THE MECHANISM OF THE INFLUENCE OF ACIDS AND ALKALIES ON THE DIGESTION OF PROTEINS BY PEPsin OR TRYPsin.

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It has long been known that pepsin digestion occurs only in acid solution while trypsin digestion takes place in neutral or alkaline solutions. This marked influence of the reaction on the rate of hydrolysis is a characteristic property of enzymes in general. A great advance in the explanation of this phenomenon was made by Sörensen (1) who was able to show in the case of a number of enzymes that the controlling factor was the hydrogen ion concentration, and that the other ions present were of secondary importance. Michaelis (2) found that the curves expressing the rate of hydrolysis as a function of the pH could be accurately calculated on the assumption that the enzyme was a weak acid or base, and that the activity was due either to the ion or to the undissociated molecule, depending on the nature of the enzyme. Michaelis' experiments leave little doubt that the experimental curve is very similar to a dissociation curve. The weak point in the hypothesis is the lack of direct or independent evidence for the ionic character of the enzymes and the fact that migration experiments (3) on pure pepsin appear to contradict some of the assumptions used in the calculations. It had been suggested by Euler (4) and by Arrhenius (5) that the effect of the acid was upon the substrate rather than on the enzyme. The writer was able to show (6) that in the case of pepsin this mechanism served very well to explain the results, since it was found that the rate of hydrolysis could be quite accurately predicted from a measurement of the concentration of ionized protein and also that the pH for minimum digestion shifted with the isoelectric point of the protein. This indicates that the effect is primarily on the protein. The present paper contains
the results of experiments designed to test this assumption more fully and to extend the observations to trypsin. It has been found that the rate of digestion of gelatin, casein, and hemoglobin by pepsin or trypsin may be predicted from the amount of protein ion present. The acid salt of these proteins is rapidly attacked by pepsin, and the alkali salt by trypsin. The curve for the rate of digestion plotted as a function of the pH is therefore nearly identical with the titration (dissociation) curve of the proteins. The same result has been found with trypsin, edestin, and globin in that the alkaline titration curve of these proteins agrees with the curve for the rate of trypsin hydrolysis.

**Experimental Procedure.**

*Regulation and Measurement of the pH.*—The measurements were made with the gas chain at 33°C, using 0.10 N HCl as the standard. The pH of this acid was taken as that given by the conductivity ratio: 1.036.

*Buffers.*—In the pepsin experiments no buffer was used; the protein solution was simply titrated to the desired pH with acid (HCl). There was no significant change in pH during the reaction. Trypsin hydrolysis causes a very large change in pH and it is necessary to use a powerful buffer. It was found that a combination of phosphate, borate, and citrate would furnish a solution having a high buffer value over a wide range of pH. It has the further advantage that the nature of the ions does not vary. The titration curve of this buffer is given in Fig. 1. It is not intended to be used as a standard and for accurate values the solutions should be checked in some way. The final concentration of the buffer was usually one-quarter that of the original solution.

*Pepsin.*—The pepsin used was a sample of specially active Fairchild's pepsin (U.S.P. 1:20,000).

*Trypsin.*—Fairchild's trypsin was used. It was purified by dialysis, as described in a preceding paper (7).

*Temperature.*—All experiments were made at 33°C ± 0.5°C.

*Determination of the Rate of Digestion.*—The rate of hydrolysis was determined by noting the time for a sample to reach a definite degree of liquefaction, as previously described (6).

*Casein.*—The casein (8) was dissolved in dilute NaOH and brought to the required pH by addition of the appropriate buffer. An aliquot portion of the solution was removed, brought to approximately pH 4.7 and added to 3 volumes of acetate buffer pH 4.7. The precipitated casein was then filtered, washed with water, dried at 100°C and weighed. The curves obtained in this way were plotted and the time required to dissolve about 10 per cent of the total casein read off. This value has been taken as the reciprocal of the velocity of hydrolysis.
Hemoglobin.—The hydrolysis was followed by means of the increase in amino nitrogen, by the Van Slyke method (9).

Trypsin. Casein.—The same method was used as that described above. The hydrolysis was also followed by the Van Slyke method for amino nitrogen.

Gelatin.—The hydrolysis was followed by the increase in formol titration (10) and also by the Van Slyke method.

Globin.—The globin was prepared as described by Robertson (11). The rate of hydrolysis was followed by bringing the solution to the isoelectric point (about pH 6) and filtering off and weighing the undissolved protein.

Edastin.—The writer is indebted to Dr. Hitchcock for the edestin used in this experiment. The rate of hydrolysis was determined by the Van Slyke method and also by precipitating and weighing the unattacked protein.
Titration Curves.—The titration curves were determined by adding a known amount of alkali or acid to the protein solution and determining the pH. A similar series was carried out in which the protein was omitted. The amount of protein combined with the acid (or alkali) was then determined by subtracting the cubic centimeters of acid required to bring water to any given pH from the cubic centimeters of acid required to bring an equal volume of the protein solution to this pH. This correction is negligible between pH 3 and 9. The curves obtained were identical with those given by Loeb.

In the case of casein solution it was found that two entirely different pH curves were obtained (in the region near the isoelectric point), depending on whether the supernatant fluid or the whole suspension was measured. This behavior had also been noted by Cohn. If the measurement was made on the whole suspension, the curve rises much more steeply near the isoelectric point and is similar to the curve given by Van Slyke and Baker (8). It also resembles closely the curve for gelatin and does not conform to the typical titration curve. Loeb (12) has recently shown that this effect is due to the fact that the pH of a suspension is intermediate between that of the supernatant liquid and that of the particle. The correct curve is that of the supernatant fluid.

Conductivity measurements (6) show that the protein salt is completely dissociated at least until a very large excess of the common ion is added. The titration curve, therefore, gives directly the amount of ionized protein up to the maximum value; beyond this point the amount of ionized protein decreases whereas the amount of combined acid remains constant. This decrease in the ionized protein can be followed in the case of gelatin (on the acid side) as described in a previous paper (6). In the case of the other proteins, however, the point lies in the region of such strong acid that the measurement is very uncertain, owing to the very large value of the correction for the free acid. The determination of this value was, therefore, not attempted in the case of the other proteins. On the alkaline side the conditions are more complicated, owing to the fact that there are apparently two alkaline salts formed, i.e., there is a group of acid radicals that is completely neutralized at pH 9 and another group that is not neutralized until a pH of 11 or 12 is reached. This is very marked in the case of gelatin and casein. Hemoglobin, however, shows only a very slight flattening of the curve at pH 7.5; the second group of acid radicals evidently begins to combine with the alkali before the first group is completely neutralized.
Effect of the Amount of Enzyme.—It has been found by the writer (13) and also by Ringer (14) that trypsin is very rapidly destroyed in solutions of pH greater than 10. In order to reduce this effect, it is necessary to use relatively large amounts of trypsin so that the experiment is completed in as short a time as is permitted by the experimental limitations. If this is not done the results are complicated by the influence of the pH on the trypsin itself. This effect would evidently cause the optimum to shift to the acid side and would be more marked the more dilute the trypsin; i.e., the longer the experiment is allowed to run. Several experiments were made to test this point and it was found that, with the amounts of trypsin used in these experiments, the position of the optimum was independent of the amount of trypsin used. The drop in the rate of digestion in strong alkali, therefore, cannot be attributed to the destruction of the trypsin by the alkali. This complication does not enter in the case of pepsin, since this enzyme is stable, even in strong acid.

Presentation of Results.—For the sake of brevity the original curves obtained for the increase in the digestion with time are not given, but only the rates interpolated from these curves. The results were plotted on a large scale and the time (in hours) required to hydrolyze 10, 20, and 30 per cent of the protein read off from the curves. The reciprocal of these times is taken as a measure of the rate of digestion. It was found that the values obtained in this way were the same, within the rather large experimental error, independent of the percentage hydrolysis which was used as the endpoint. In other words, the rate of hydrolysis-pH curve is independent of the stage of hydrolysis, in the beginning. If the results are taken from a much later stage of hydrolysis, the curve is changed slightly, as would be expected, since the secondary reactions become more predominant and the effect of the pH on the destruction of the enzyme more pronounced. No significant effect of different enzyme concentration could be noted, nor did the different methods of following the hydrolysis give results which differed more than the experimental error. It is unnecessary, therefore, to distinguish between the different experiments.
In order to reduce all the experiments to a comparative basis, the results have been calculated to a percentage scale; i.e., the maximum rate of hydrolysis has been taken as 100 and the rates at other pH expressed as percentages of this maximum.

Comparison of the Titration Curve and the Rate of Hydrolysis.—In order to compare the rate of hydrolysis with the amount of protein salt found, it is necessary to express both quantities in the same units. This has been done by calculating the titration curve also to a percentage basis. The largest amount of alkali or acid which is found to combine with the protein has been taken as 100 and the amounts at other pH expressed as per cent of this figure. The maximum on the acid side is well defined and there is no difficulty in deciding at which pH the protein is all present as the acid salt. On the alkaline side, as mentioned above, the titration curve shows that there are two alkaline salts formed. It could not be told a priori from the titration curve alone which of the salts was attacked by the trypsin. Comparison with the hydrolysis pH curve, however, shows that, except in the case of globin, the maximum rate of hydrolysis occurs at the same pH as does the maximum formation of the first alkali salt. The point at which the greatest amount of this salt is formed has therefore been taken as 100. In the case of globin the hydrolysis increases with the second rise in the titration curve, indicating that in this case the second salt formed is still more easily attacked. The second maximum in the titration curve has therefore been taken as 100 in the case of this protein. The position of this maximum is much more difficult to determine and it is possible that the quantitative agreement of the titration and rate of hydrolysis curves of globin is partly due to an incorrect value for the maximum point of the titration curve. There is no doubt, however, that the curves agree qualitatively in that they both have a flat portion between pH 8 to 9, followed by a second rise; the only uncertainty is as to the exact coincidence of this second maximum. The hydrolysis curve cannot be carried further, owing to the destruction of the trypsin.

Results of the Experiments. Influence of the Ions Other Than H and OH and the Influence of the Physical Properties of the Substrate.—The work of Sörensen (1), Michaelis and his coworkers (2), and of the writer (6, 13, 15) has shown conclusively that the hydrogen or
hydroxyl ion is the determining factor and that the nature and valence of the other ion is of very minor importance as is the physical condition of the substrate. Nevertheless the question of the influence of the "degree of hydration" or swelling of the protein has again been raised by Ostwald (16). This idea may be traced back to Brücke (1859). Brücke (17) was a pioneer worker and it is easy to understand his suggestion that swelling and digestion are connected since both increase with increasing amounts of acid. At present, however, it would seem to be difficult to add anything to the proof already existent that the swelling, osmotic pressure, "degree of hydration," etc., have no direct connection with the hydrolysis of proteins by either pepsin or trypsin. Since, however, this is a fundamental point in the study of these enzymes, some of the experiments referred to were repeated. The critical experiment is to compare the rate of hydrolysis of a protein in the presence of sulfate and chloride ion and in the presence of Na and Ba. It has been shown by Loeb (18) that the osmotic pressure, viscosity, and swelling of a protein are very much less in the presence of bivalent ions than in the presence of monovalent ions at the same pH. It has also been shown that the amount of acid required to bring the protein solution to a given pH is independent of the nature and valence of the anion. In other words the chemical effect of all acids is the same, but the effect on the physical properties is different for various acids. It has also been shown by Loeb that this effect on the physical properties has nothing to do with the degree of hydration, but is the result of an equilibrium governed by the law of mass action, the theory of which was stated by Donnan (12). If the rate of hydrolysis, then, depends on any physical property of the protein, there should be a marked difference in the rate of hydrolysis of gelatin in the presence of sulfuric and of hydrochloric acid. The results of an experiment made to test this point are shown in Fig. 2. The figure shows the rate of hydrolysis of gelatin by pepsin in the presence of sulfuric acid and of HCl, and of gelatin and casein by trypsin in the presence of Ba and Na. As the figure shows, there is no difference whatever in the rate of hydrolysis in sulfuric acid as compared to HCl solution nor in Ba(OH)$_2$ as compared with NaOH (of the same pH), although the swelling, viscosity, and osmotic pressure are very much less in the presence of the divalent ions.
In the case of two solutions in one of which the protein is soluble and in the other insoluble, it would be expected that there would be a difference in the rate of digestion, no matter what mechanism is assumed to govern the reaction, since the concentration of the protein in the solution is entirely different in the two cases. In the insoluble case the protein salt is concentrated at the bottom of the vessel while in the soluble case it is distributed throughout the solution.

Even here, however, there is no striking difference in the rate of digestion, as shown by the writer’s experiments (19) with edestin and casein in hydrochloric and sulfuric acids and Gyemant’s (20) experiments with sulfosalicylic acid.

Relation between the Titration Curve and the Rate of Hydrolysis.—The experiments described above show that the effect of the acid and alkali is primarily on the chemical condition of the protein. Increasing addition of acid or alkali causes the formation of increasing amounts of protein salt. The increase in this amount of salt is shown
by the titration curve. If the increase in the rate of hydrolysis is
due to the fact that the enzyme reacts with the ionized protein, the
increase in the rate of hydrolysis with increasing acid or alkalis should
be identical with the titration curve. The results of the experiments
are shown in Figs. 3 and 4. The figures show that in general the
results agree very well with the assumption that the ionized protein is more readily attacked by the enzyme. The agreement in the case of casein is striking. In every case the solid line is the titration curve and the points represent the results of the hydrolysis experiments. It must be kept in mind that the titration curves give the amount of acid combined and not the amount of ionized protein. The two figures are the same up to the maximum, but beyond that point the ionization will decrease, due to the effect of the common ion. This decrease is actually shown in the case of gelatin in acid solution where it is possible to measure the ionization by conductivity measurements. The experiment shows that this decrease is in quantitative agreement with the decrease in the rate of hydrolysis. Another complication is added by the fact that the hydrolysis experiments with trypsin are necessarily carried out in fairly high concentration of salt which tends to decrease the amount of ionized protein, especially near the isoelectric point. The curves also show that the pH of minimum digestion is shifted with the isoelectric point of the protein. This is shown more clearly in Fig. 5 in which the pH rate of hydrolysis curves of all the proteins studied have been plotted. The figure also shows that the descending branch of the curves varies with the protein as well as the ascending branch. If the decrease in hydrolysis found on adding excess alkali or acid were due to the
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effect on the enzyme, as has been proposed by Gyemant (20) it would be expected that the descending curves would all be the same.

The experiments as a whole substantiate the idea that the reaction of the protein and enzyme depends on the concentration of ionized protein, although the experiments do not all show quantitative agreement with this mechanism. Pepsin reacts with positively charged protein ions and trypsin with negatively charged protein ions. This view is also supported by the fact that when the protein is insoluble, it combines with pepsin only when the protein is positive (21). It is also in agreement with the result of Pekelharing and Ringer (3) who found that pure pepsin is negatively charged.

A marked difference between the rate of reaction of ionized and unionized substances is the general rule in all chemical reactions and has been found to be at the basis of many reactions; it is not surprising, therefore, that it should be found to hold also in the case of protein hydrolysis. It may be remarked that the writer (10) has found that in the case of gelatin hydrolyzed by acid or alkali without any enzyme, the relative ease of hydrolysis is reversed and the results can be accounted for on the assumption that the unionized gelatin is more rapidly attacked.

An objection to this point of view which presents itself is the fact that in the case of sugar and other substances, it can hardly be supposed that the acid or alkali could affect the substrate in such a way as to give a titration curve. Michaelis and Rothstein (22) have recently suggested that in the case of sugar, a compound is formed between the invertase and the sugar, and that it is the dissociation of this compound that regulates the hydrolysis.

SUMMARY.

1. The effect of the addition of acid on the amount of ionized protein has been compared with the effect on the rate of digestion of gelatin, casein, and hemoglobin by pepsin.

2. A similar comparison has been made of the addition of alkali in the case of trypsin with gelatin, casein, hemoglobin, globin, and edestin.

3. In general, the rate of digestion may be predicted from the amount of ionized protein as determined by the titration curve or
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conductivity. The rate of digestion is a minimum at the isoelectric point of the protein and a maximum at that pH at which the protein is completely combined with acid or alkali to form a salt.

4. The physical properties of the protein solution have little or no effect on the rate of digestion.

BIBLIOGRAPHY.