS [1]

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ABSTRACT

The rates of hydrolysis by dilute acids of both a dissolved protein (egg albumin) and an insoluble protein (wool) are shown to depend not only on the temperature and acidity but also on the acid used. When hydrolyzed at 65° C by certain strong monobasic acids of high molecular weight, the amide and the peptide bonds are broken over 100 times as fast as when they are hydrolyzed with hydrochloric acid. Even among the common mineral acids, large differences appear. These differences in hydrolytic effectiveness parallel differences in the affinities of the anions of the acids for protein. A further reason for attributing this effect to the anions is the attainment, with anions of high affinity, of a maximum rate of amide hydrolysis at relatively low concentrations, stoichiometrically equivalent to the sum of the amino plus the amide groups. A similar limiting anion concentration or maximum rate of hydrolysis of the much more numerous peptide groups is not observed. On the basis of details of the dependence of the rate of hydrolysis on concentration of effective anions and hydrogen ions, a mechanism which involves combination of the groups hydrolyzed with hydrogen ions, is proposed.

At low concentrations of effective anions, amide hydrolysis is catalyzed more strongly than peptide hydrolysis. By keeping the concentration slightly below stoichiometric equivalence to the sum of the amino plus amide groups the amide groups may be rapidly hydrolyzed without extensive hydrolysis of the peptide bonds in the protein. Practical applications are suggested.

CONTENTS

[1] A brief account of this work was presented at the Boston meeting of the American Society of Biological Chemists in April 1942.

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I. INTRODUCTION

The rate of hydrolysis of proteins in dilute solutions of strong acids has long been known to depend on temperature and on concentration of acid. Recently it has been found that the rates of hydrolysis of both an insoluble protein (wool) and a soluble protein (egg albumin) also depend on the choice of hydrolyzing acid [7].[3] Since large differences in hydrolytic effectiveness appear among acids which are totally dissociated, it is evident that the rate of hydrolysis is influenced strongly by anions. This catalytic influence is further demonstrated by other experiments described in the present paper. The results yield information concerning the mechanism of hydrolysis, and suggest that proteins can be hydrolyzed under conditions considerably milder than those customarily employed.

II. EXPERIMENTAL PROCEDURE

1. EXPERIMENTAL CONDITIONS

Two proteins, purified wool and crystallized egg albumin, were hydrolyzed at two temperatures (65° C and 75° C, $\pm 0.1°$). Details of the purification of both proteins and, with few exceptions,[4] of the reagents used, have been described elsewhere [8, 9]. The kinetic procedures were as follows:

For each measurement with wool a portion was immersed in a quantity of solution, previously brought to the temperature of the thermostat. The proportion of 98 ml of solution per g of dry wool was always employed. With egg albumin, the concentration of protein, after mixing a stock solution with the reagents, was 0.71 percent. Glass-stoppered flasks were used; they were sealed as soon as the wool was thoroughly wetted, to prevent evaporation. After the desired time had elapsed, the flasks were cooled rapidly, opened, and aliquots of 5 or 10 ml were removed and analyzed as described in the next section.

Although the egg albumin is almost instantly denatured at the concentration of acid (0.053 *M*) used in the experiments with this protein, it remains in solution at all concentrations of sodium dodecylsulfonate above 0.01 *M*. However, as the products of hydrolysis accumulate, they precipitate, unless the concentration of dodecylsulfonate is above 0.016 *M*.

2. ANALYTICAL METHODS

A distinction has been made experimentally between the hydrolysis of amide bonds ($RCO\text{-}NH_2$) and of peptide bonds ($RCO\text{-}NHR'$). The first may be followed with both proteins by measuring the evolution of ammonia, whereas the second may be studied by a direct method (the formol titration) only with the soluble protein. However, other methods which indicate the extent of peptide hydrolysis are available, and were used with both proteins, as described later.

The ammonia evolved was adsorbed on permutit before distillation to remove it from much of the other dissolved material present, thus decreasing the danger of secondary production of ammonia during the

[3] Figures in brackets indicate the literature references at the end of this paper.

[4] The sulfate half-esters ($ROSO_3H$) were supplied through the courtesy of E. I. du Pont de Nemours & Co., Inc. Mixtures of these half-esters have been used to denature proteins [1].

distillation with alkali. In the experiments with egg albumin, determinations of ammonia were also made on protein-free filtrates, which were obtained by precipitating the unchanged protein with trichloroacetic acid as explained below. The results obtained by the two methods agreed closely.

The hydrolysis of peptide bonds in egg albumin was measured by determining the increase in the formol titration value, as in Northrop's modification [4] of Sörensen's method. Since the increase corresponds to the number of carboxyl groups liberated by the hydrolysis of both amide and peptide groups, the amount of hydrolysis of the latter is given by the difference between the increase in the formol titer and the amount of ammonia evolved.

As its peptide bonds are hydrolyzed, part of the wool dissolves. The amount dissolved, expressed in terms of its nitrogen content as determined by Kjeldahl analysis, has therefore been used as a convenient measure of the total extent of hydrolysis of the wool. Subtraction of the ammonia evolved gives a quantity designated as "nonammonia nitrogen," which depends only on the hydrolysis of peptide bonds. Since partially hydrolyzed egg albumin is not precipitated by trichloroacetic acid, Kjeldahl analyses of the solutions of this protein, after mixing with trichloroacetic acid, have been used as an additional indication of the extent of peptide hydrolysis in this protein. The term "dissolved nitrogen" is also applied to this experimental quantity.

The presence of sodium dodecylsulfonate and certain other salts interfers with the precipitation of protein by trichloroacetic acid in the concentrations of this reagent customarily used. However, tests showed that in the presence of 0.03 *M* sodium dodecylsulfonate, the amount of protein precipitated was practically independent of the concentration of trichloroacetic acid at concentrations above 0.47 *M*. This concentration has been used throughout. Whenever the concentration of sodium dodecylsulfonate was below 0.03 *M*, enough of the salt was added to the solution just before sampling it to bring the concentration up to this value before addition of the precipitant.

III. RESULTS AND DISCUSSION

1. RELATIVE EFFECTIVENESS OF DIFFERENT ACIDS

The relative effectiveness of various anions has been chiefly determined by measuring the rate at which ammonia is liberated by hydrolysis of amide linkages. This is the rate of a definite chemical reaction involving the breaking of only one bond. The results of many such measurements made with wool are shown in figure 1.

It is obvious that different acids vary enormously in their effectiveness in hydrolyzing the amide bonds of this protein. Thus the rates given by hydrochloric acid and cetylsulfonic acid,[5] differ by a factor of 114. All degrees of effectiveness between these extremes are represented by the other acids studied. Their relative effectiveness as hydrolyzing agents tends to parallel the affinity of their anions for proteins, as previously reported [9].

A number of other acids have been studied which are not included

[5] Because of its low solubility, cetylsulfonic acid was tested at a lower concentration (0.02 *M*), but the total concentration of acid was 0.05 *M*, as in the other experiments.

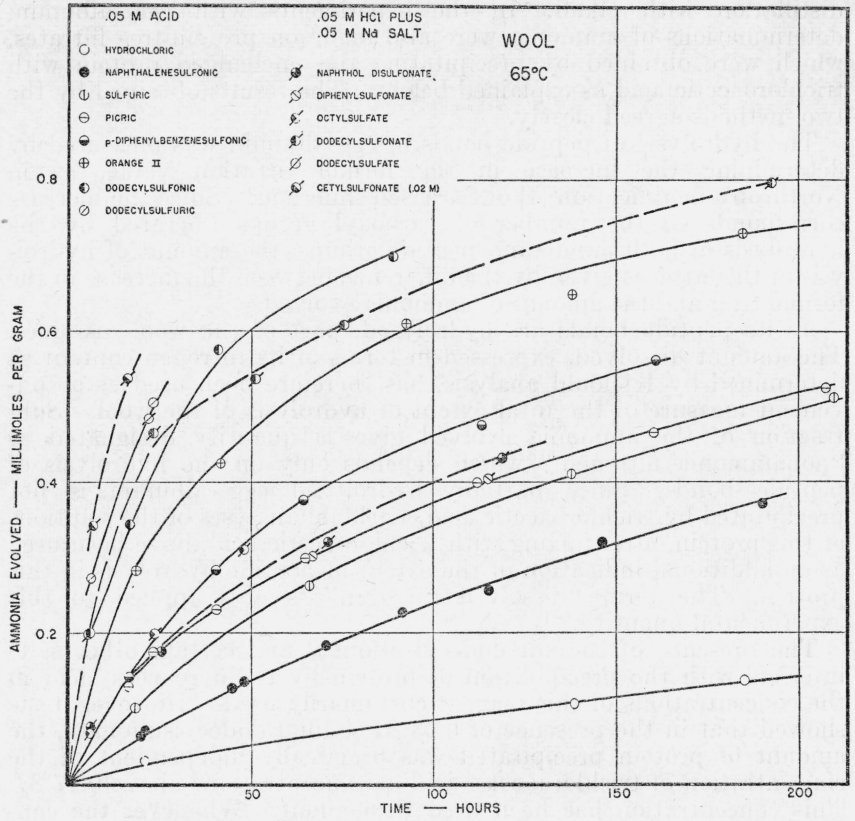


FIGURE 1.—*Relative effectiveness of various strong acids in hydrolyzing the primary amide bonds of wool.*

All the acids are compared at the same concentration of hydrogen ion (0.05 *M*) and at the same temperature (65° C). Points crossed by a shilling mark represent measurements obtained with equimolar mixtures with hydrochloric acid of the sodium salts of the anions indicated.

in figure 1. Among them is the homologous series of sulfate half-esters ($ROSO_3H$), for which comparative data are given in table 1. Here a maximum hydrolytic effect appears to be reached with the 14-carbon acid. This effect is a consequence of the lower hydrogen-ion concentration in solutions of the higher homologues, a result of an increasing tendency to form molecular aggregates as their molecular weight increases [10]. The results obtained with the sulfate half-esters are further complicated by the fact that as their molecular weights increase they are increasingly rapidly hydrolyzed at 65° C to give sulfuric acid and the alcohols.[6] Thus with these acids the rate of the hydrolysis is affected by a simultaneous increase in acidity and decrease in the concentration of effective anions as hydrolysis proceeds. This complication does not affect the data obtained with the sulfonic acids (RSO_3H), which are shown in figure 1.

[6] The half periods for this self-hydrolysis, in the case of the highest members of the series, are less than 24 hours.

TABLE 1.—*Relative effectiveness of various sulfate half-esters in accelerating the acid hydrolysis of wool at 65° C*

All the solutions contained 0.05 *M* HCl plus 0.05 *M* sodium salt of the half-esters

Sulfate half-ester	Amide hydrolysis. Time required to liberate 0.3 millimole ammonia per gram	Peptide hydrolysis. Time required to liberate 1.0 millimole of nonammonia nitrogen per gram
	Hours	*Hours*
n-Octyl-	45.2	100
n-Decyl-	11.4	26
n-Dodecyl-	9.8	17
n-Tetradecyl-	9.3	15
n-Hexadecyl-	10.3	21
n-Octadecyl-	12.2	29

2. DEPENDENCE OF REACTION RATE ON CONCENTRATION OF CATALYTICALLY EFFECTIVE ANIONS

The influence of the more effective anions may be appraised, apart from the simultaneous influence of hydrogen ions, by varying the concentration of the sodium salt of a strongly effective anion, at a fixed concentration of one of the least effective acids, i. e., hydrochloric acid. This procedure is justified because the effect of the presence of sodium and chloride ions is relatively small (cf. data for dodecylsulfonate in figure 1).

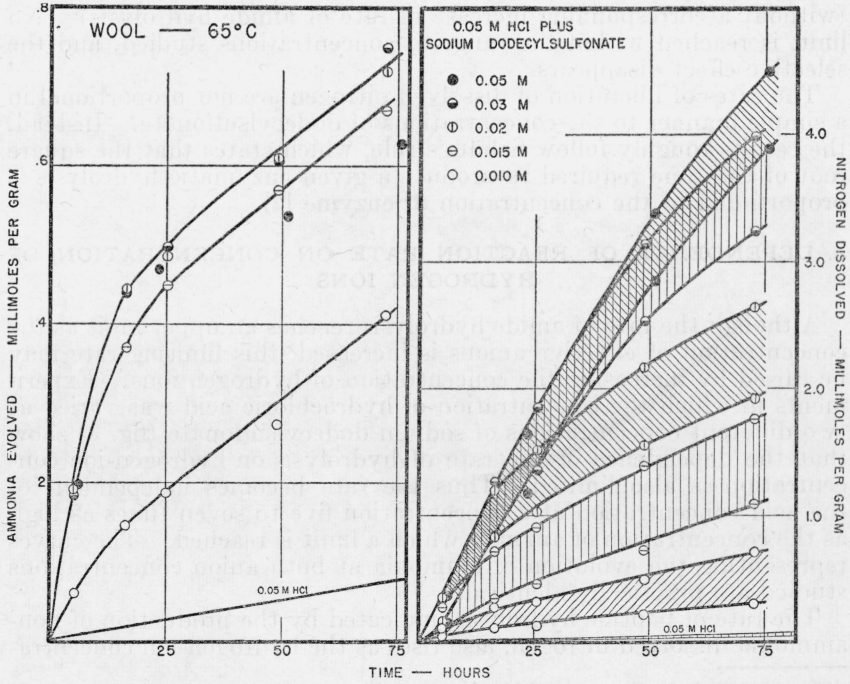


FIGURE 2.—*Hydrolysis of wool in 0.05 M hydrochloric acid at 65° C, as affected by the concentration of dodecylsulfonate ion.*

The hydrolysis of amide bonds is represented in the left-hand frame, and the evolution of dissolved nitrogen, which is a function of the hydrolysis of peptide as well as amide bonds, is represented in the frame on the right. In the latter, experiments with each concentration of anions are represented by a pair of curves, the area between which is shaded. The upper curve of each pair represents the total dissolved nitrogen, and the lower curve represents the nonammonia nitrogen, which refers to the hydrolysis of peptide bonds only.

The results of one such experiment with sodium dodecylsulfonate at 65° C are shown in figure 2. The measurements of amide hydrolysis (left-hand frame) are particularly significant. The rate of hydrolysis increases sharply as the concentration of dodecylsulfonate is increased until a limit is reached at a concentration between 0.015 *M* and 0.02 *M*. Beyond 0.02 *M* no increase in rate occurs.[7] Another limit exists at very low concentrations (<0.008 *M*, not shown in the figure). This limit is practically identical with the curve shown in the figure for hydrochloric acid alone. The lowest concentration at which the rate is appreciably affected by the presence of dodecylsulfonate is stoichiometrically equivalent to the amount of strongly basic groups in the protein.[8] The concentration at which the upper limiting rate is reached corresponds roughly to the sum of the amino groups (0.8 millimole per g) plus the amide groups (0.8 to 0.9 millimole per g) of the wool.

The rate of peptide hydrolysis, as measured by the evolution of nonammonia nitrogen (right-hand frame of fig. 2), is affected by the same range of dodecylsulfonate concentrations in a different way. Low concentrations (0.01 to 0.02 *M*) cause only a small increase in the rate of liberation of nonammonia nitrogen as compared with their effect on the rate of evolution of ammonia. Thus the addition of small amounts of this anion to solutions of hydrochloric acid selectively favors the hydrolysis of amide linkages. With larger amounts the rate of production of nonammonia nitrogen steadily increases (without a corresponding increase in rate of amide hydrolysis). No limit is reached within the range of concentrations studied, and the selective effect disappears.

The rates of liberation of dissolved nitrogen are not proportional in a simple manner to the concentrations of dodecylsulfonate. Instead, the results roughly follow Schütz's rule, which states that the square root of the time required to produce a given enzymatic hydrolysis is proportional to the concentration of enzyme [2].

3. DEPENDENCE OF REACTION RATE ON CONCENTRATION OF HYDROGEN IONS

Although the rate of amide hydrolysis reaches an upper limit as the concentration of effective anions is increased, this limiting rate may be raised by increasing the concentration of hydrogen ions. Experiments in which the concentration of hydrochloric acid was varied at two different concentrations of sodium dodecylsulfonate (fig. 3) show that the dependence of the rate of hydrolysis on hydrogen-ion concentration is also limited. Thus the rate becomes independent of the acid concentration at a concentration five to seven times as high as the concentration of anion at which a limit is reached. The curves representing the evolution of ammonia at both anion concentrations studied are practically identical.

The rate of peptide hydrolysis, indicated by the production of nonammonia dissolved nitrogen, also rises as the hydrogen-ion concentra-

[7] The slight diminution in rate at 0.05 *M* is due to the slightly lower hydrogen-ion concentration of this solution, in which decomposition products more quickly accumulate.

[8] About 0.8 millimole per g, corresponding to about 0.008 *M* at the ratio of wool to solution in these experiments.

tion is increased. Extrapolation of the data appears to indicate that a limiting rate is reached at concentrations of acid somewhat higher than those studied.

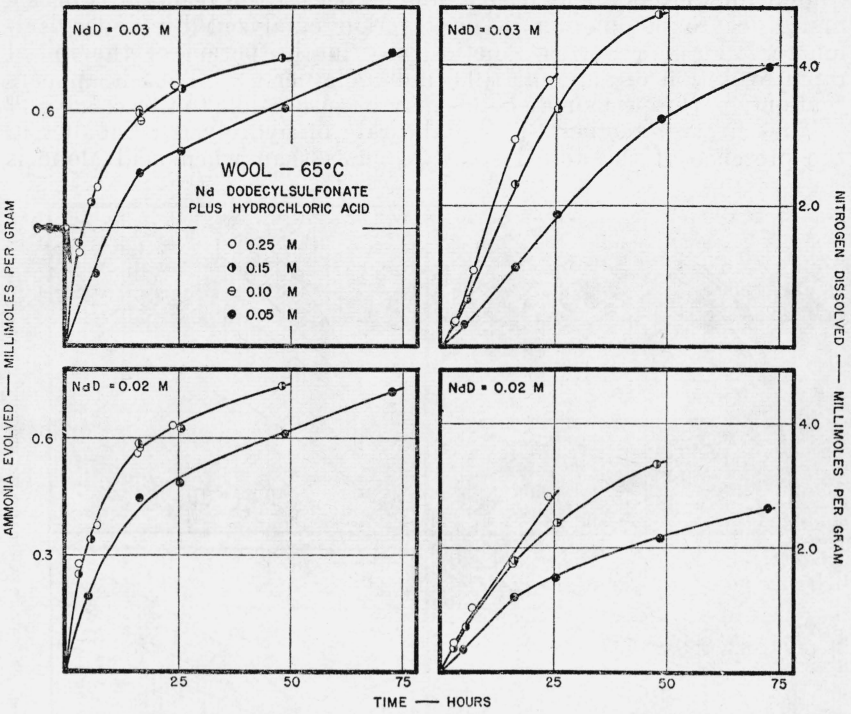


FIGURE 3.—*Relative rates of anion-catalyzed hydrolysis of wool at 65° C, as affected by the concentration of acid.*

Unlike figures 2 and 4, the curves represent the results obtained with different concentrations of acid instead of different concentrations of salt. Results with two different concentrations of dodecylsulfonate are given. Both concentrations are above the level at which the limiting rate of amide hydrolysis in 0.05 *M* acid is attained.

4. DEPENDENCE OF REACTION RATE ON CONCENTRATION OF EFFECTIVE ANIONS AND ON TEMPERATURE AT HIGHER CONCENTRATIONS OF ACID

The course of hydrolysis with respect to time, shown in figures 1 and 2, is affected by changes in pH and in concentration of effective anions, as the products of hydrolysis accumulate. These effects may be reduced by working with higher concentrations of acid. The results of experiments made with three times the concentration of acid represented in figure 2 are shown in figure 4. Data are given for two temperatures.

Owing to the high initial concentration, the change in pH in these experiments never exceeded 0.04 unit. This results in somewhat simpler chemical kinetic relations. The rate of liberation of nonammonia dissolved nitrogen is directly proportional to the concentration of catalytically effective anions, with good approximation. However, the time course of the hydrolysis of amide bonds remains complex. A rapid initial evolution of ammonia (about 0.6 millimole per g) is

followed by a much more gradual reaction, which is hardly faster than when no dodecylsulfonate is present. The course of the reaction in solutions of hydrochloric acid alone appears to be simple; if the amide content of wool is about 0.84 millimole per g,[9] the reaction is of the first order to completion. The reaction catalyzed by dodecylsulfonate follows first-order kinetics only in the period of the initial rapid evolution of ammonia (0.6 millimole per g). Thus it appears that only part of the amide bonds may be susceptible to this catalyst.[10]

The effect of temperature on the rate of hydrolysis is smaller in the presence of the added salt (Q_{10}=2.3) than when acid alone is

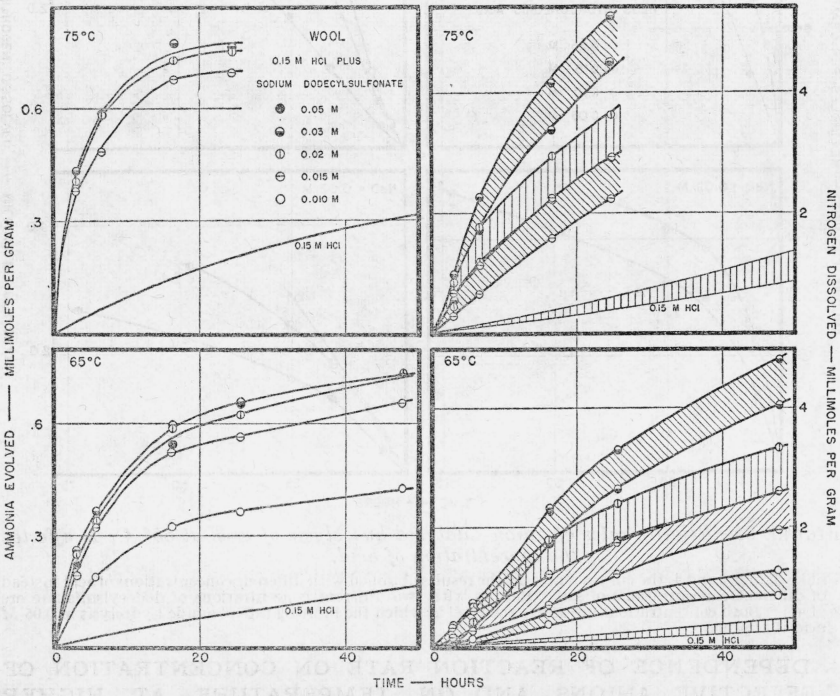


FIGURE 4.—*Hydrolysis of wool in 0.15 M hydrochloric acid, as affected by the concentration of dodecylsulfonate ion and by the temperature.*

used (Q_{10}=3.0). This difference agrees with the common rule that the activation energies of catalyzed reactions are lower than those of the same reactions in the absence of a catalyst. Because of this difference, the catalytic coefficients of the anion (the factor by which it increases the rates) is lower at the higher temperature.

The catalytic coefficient is also lower at 0.15 *M* acid than at 0.05 *M*. Thus, at still higher concentrations, such as those normally used for protein hydrolysis, the catalytic effect of dodecylsulfonate ions may be entirely negligible (cf. section IV).

[9] This value (1.18%) is close to others obtained in this laboratory by a number of different methods.

[10] Of the two amino-acids in wool which may be present in the form of amides, glutamic acid is found in a quantity which corresponds closely with the rapidly evolved portion of the ammonia.

5. RESULTS OBTAINED WITH A SOLUBLE PROTEIN

It is of great interest to determine whether the specific hydrolytic effects of anions which manifest themselves with wool also affect other proteins. Egg albumin has been chosen for investigation as an example of a soluble protein, principally because the careful work of Shore, Wilson, and Stueck [6] provides a close estimate of its amide content.

The results obtained in a series of experiments analogous to those performed with wool (fig. 2) are shown in figure 5. In most important

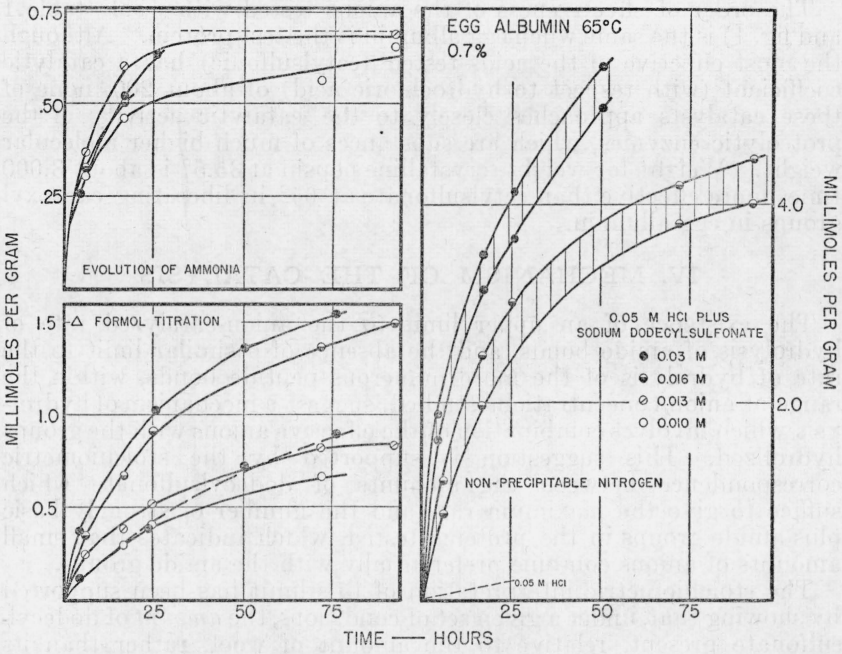


FIGURE 5.—*Hydrolysis of four times crystallized egg albumin in 0.05 M hydrochloric acid at 65° C., as affected by the concentration of dodecylsulfonate ion.*

The horizontal broken line in the upper left-hand frame represents the amide content of egg albumin [6]. The lower left-hand frame represents the change in the formol titer as the result of liberation of carboxyl groups in the protein. The broken line in this frame represents the hydrolysis of peptide bonds at the highest concentration of dodecylsulfonate represented. It was obtained by subtracting the appropriate curve for ammonia evolved from the top curve in this figure.

particulars they are entirely parallel with those obtained with wool. The rate of evolution of ammonia becomes independent of the dodecylsulfonate concentration at a lower concentration (0.011 *M*) than with wool, corresponding to stoichiometric equivalence to the sum of the basic groups plus amide groups of these protein solutions.[11] Unlike wool, egg albumin liberates practically all of its amide nitrogen very quickly; the experimental values thus soon approach those previously reported [6]. Although the state of dispersion of the two proteins is entirely different, the *initial* rate of ammonia evolution is only slightly higher with egg albumin than with wool.

[11] The titration data of Kekwick and Cannan [5] indicate that egg albumin contains per gram about 0.9 millimole of amino and guanidino groups. The amide content [6] is about 0.7 millimole per g. The sum, 1.6 millimoles per g, corresponds to a concentration of about 0.011 *M* at the concentration of protein used in these experiments.

With this soluble protein, the formol titration gives a direct measure of the total number of bonds hydrolyzed. Data obtained by this method, with several concentrations of dodecylsulfonate, are shown in figure 5. The rate of hydrolysis increases without limit within the range of concentrations studied. At the lowest concentration the hydrolysis indicated by the formol titration is hardly larger than that due to breaking amide linkages only, but at higher concentrations the increased rate is obviously due to increased peptide hydrolysis. The selective effect of low concentrations of anions, indirectly indicated by the work with wool, is here directly shown.[12]

The order of effectiveness of the anions tested with wool (table 1 and fig. 1) is the same when egg albumin is the test protein. Although the most effective of the acids tested (cetylsulfonic) has a catalytic coefficient (with respect to hydrochloric acid) of about 200, none of these catalysts approaches closely to the catalytic activity of the proteolytic enzymes, which are substances of much higher molecular weight. Weight for weight, crystalline pepsin at 35.5° is about 3,000 times more effective than cetylsulfonate at 65° in liberating carboxyl groups in egg albumin.

IV. MECHANISM OF THE CATALYSIS

The existence of an upper limit to the anion-catalyzed rate of hydrolysis of amide bonds, and the absence of a similar limit to the rate of hydrolysis of the more numerous peptide bonds, within the range of anion concentrations studied, suggest a mechanism of hydrolysis which involves combination of the effective anions with the groups hydrolized. This suggestion is supported by the stoichiometric correspondence between the amounts of dodecylsulfonate which suffice to give the maximum rate and the number of strongly basic plus amide groups in the proteins tested, which indicates that small amounts of anions combine preferentially with the amide groups.

The stoichiometric interpretation of this limit has been supported by showing that, under a given set of conditions, the *amount* of dodecylsulfonate present, relative to the amount of wool, rather than its *concentration* in solution, determines the hydrolytic rate. One of the solutions represented in figure 2 (0.02 *M* in sodium dodecylsulfonate, and 0.05 *M* in hydrochloric acid) was diluted with an equal volume of 0.05 *M* hydrochloric acid. The *concentration* of effective anion thus fell below that required for the attainment of the maximum rate of amide hydrolysis in the earlier experiments, although the total amount remained unchanged. The rate of production of ammonia remained as high as if the dodecylsulfonate had not been diluted.

The result of the experiment just described depends on the fact that dodecylsulfonate ion is almost quantitatively transferred from the solution to the fibers [9]. With anions having a lower affinity for wool, the concentration at which a limiting rate is attained should be higher than with dodecylsulfonate, and should not be assigned stoichiometric significance. Data obtained with an anion of only moderate affinity for wool, octylsulfate, are shown in figure 6.

[12] The formol data also indicate that only about 10 percent of the peptide bonds in the protein were hydrolyzed when two-thirds of the protein was not precipitated with trichloroacetic acid.

The concentration of octylsulfate at which a maximum rate of amide hydrolysis is attained is at least five times as great as in the experiments with the ion of higher affinity. However, the maximum rate obtained with octylsulfate is only slightly lower than the maximum attained with dodecylsulfonate. Thus, the two anions, when *combined with the protein*, are almost equally effective.[13]

Since the maximum anion-catalyzed rates attained at a given acid concentration may be increased by raising the concentration of acid, it is apparent that hydrogen ions, as well as anions, participate in the

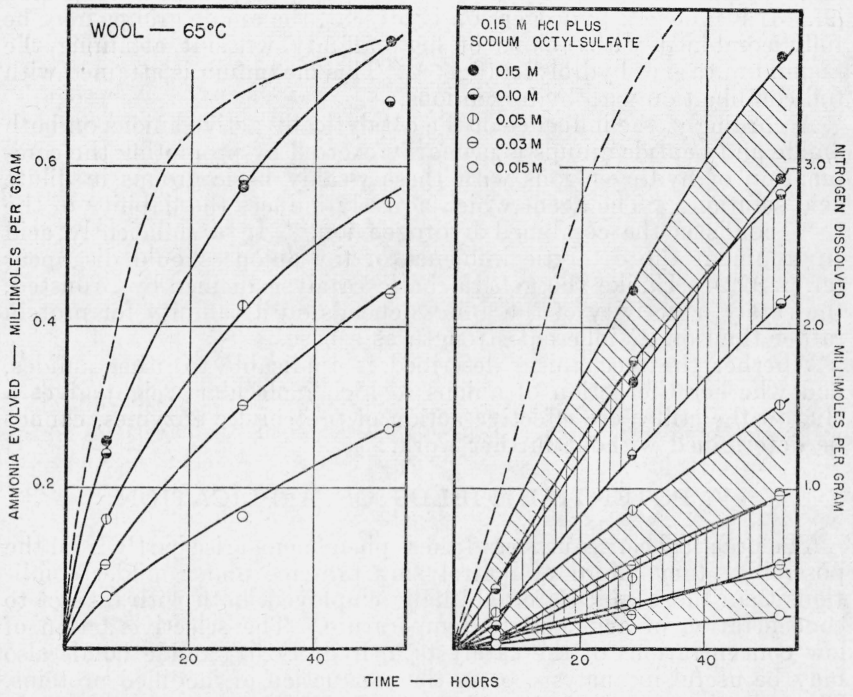


FIGURE 6.—*Hydrolysis of wool at 65°C, as affected by the concentration of octylsulfate ion.*

The concentration of acid (0.15 *M*) is higher than in the experiments represented in figure 2. The broken lines, included for comparison, represent data obtained with the same concentration of acid, and 0.03 *M* sodium dodecylsulfonate, a concentration well in excess of the amount required to attain the maximum rate of amide hydrolysis with this anion.

reaction. However, the fact that the effect of varying the concentration of acid is also limited to relatively low concentrations (<0.1 *M*) shows that its influence is not a common case of simple hydrogen-ion catalysis. In the latter, only an exceedingly small fraction of the hydrogen ions combine with the substrate molecules. In the present case, a very considerable part must combine since saturation of the combining groups in the protein is evidently attained at relatively low concentrations. Under ordinary conditions, amide and peptide groups are too weakly basic to combine to any appreciable extent with hydrogen ions at these concentrations of acid, but their

[13] The small residual differences between the limiting effects of various anions may be attributed to a kinetic salt effect of the uncombined ions.

readiness to combine with hydrogen ions may be greatly increased by acquiring a negative charge by combination with an anion. Similar effects of charge on the strengths of acids and bases have been discussed by the present authors [8, 9] and have been treated theoretically by many others.

The fact that much more hydrogen ion than dodecylsulfonate ion must be present to attain the maximum rate of amide hydrolysis indicates that the proteins combine with hydrogen ion less readily than with dodecylsulfonate. This indication is consistent with the relative affinities for wool of these two ions, as previously reported [9]. If insufficient hydrogen ion is present, the amide groups may be fully combined with anions of high affinity without attaining the maximum rate of hydrolysis (fig. 3). This maximum is attained with full combination with hydrogen ions.

Accordingly, the influence of the catalytically active anions on both amide and peptide groups is primarily exerted by promoting the combination of hydrogen ions with these weakly basic groups in dilute acid solutions. The agent which actively induces the liability of the C-N bonds is the combined hydrogen ion.[14] In a sufficiently acid environment the catalytic influence of the anions should disappear (cf. p. 322). Unlike the general basic catalysis defined by Brønsted, the catalytic activity of the anion depends on its affinity for protein rather than on its inherent strength as a base.

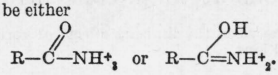
Whether the mechanism described is applicable to other amides, and whether the action of anions of high molecular weight gives a clue to the still more effective action of proteolytic enzymes, cannot be determined without further work.

V. POTENTIAL FIELDS OF APPLICATION

The practical consequences of these phenomena arise partly from the possibility they afford of hydrolyzing proteins under milder conditions than those which are habitually employed, both with respect to concentration of acid and to temperature. The selective action of low concentrations of the catalysts in hydrolyzing amide bonds also may be useful in analysis, or in the production of modified proteins. This selective effect is favored by (a) use of anion concentrations only slightly greater than are required for combination with the amino and guanidino groups of the protein, (b) low acidities, such as 0.05 *M*, (c) relatively low temperatures, such as 65° C. The result is also influenced by the choice of anion, a higher selective action resulting from the use of cetylsulfonate or diphenylbenzenesulfonate than from the use of any other ions tested.

Mild methods of protein hydrolysis are of value in the preparation of hydrolysates for intravenous or oral use in medicine [3], because they are less likely to impair the nutritive value of the digested material. Likewise, it is sometimes desirable to dissolve or destroy protein materials without affecting other materials which may be associated with them. The removal of glues and albuminous coatings from cellulosic materials is one instance; the purification of enzymes and other biological products is another.

[14] The reactive intermediate may be either



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