Experiments with crystalline pepsin already described (1–3) show that the crystalline protein has a constant and characteristic proteolytic activity. The proteolytic activity of various preparations is the same and cannot be changed by repeated recrystallization, partial solution in various salt concentrations, or by fractional diffusion. The loss of activity when the protein is denatured by alkali corresponds to the amount of denatured protein formed and when this denatured protein is changed back to native protein the original activity is regained. These results render it very improbable that the activity is due to the presence of any non-protein molecule adsorbed on the protein molecule since adsorption complexes in general do not have constant properties. They indicate that the proteolytic activity is a property of this molecular species of protein, just as the peculiar properties of hemoglobin are attributes of the molecule of the protein, hemoglobin. In both cases the characteristic property is lost when the protein is denatured and regained when the protein is again brought into the native form (2, 4). In the case of hemoglobin it is known that the characteristic properties are due to the presence of a special prosthetic group in the molecule and it is probable that the pepsin protein also contains some characteristic group to which its activity is due. There is at present no evidence as to the nature or even the existence of such a characteristic group and, owing to the difficulty of determining the structure in the case of proteins, it is difficult to obtain such evidence by the ordinary methods of structural chemistry. The problem would be greatly simplified if a part of the protein molecule possessing even very slight activity could be split
off. The attempt was therefore made to hydrolyze pepsin under conditions which would not completely destroy the activity and to determine whether a solution could be obtained which was more active than would be expected from its content of protein. If any of the split products of the protein were appreciably active compared to the original protein molecule the total activity of the solution would be higher than that calculated from its protein content.

Unfortunately the methods of hydrolyzing the protein are limited practically to dilute acid. Several attempts were made to hydrolyze pepsin with trypsin at pH 6 which is as far on the alkaline side as it is possible to work without causing alkali inactivation. The trypsin is, however, very rapidly destroyed by the pepsin and no hydrolysis of the pepsin could be detected so that there was no change in either the protein concentration or the activity when trypsin was added to pepsin solutions. Attempts to hydrolyze the protein with papain were also unsuccessful. No hydrolysis of the pepsin protein or decrease in peptic activity could be detected. Most of the protein present in the papain preparation used was very rapidly digested and there was a slight loss in papain activity so that the results indicate that papain is digested by pepsin although very much more slowly than is trypsin.

Hydrolysis in Dilute Acid

It is known that pepsin solutions lose their activity slowly when allowed to stand in dilute acid and it was shown in the first paper (1) that this loss in activity was exactly equivalent to the amount of native protein destroyed, since if a saturated solution of the protein crystals is partially inactivated and then saturated again with the crystals, the activity of the solution returns exactly to its original value while the total nitrogen content increases to just the extent calculated from this increase in activity. This is a very sensitive method since the solubility is probably the most specific property of a protein. It cannot be used, however, to follow the hydrolysis very far since the products formed by the hydrolysis, when present in considerable quantities, begin to affect the solubility of the crystals. In the present experiments, therefore, the concentration of protein nitrogen in the solution was determined directly by precipitating the protein with 2.5 per cent trichloracetic acid and also by determining
the quantity of protein which becomes denatured and insoluble when
the solution is made alkaline. This effect of alkali is a characteristic
property of the pepsin protein and it seemed possible that the figure
obtained in this way might differ from that obtained with 2.5 per
cent trichloracetic acid since the latter reagent might precipitate other
substances than the unchanged protein itself. However, the results
showed that exactly the same amount of protein nitrogen was deter-
mined by both methods. In addition, the amount of nitrogen precipi-
tated by phosphotungstic acid was also determined. The loss in
activity was determined by several methods.

TABLE 1

Decrease in Activity and Protein Nitrogen in Pepsin Solutions at Different pH

<table>
<thead>
<tr>
<th>Concentration HCl mols/l</th>
<th>0.01</th>
<th>0.05</th>
<th>0.20</th>
<th>1.0</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approximate pH</td>
<td>4.0</td>
<td>4.5</td>
<td>5.0</td>
<td>6.0</td>
<td>6.5</td>
</tr>
<tr>
<td>Hrs. at 35°C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P.U/</td>
<td>P.U/</td>
<td>P.U/</td>
<td>P.U/</td>
<td>P.U/</td>
</tr>
<tr>
<td></td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
<td>ml.</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>780</td>
<td>1.82</td>
<td>770</td>
<td>0.81</td>
</tr>
<tr>
<td>24</td>
<td>9</td>
<td>720</td>
<td>0.52</td>
<td>620</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Effect of pH

As a preliminary experiment a series of solutions of crystalline pep-
sin containing different concentrations of hydrochloric acid were kept
at 35°C. for 24 hours and the protein nitrogen per ml. of solution pre-
cipitated with 2.5 per cent trichloracetic acid was determined at the
beginning and the end of the experiment. The results of this experi-
ment are shown in Table I. The table shows that the more acid the
solution the more rapidly the protein is hydrolyzed so that with 2
molar hydrochloric acid only about 10 per cent of the original protein
is present after 24 hours. The table also shows that the decrease in
activity is proportional to the decrease in protein nitrogen. The effect of pH on the stability of the protein therefore is similar to that already found for crude pepsin preparations (5).

Mechanism of the Reaction

There are several possible mechanisms which could account for the hydrolysis of the protein under these conditions.

1. The reaction is analogous to ordinary acid hydrolysis of proteins and differs only in that it proceeds more rapidly than is the case with other proteins.

2. The first step in the reaction is the formation of denatured protein which is then hydrolyzed by the acid.

3. The protein digests itself, or denatured protein is formed, by the acid and this denatured protein is then digested by the remaining active protein.

The results as a whole indicate that the reaction is simply a case of ordinary acid hydrolysis and that no denatured protein is formed. The denatured protein is completely insoluble in solutions more acid than pH 3 and if such solutions are heated to boiling rapidly the protein is completely precipitated. No precipitate appears in any of the solutions in the preceding experiment and therefore no appreciable amount of denatured protein is present. In general, there is an optimum pH for the digestion of proteins by pepsin, while the denaturation of proteins increases with the concentration of acid and no optimum is obtained. If, therefore, the first step in the reaction were the formation of denatured protein by the acid and this denatured protein were subsequently hydrolyzed by the active enzyme, it would be expected that in very strong acid solution denatured protein would accumulate in the solution since there should be a point on the acidity curve where the rate of digestion would be decreased by increasing acid while the formation of denatured protein would increase. This is not the case and indicates quite strongly that the observed hydrolysis is not due to enzyme activity. The evidence is not so clear in regard to the possibility that denatured protein is the first step in acid hydrolysis itself. It would be expected that the rate of hydrolysis of denatured protein would increase with the acidity just as does the formation of denatured protein. If it is assumed,
therefore, that the denatured protein is more rapidly hydrolyzed than the native protein, then there would be no accumulation of denatured protein at any degree of acidity and the results would agree with this mechanism. As a matter of fact, if the protein is denatured by boiling and then kept at 50°C, it is found to hydrolyze less rapidly than a similar solution which has not been boiled. The boiled protein, however, is present in the form of a precipitate and it could be supposed that this was the reason for the slow rate of hydrolysis. On the other hand, there is no evidence for the existence of any denatured protein in any of the solutions at any time so that the assumption that the first step in the reaction consists in denaturation is hardly justified. For the time being, therefore, the reaction will be considered simply as a case of ordinary acid hydrolysis.

Decrease in Protein Nitrogen and Activity at pH 1.8

If any of the products of the hydrolysis of the pepsin were active their presence might be shown by changes in the relative velocity with which the solution hydrolyzes different proteins as well as by a change in the specific activity of the protein in the solution with regard to one substrate. In order to determine whether such changes, either in specific activity or comparative activity with different proteins, occur during the hydrolysis of the pepsin an experiment was carried out at about pH 1.8 and at 50°C. in which the decrease in protein nitrogen and the decrease in activity were determined. The activity was measured by the change in viscosity of gelatin, casein, or edestin, the rennet action on milk, the formation of non-protein nitrogen from casein and edestin, the rate of increase in formol titration of edestin and gelatin, and also the digestion of hemoglobin.

About 2 gm. of four times recrystallized pepsin was stirred into 50 ml. of water and dissolved by the careful addition of 1 ml. m/1 sodium hydroxide. 10 ml. of m/1 hydrochloric acid was then added rapidly and the solution placed at 50°C. 10 ml samples were withdrawn at various time intervals and 2 ml. m/1 sodium acetate added in order to bring the pH to about 4. The samples were stored in the ice box until the experiment was completed and then analyzed for protein nitrogen and for activity by the various methods mentioned above.

The results of the experiment are shown in Table II. The table shows that the protein nitrogen, as determined by either alkali de-
naturation or precipitation with 2.5 per cent trichloracetic acid decreases from 1.9 mg. per ml., its original value, to 0.06 while the protein nitrogen, as determined by precipitation with phosphotungstic acid decreases only to about 0.5 mg. per ml. The activity of the solution as measured by any of the methods decreases in almost exact proportion to the decrease in the concentration of protein nitrogen as determined by 2.5 per cent trichloracetic acid. This fact may be best shown by expressing the results in terms of the specific activity per milligram protein nitrogen. This figure is obtained by dividing the observed activity per ml. of solution by the number of milligrams of protein nitrogen per ml. of solution. The results of the experiment calculated in this way are shown in Table II. The table shows that

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein nitrogen per ml.</th>
<th>[P.U./mg. P.N.] (Specific activity of protein N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.90</td>
<td>1.80</td>
</tr>
<tr>
<td>3</td>
<td>0.62</td>
<td>0.60</td>
</tr>
<tr>
<td>5</td>
<td>0.20</td>
<td>0.21</td>
</tr>
<tr>
<td>6</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

the specific activity, as measured by any of the eleven methods used remains constant, within the limits of error of the method, throughout the entire experiment. They prove, therefore, that the activity is entirely due to the original protein remaining in solution and that if any of the split products of the protein possess any activity it must be less than 5 per cent of that possessed by the protein molecule itself. The methods are not sufficiently accurate to detect changes in activity of less than 5 per cent and it is still possible that some of the split products may have a very low activity. In addition to the constant activity the figures also show that there is no change in the relative activity as determined with different proteins. The experiment cannot be continued further since five hundredths of a milligram protein nitrogen per ml. approaches the limit of accuracy of protein nitrogen
determinations although the activity measurements are very much more sensitive. It is necessary, however, to be able to compare the activity with the protein nitrogen content and no information can be obtained from activity measurements made on a solution containing too little protein nitrogen to determine quantitatively.

EXPERIMENTAL METHODS

Activity Measurements were made as already described (6).

Protein Nitrogen Methods

2.5 Per Cent Trichloracetic Acid.—1 ml. of solution is added to 10 ml. of 2.5 trichloracetic acid, heated to 80°C. for 10 minutes, and then cooled. The precipitate is centrifuged, washed three times with 2.5 trichloracetic acid, dissolved in dilute sodium hydroxide. The solution is washed into the Kjeldahl flask and the total nitrogen determined.

Alkali Protein.—2 ml. of the solution is added to 2 ml. M/5 sodium hydroxide, 2 ml. K/5 hydrochloric acid added and then 5 ml. of a solution of M/2 sodium sulfate and M/200 sulfuric acid. The suspension is centrifuged, washed three times with the sodium sulfate solution, the precipitate dissolved in dilute sodium hydroxide, and the total nitrogen content determined by the Kjeldahl method.

Phosphotungstic Acid.—Reagent—1.5 gm. of phosphotungstic acid dissolved in 5 ml. concentrated hydrochloric acid, made up to 100 ml. with ethyl alcohol, and then made up to 500 ml. with water. 1 ml. of the solution is added to 5 ml. of this reagent, the suspension centrifuged, washed three times with the reagent, the precipitate dissolved in dilute sodium hydroxide and the total nitrogen content determined by the Kjeldahl method.

SUMMARY

The decrease in protein nitrogen and in the activity of solutions of crystalline pepsin at pH 1.8 and 45°C. has been determined. The decrease in activity, as measured with eleven different methods, is in exact proportion to the decrease of protein nitrogen of the solution. The measurements were continued until less than 5 per cent of the original protein remained. These results indicate that none of the split products of the protein molecule possess any appreciable activity compared to that of the original protein.

BIBLIOGRAPHY

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