THE SIGNIFICANCE OF THE HYDROGEN ION CONCENTRATION FOR THE DIGESTION OF PROTEINS BY PEPSIN.

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One of the most striking peculiarities of enzyme action is the fact that the activity of the enzyme is limited to a definite range of acidity. If the solution is more or less acid than this the enzyme is practically inactive. Sörensen\(^1\) showed that for a number of enzymes the determining factor was the hydrogen ion concentration and not the total acidity of the solution.

In attempting to account for this phenomenon it has usually been assumed that the influence of the hydrogen ion concentration was exerted upon the enzyme. Michaelis\(^2\) pointed out, in the case of many enzymes, that if the activity of the enzyme was plotted against the hydrogen ion concentration of the solution the curve resembled strikingly that obtained when the ionization of a salt of a weak base and a strong acid was plotted in the same way. He concluded therefore that enzymes were weak bases or acids which formed salts with the acids or bases of the solution. These salts then dissociated into ions and the ions so formed were the active agents in the reaction. A similar explanation had already been proposed independently by Loeb\(^3\) and by Nasse\(^4\). Michaelis' work rendered the hypothesis quite plausible. In the case of pepsin, however, it meets with several serious objections. In the first place, one of the strongest points of Michaelis' experiments was the fact that pepsin was found to have an isoelectric point at about pH 3.0 which agreed fairly well with the

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theory. Pekelharing and Ringer, however, showed that in solutions of pure pepsin (prepared by Pekelharing's method from gastric juice) the pepsin was always negatively charged. This objection may of course be met by the statement that the pepsin under the actual conditions of hydrolysis (i.e. when in the protein solution) is not pure but is combined with some other substance and it is the ionization of this compound which determines the activity of the enzyme. An explanation similar to this has been offered by Michaelis. The author has shown, however, that pepsin combined with peptone or other decomposition products of the proteins is inactive and that it is only the free pepsin which takes part in the reaction. It was also found that no positively charged pepsin could be found on the alkaline side of pH 3.3. Pepsin retains its activity up to pH 5, however, so that it seems unlikely that only positively charged pepsin is active, as assumed by Michaelis.

A second objection to Michaelis' view is the fact that the optimum hydrogen ion concentration for the activity of pepsin is found to vary with the substrate. This point has been emphasized by Long and Hull (for trypsin) and by Ringer. From Michaelis' point of view it is difficult to see how this can be. Neither of these objections, however, can in the author's opinion be considered as conclusive evidence against Michaelis' hypothesis. It could be stated for instance that pepsin contained several enzymes, one for each substrate and each with a different optimum. It seems simpler, however, to assume that the hydrogen ion concentration affects the condition of the substrate rather than the enzyme. This hypothesis has the advantage that it also accounts for the peculiar relation between the concent-

tration of the substrate and the rate of hydrolysis. Experiments described in a former paper show that the rate of hydrolysis of protein solutions of varying concentration but the same pH was directly proportional to the amount of ionized protein present in the solution but not to the total concentration of protein. They agree therefore with the hypothesis that the ionized protein is the form which takes part in the reaction. If this explanation is correct it follows that the optimum hydrogen ion concentration for the activity of pepsin is also due to the increased ionization of the protein and must coincide with the hydrogen ion concentration at which the protein solution contains the greatest number of protein ions. (It was first suggested by Pauli that the ionized protein was the form which was attacked. Euler and Arrhenius have made a similar suggestion. Ringer considers also that the ionization of the substrate has an influence on the rate of digestion at least in the later stages.) It should be possible therefore to determine the optimum degree of acidity for pepsin digestion by measuring the conductivity of the protein solution. It will be shown below that this is true. It will further be shown that the range of hydrogen ion concentration in which the enzyme is active shifts in the same sense as the conductivity of the protein solution when a protein of different isoelectric point is used, and also that when the protein is insoluble the enzyme combines with it only over that range of hydrogen ion concentration in which the enzyme is active and in which the protein is ionized.

The Influence of the Isoelectric Point of the Protein on the Activity of Pepsin at Different Hydrogen Ion Concentrations.

Ringer has already shown that the optimum hydrogen ion concentration for the digestion of proteins by pepsin varies somewhat with the protein hydrolyzed and with the acid used. He accounts for this phenomenon by the assumption that the hydration of the

12 Pauli, W., Arch. ges. Physiol., 1910, cxxvi, 483.
14 Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 44.
protein determines the ease with which it is attacked by the enzyme. The viscosity is assumed to be a measure of the hydration. The same explanation has been proposed by Brücke,16 by Pfliederer,17 and recently by Traube.18 The writer has been able to show,19 however, that gelatin digests at the same rate in sulfuric or hydrochloric acid solution (provided the pH is the same) although the swelling, which Ringer considers a measure of the hydration, is much greater in hydrochloric than in sulfuric acid. It was also found11 that the rate of digestion of egg albumin solutions decreased as the viscosity increased with the age of the solution instead of increasing as would be expected if the rate of digestion was determined by the hydration of the protein (as shown by the viscosity). Loeb20 has shown that the ionization of the protein and the viscosity and swelling are all approximately proportional for a small range of acidity to the acid side of the isoelectric point. The maximum for the swelling and viscosity, however, occurs at about pH 3.4 whereas that for the ionization is much further to the acid side and agrees very well for that of the rate of digestion. This question will be discussed more fully below. It is clear, however, that in certain cases the swelling or viscosity and the ionization and rate of digestion may all be proportional. It would seem from the experiments described here that the determining factor for the rate of digestion is the ionization of the protein, and the swelling and viscosity are secondary characteristics which are probably also connected with the ionization.

It is known that, with most proteins, pepsin becomes inactive at a pH of about 4.5. This cannot be ascribed to the destruction of the enzyme since the author8 found pepsin to be more stable in this range of acidity than at any other. The ionization of most proteins is very slight at this pH, however, so that it would be expected (from the hypothesis that it is the protein ion which is attacked by the enzyme) that little or no hydrolysis should occur at this point. Oxy-

17 Pfliederer, R., Arch. ges. Physiol., 1897, lxvi, 605.
16 Traube, M., Deutsch. med. Woch., 1919, xxvii,
20 Loeb, J., J. Gen. Physiol., 1918-19, i, 39; 1920-21, iii, 85.
hemoglobin, however, is isoelectric at a pH of about 6.8 (Michaelis) so that it must be quite largely present as a salt and therefore ionized at a pH of 4.5. It would be predicted then, according to the hypothesis that the amount of protein ions present determines the rate of digestion of the protein, that hemoglobin should be digested by pepsin at pH 4.5 more rapidly than is egg albumin or gelatin at the same pH.

In order to test this prediction, parallel experiments were made to determine the rate of digestion and the conductivity of hemoglobin and egg albumin solutions at various hydrogen ion concentrations. The results of such an experiment are shown graphically in Fig. 1. It is clear that the conductivity and digestion curves, for each protein, as plotted against the pH of the solution are approximately parallel.
and also that the curves for the digestion and conductivity of the hemoglobin fall further to the left (i.e. to the alkaline side) than do the curves for the egg albumin.

The experiments cannot be considered as showing quantitative agreement between the rate of digestion and the conductivity of the solution since the digestion curve is given as the amount of protein decomposed in a certain time—a quantity which is not connected in any simple way with the rate of digestion. They are further complicated by the fact that the digestion in the region of the optimum acidity represents approximately 50 per cent of the complete digestion of the protein and therefore probably includes the secondary splitting of some of the primary products of the hydrolysis, and not purely the action on the protein itself. The conductivity on the other hand was measured on the protein solution itself. It is not possible to carry the digestion curve much beyond pH 5.0 owing to the rapid destruction of the enzyme.

EXPERIMENTAL.

Egg Albumin.—The egg albumin was crystallized three times as described by Hopkins and Pinkus\(^{21}\) and then dialyzed under pressure of about 150 cm. of water at pH 4.8 until the specific conductivity of the solution was less than \(1 \times 10^{-4}\) reciprocal ohms. The solution was then diluted to 2 per cent with water. Increasing amounts of HCl were added to a series of 50 cc. portions of this solution and the total volume made up to 100 cc. 1 cc. of 2 per cent pepsin was then added to 25 cc. of these solutions and placed at 25\(^\circ\)C. 1 cc. of the solution was analyzed by the Van Slyke\(^{22}\) method for amino nitrogen after 0, 8, 24, and 36 hours. The curve given is the increase in cubic centimeters of amino nitrogen per cubic centimeter of solution after 24 hours. The 8 and 36 hour curves were similar.

Conductivity.—1 cc. of inactivated pepsin was added to another 25 cc. portion of the above solutions and the conductivity and pH of the solution were measured at 25\(^\circ\)C. The conductivity of the egg albumin salt was determined from the conductivity of the solution by subtracting from the observed conductivity the conductivity of HCl of the same pH (Northrop\(^{19}\)).

Oxyhemoglobin.—Erythrocytes from fresh defibrinated ox blood were washed with 7.8 per cent glucose solution until the conductivity of the suspension was less than \(1 \times 10^{-4}\) reciprocal ohms. The cells were then laked with ether, separated from the excess ether, and the ether in the solution removed \textit{in vacuo}. The solution was then diluted to contain about 1 cc. of amino nitrogen per cubic centimeter as determined by the Van Slyke method. The conductivity of this


solution was about $1 \times 10^{-5}$ reciprocal ohms. Increasing amounts of HCl were added to 50 cc. portions of this solution and the total volume made up to 100 cc. The conductivity and digestion of the solution were then determined as described for the egg albumin.

The Optimum Hydrogen Ion Concentration for Pepsin Digestion.

The optimum hydrogen ion concentration for the activity of pepsin has been determined many times. All the methods used for following the digestion, however, have depended on the change in some physical property of the protein. It seemed of interest therefore to determine the optimum degree of acidity for the reaction when the hydrolysis was followed by means of the increase in amino nitrogen, which probably represents correctly the actual course of the digestion. The method has the disadvantage, however, that only comparatively large changes can be followed. The results of an experiment made with egg albumin solutions of different pH (adjusted with HCl) are given in Fig. 2.

The time of digestion was 4 hours. The figure shows that the optimum acidity for the digestion as determined by the increase in amino nitrogen is at about pH 1.0 (0.1 N). This is slightly more acid than that found by Sörensen, Michaelis and Mendelssohn, or Okada, and much more acid than that found by Ringer. It must be remembered,

![Fig. 2. Influence of pH on the rate of digestion of egg albumin.](image)

however, that the chemical changes followed by the increase in amino nitrogen represent much more complete hydrolysis than those followed by the other authors. The curve therefore probably does not represent the correct optimum for the digestion of the protein itself but probably also the digestion of some of the primary products. The careful work of Ringer has shown that the optimum zone for the digestion of these products extends further to the acid side than the zone for the digestion of the protein itself. This probably accounts for the difference in the optimum found by the different methods and agrees with the results of Sörensen who found that the optimum shifts to the acid side with more complete digestion.

The Effect of Adding Salt with a Common Ion to a Solution Already Containing the Optimum Amount of Acid.

It will be noted from the curve (Fig. 2) that the amount of digestion increases with increasing amounts of acid in the solution until the hydrogen ion concentration is about 0.1 N and then decreases. According to the hypothesis that it is the ionized protein which is hydrolyzed by the pepsin, the increase in digestion from pH 4.0 to 1.0 is due to the fact that as acid is added to the albumin more protein salt and hence more protein ions are formed in the solution, until all the albumin is present as salt. The addition of a further amount of acid serves to depress the concentration of protein ions again due to the effect of the common ion. According to this mechanism the hydrogen ion concentration is the determining factor on the alkaline side of the optimum while on the acid side the concentration of the anion is the determining factor. It can be predicted therefore that if a solution of a salt (having the same anion as the acid) is added to a solution of the protein which already contains the optimum amount of acid, the depressing effect of the salt on the digestion should be the same as if excess acid had been added, provided the final anion concentration is the same. The conductivity of the albumin salt should also be diminished. In the case of egg albumin this cannot be experimentally verified owing to the fact that the albumin precipitates under these conditions, and also since the conductivity of the protein in such strongly acid solutions is so small, compared to
the total conductivity, as to render the measurement very uncertain. It will be shown later, however, that in the case of gelatin the decrease in conductivity can be followed and is proportional to the decrease in the rate of digestion.

**TABLE I.**

*Increase in Amino Nitrogen per Cc. of Solution Containing Normal Total Chlorine Concentration Furnished by Different Salts.*

<table>
<thead>
<tr>
<th>Salt</th>
<th>pH</th>
<th>Increase in NH₃ nitrogen per cc. after 6 hrs. at 25°C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>0.42</td>
<td>0.25, 0.26</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.40</td>
<td>0.15</td>
</tr>
<tr>
<td>KCl</td>
<td>0.42</td>
<td>0.15, 0.15</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.41</td>
<td>0.17, 0.15</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.42</td>
<td>0.12, 0.11</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>0.42</td>
<td>0.17, 0.18</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>0.40</td>
<td>0.10, 0.11</td>
</tr>
<tr>
<td>HCl</td>
<td>0.13</td>
<td>0.13, 0.14</td>
</tr>
</tbody>
</table>

Table I contains a summary of the results of such an experiment in which a series of egg albumin solutions all containing a total chlorine ion concentration of 0.5 N and at a pH of 0.42 were brought to a total chlorine ion concentration of 1.0 N by the addition of the salts noted or excess acid. The final solutions therefore were all
1.0 N in respect to the chlorine ion but those which had been brought to this concentration by the addition of salts were of course much less acid than the one to which excess acid had been added. The amount of digestion in all the solutions containing the same chlorine ion concentration was approximately the same, however. This result indicates that the controlling factor on the acid side of the optimum is the anion concentration and not the hydrogen ion concentration. As a corollary of this it can be stated that the addition of salt to a protein solution will cause the optimum hydrogen ion concentration for digestion to be shifted to the alkaline side. This was the effect noted by Michaelis and Mendelsohn.  

The above question has recently been examined by Gyemant. This author found, however, the optimum pH for digestion remained at about pH 2.0 even though the anion concentration was the same in all the solutions. He concludes therefore that the decrease in the rate of digestion on the acid side of the optimum is due to the influence of the hydrogen ion on the pepsin as proposed by Michaelis.

The experiments described in this paper are complicated by the fact that the egg albumin was partially precipitated by the high concentrations of salt and acid used. This may account for the difference between the present results and those of Gyemant. The discrepancy may also be due to the fact that Gyemant followed the reaction by means of the increase in non-protein nitrogen whereas the author used the increase in amino nitrogen. In view of Gyemant's results and of the complicating factor of precipitation in the present experiments, they cannot be considered as conclusive evidence in favor of the view that the anion alone affects the digestion on the acid side of the optimum. It is possible that both ions are active. It appears to the author, however, that the action is exerted on the protein rather than the enzyme in view of the fact that different proteins show slightly different optimum pH, and of the close connection between the conductivity and rate of digestion of gelatin solutions (as described below in this paper).

The Conductivity and Rate of Digestion of Gelatin Solutions.

It was mentioned above that determinations of the conductivity of egg albumin solutions in strongly acid solution were made uncertain owing to the precipitation of the protein. This difficulty is not encountered with gelatin. Gelatin possesses the further advantage that the rate of digestion in the very early stages may be easily fol-

followed by noting the time necessary to cause a certain degree of liquefaction of the gelatin.

A series of gelatin solutions, containing 5 per cent dry weight of gelatin and adjusted to various hydrogen ion concentrations by means of HCl, were prepared. The gelatin had previously been purified as described by Loeb.26 The conductivity of the solutions and the time necessary for them to reach an easily determined degree of liquefaction were then determined. The reciprocal of this time is plotted in the curve as the rate. Fig. 3 and Table II show the result of a typical experiment of this kind. It is clear that the rate of digestion and the conductivity of the solution have their maximum value at the same hydrogen ion concentration, and that the curves are nearly parallel throughout. The rate of digestion decreases slightly more rapidly than the conductivity of the solution on the alkaline side of the optimum and slightly less rapidly on the acid side. This peculiarity was noted in all the experiments made and can hardly be ascribed to experimental errors. It shows that the digestion on the alkaline side of the optimum is slightly less rapid than would be predicted from the conductivity data and that it is slightly more rapid on the acid side. The divergence on the acid side is due to the fact that in such strongly acid solutions the acid itself has some action on the protein as was shown by control experiments without any pepsin. The correction is too uncertain to be applied to the figures, however.

It can only be said that such a correction is necessary and that it would be in the right sense. The divergence on the alkaline side is probably due to the fact that the amount of hydrolysis selected as the end-point represented too great a percentage change in the original substrate concentration to assume that the substrate concentration remained constant during the course of the experiment.

### TABLE II.

**pH, Conductivity, and Rate of Digestion of Gelatin Solutions.**

Gelatin, 5 per cent dry weight in solution of total (approximate) concentration of HCl noted. Temperature, 37°C.

<table>
<thead>
<tr>
<th>Approximate total concentration of HCl</th>
<th>pH</th>
<th>C&lt;sub&gt;H&lt;/sub&gt; &lt;sup&gt;× 10&lt;/sup&gt;&lt;sup&gt;4&lt;/sup&gt;</th>
<th>Specific conductivity of HCl of same pH as solution (Reciprocal ohms &lt;sup&gt;× 10&lt;/sup&gt;&lt;sup&gt;4&lt;/sup&gt;)</th>
<th>Gelatin chloride (= a solution of HCl)</th>
<th>Time for gelatin to liquefy (Hours)</th>
<th>Rate (hrs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>4.23</td>
<td>0.60</td>
<td>17.2</td>
<td>0.27</td>
<td>17.0</td>
<td>4.5</td>
</tr>
<tr>
<td>0.04</td>
<td>3.50</td>
<td>3.16</td>
<td>33.1</td>
<td>1.47</td>
<td>31.6</td>
<td>1.1</td>
</tr>
<tr>
<td>0.06</td>
<td>2.78</td>
<td>16.6</td>
<td>48.2</td>
<td>7.7</td>
<td>40.5</td>
<td>0.40</td>
</tr>
<tr>
<td>0.08</td>
<td>1.78</td>
<td>166.0</td>
<td>110.0</td>
<td>76.0</td>
<td>34.0</td>
<td>0.42</td>
</tr>
<tr>
<td>0.10</td>
<td>1.48</td>
<td>331.0</td>
<td>175.0</td>
<td>151.0</td>
<td>24.0</td>
<td>0.65</td>
</tr>
<tr>
<td>0.12</td>
<td>1.26</td>
<td>550.0</td>
<td>260.0</td>
<td>245.0</td>
<td>15.0</td>
<td>0.92</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL.**

50 gm. (dry weight) of purified isoelectric gelatin were dissolved in warm water and the volume was made up to 500 cc. Increasing amounts of HCl were then added to a series of 50 cc. portions of this solution and the volume of each portion was then made up to 100 cc. 2 cc. of 2 per cent pepsin solution were then added to 75 cc. of each of the above solutions and the solutions put in the water bath at 37°C. At short intervals 5 cc. samples were pipetted from each of the solutions into a series of test-tubes containing 2 cc. of water. These tubes were then placed in a water bath at 2°C. for 10 minutes, taken out, and the degree of liquefaction was compared with that of a standard tube. (This is a slight modification of the method of Fermi as described by Dernby<sup>27</sup>) This procedure was repeated until a sample from each of the tubes showed the same degree of liquefaction as the standard tube. In this way the time necessary to produce a certain degree of liquefaction can be accurately and easily determined. The pH and con-

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<sup>27</sup> Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.
ductivity of the solution were determined on the remaining 25 cc. of solution to which had been added the equivalent amount of inactivated pepsin. The determinations were made as described above except that the measurements were made at 37°C.

The Combination of Pepsin and Gelatin.

In a former paper it was shown that the amount of pepsin which combined with a given quantity of coagulated egg albumin depended entirely on the reaction of the solution in which the egg albumin was suspended. The greatest amount of pepsin was combined when the solution had a reaction of pH 2.5 to 3.0. It was pointed out that this was probably part of the mechanism that caused insoluble proteins to digest more rapidly at this reaction than at any other since it seems that the rate of digestion must depend on the amount of pepsin in the solid protein.

These experiments have been repeated with gelatin and show in general the same result. The results of such an experiment are given in Fig. 4 and Table III. The figures show that a greater amount

\[\text{Fig. 4. Influence of pH on the combination of pepsin and gelatin.}\]

\[\text{Northrop, J. H., } J. \text{ Gen. Physiol., } 1919-20, \text{ ii, 113.}\]
of gelatin and pepsin is combined at about pH 3.0 than in either more or less acid solutions. In the case of gelatin the volume varies greatly with the reaction owing to the effect of the acid on the swelling of gelatin. The swelling is greatest at about pH 3.4 (cf. Loeb\textsuperscript{26}). It might be supposed therefore that more pepsin was combined with the gelatin at about this degree of acidity simply because there was

### TABLE III.

**Combination of Gelatin and Pepsin.**

5 gm. of isoelectric purified gelatin (= 0.75 gm. of dry weight) suspended in 200 cc. of HCl of strength noted and left 16 hours at 2°C. Filtered and washed twice with 100 cc. of water (5°C.) and total volume made up to 75 cc. 5 cc. of 2 per cent pepsin added. Allowed to stand 20 min. at 5° with occasional stirring. 4 cc. of supernatant fluid pipetted off and pepsin determined\* in 1 cc. of this sample. Gelatin filtered and volume of filtrate measured. Gelatin melted and pH determined of this and of the filtrate.

<table>
<thead>
<tr>
<th>Concentration of HCl</th>
<th>pH of Filt.</th>
<th>Volume of Filt. (cc.)</th>
<th>Volume of Gelatin (= 80 cc. of filtrate)</th>
<th>Pepsin in Filt. (cc.)</th>
<th>Units of pepsin</th>
<th>Total Pepsin in Gelatin (115 cc.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.3</td>
<td>46</td>
<td>50</td>
<td>1.7</td>
<td>107.0</td>
<td>8</td>
</tr>
<tr>
<td>M 256</td>
<td>3.6</td>
<td>42</td>
<td>57</td>
<td>23</td>
<td>0.65</td>
<td>37.0</td>
</tr>
<tr>
<td>M 64</td>
<td>3.0</td>
<td>34</td>
<td>47</td>
<td>33</td>
<td>0.52</td>
<td>25.0</td>
</tr>
<tr>
<td>M 8</td>
<td>2.4</td>
<td>29</td>
<td>47</td>
<td>33</td>
<td>0.71</td>
<td>33.0</td>
</tr>
<tr>
<td>M 4</td>
<td>1.8</td>
<td>20</td>
<td>53</td>
<td>27</td>
<td>1.17</td>
<td>62.0</td>
</tr>
<tr>
<td>Control. No gelatin.</td>
<td>5.2</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>1.44</td>
<td>115.0</td>
</tr>
</tbody>
</table>

\* Cf. Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 113. The relative amount of pepsin is taken as the reciprocal of the time in hrs. required to cause a 5 per cent change in the conductivity of a 5 per cent egg albumin solution titrated to pH 1.7 with hydrochloric acid when 1 cc. of pepsin solution is added to 25 cc. of egg albumin at 37°C. The unit of pepsin is taken as that amount which when dissolved in 1 cc. and added to 25 cc. of the egg albumin solution will cause a change of 5 per cent in the conductivity in 1 hr.
a greater volume of gelatin present at this point. It will be seen, however, from Table II and Fig. 3 that this is not true since the figures show that there is a maximum even when the results are calculated to the basis of pepsin per cubic centimeter of gelatin. That is, there is not only more pepsin combined with a gram of gelatin at this pH but also the concentration of the pepsin in the gelatin is greatest here. There is considerable uncertainty as to the pH measurements since, as the table shows, the reaction of the liquid was always considerably more acid than that of the gelatin. In most of the experiments the difference was much more marked than in the experiment given; in some cases the maximum fell at about pH 2.2. This agrees much more closely with the optimum acidity found for digestion and for the ionization of the protein. Owing to the uncertainty of the pH measurement, however, it is probably better to make no definite statement as to the exact position of the optimum acidity for the combination between the gelatin and pepsin. The determining factor in regulating the amount of pepsin which is combined with the gelatin is the chemical condition of the gelatin and pepsin and not a difference in the rate of diffusion of the pepsin since the same curve is obtained irrespective of the time (after the first few minutes) during which the gelatin is left in the solution. The simplest explanation would seem to be that the gelatin combines only with the ionized protein and the amount combined therefore is dependent on the amount of ionized gelatin present. Pepsin therefore behaves just as do the inorganic anions studied by Loeb as far as the influence of the hydrogen ion concentration on the combination is concerned.

**DISCUSSION AND SUMMARY.**

The experiments described above show that the rate of digestion and the conductivity of protein solutions are very closely parallel. If the isoelectric point of a protein is at a lower hydrogen ion concentration than that of another, the conductivity and also the rate of digestion of the first protein extends further to the alkaline side. The optimum hydrogen ion concentration for the rate of digestion and the degree of ionization (conductivity) of gelatin solutions is the same, and the curves for the ionization and rate of digestion as
plotted against the pH are nearly parallel throughout. The addition
of a salt with the same anion as the acid to a solution of protein
already containing the optimum amount of the acid has the same
depressing effect on the digestion as has the addition of the equivalent
amount of acid. These facts are in quantitative agreement with the
hypothesis that the determining factor in the digestion of proteins
by pepsin is the amount of ionized protein present in the solution.
It was shown in a previous paper\(^1\) that this would also account for
the peculiar relation between the rate of digestion and the concen-
tration of protein. The amount of ionized protein in the solution
depends on the amount of salt formed between the protein (a weak
base) and the acid. This quantity, in turn, according to the hydro-
lysis theory of the salts of weak bases and strong acids, is a function
of the hydrogen ion concentration, up to the point at which all the
protein is combined with the acid as a salt. This point is the optimum
hydrogen ion concentration for digestion, since the solution now
contains the maximum concentration of protein ions. The hydrogen
ion concentration in this range therefore is merely a convenient
indicator of the amount of ionized protein present in the solution
and takes no active part in the hydrolysis. After sufficient acid
has been added to combine with all the protein, \(i.e.\) at pH of about
2.0, the further addition of acid serves to depress the ionization of
the protein salt by increasing the concentration of the common
anion. The hydrogen ion concentration is, therefore, no longer an
indicator of the amount of ionized protein present, since this quantity is
now determined by the anion concentration. Hence on the acid side
of the optimum the addition of the same concentration of anion should
have the same influence on the rate of digestion irrespective of whether
it is combined with hydrogen or some other ion (provided, of course,
that there is no other secondary effect of the other ion). The pro-
posed mechanism is very similar to that suggested by Stieglitz and
his coworkers\(^9\) for the hydrolysis of the imido esters.

Pekelharing and Ringer\(^8\) have shown that pure pepsin in acid
solution is always negatively charged; \(i.e.,\) it is an anion. The
experiments described above show further that it behaves just as
would be expected of any anion in the presence of a salt containing
the protein ion as the cation and as has been shown by Loeb\(^9\) to be
the case with inorganic anions.

586, 719.
Nothing has been said in regard to the quantitative agreement between the increasing amounts of ionized protein found in the solution (as shown by the conductivity values) and the amount predicted by the hydrolysis theory of the formation of salts of weak bases and strong acids. There is little doubt that the values are in qualitative agreement with such a theory. In order to make a quantitative comparison, however, it would be necessary to know the ionization constant of the protein and of the protein salt and also the number of hydroxyl (or amino) groups in the protein molecule as well as the molecular weight of the protein. Since these values are not known with any degree of certainty there appears to be no value at present in attempting to apply the hydrolysis equations to the data obtained.

It is clear that the hypothesis as outlined above for the hydrolysis of proteins by pepsin cannot be extended directly to enzymes in general, since in many cases the substrate is not known to exist in an ionized condition at all. It is possible, however, that ionization is really present or that the equilibrium instead of being ionic is between two tautomeric forms of the substrate, only one of which is attacked by the enzyme. Furthermore, it is clear that even in the case of proteins there are difficulties in the way since the pepsin obtained from young animals, or a similar enzyme preparation from yeast or other microorganisms, is said to have a different optimum hydrogen ion concentration than that found for the pepsin used in these experiments. The activity of these enzyme preparations therefore would not be found to depend on the ionization of the protein. It is possible of course that the enzyme preparations mentioned may contain several proteolytic enzymes and that the action observed is a combination of the action of several enzymes. Dernby has shown that this is a very probable explanation of the action of the autolytic enzymes. The optimum hydrogen ion concentration for the activity of the pepsin used in these experiments agrees very closely with that found by Ringer for pepsin prepared by him directly from gastric juice and very carefully purified. Ringer's pepsin probably represents as pure an enzyme preparation as it is possible to prepare. There is every reason to suppose therefore that the enzyme used in this work was not a mixture of several enzymes.