COMPARATIVE HYDROLYSIS OF GELATIN BY PEP SIN, TRYPSIN, ACID, AND ALKALI.

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Gelatin and proteins in general may be hydrolyzed by either acids (hydrogen ions), alkali (hydroxyl ions) pepsin, or trypsin. In all cases the reaction consists in the rupturing of one or more of the peptide linkings with the addition of 1 molecule of water. The ease with which the various linkages are split is very different, as was shown by Fischer, in his study of the hydrolysis of the polypeptides. The same phenomenon is shown by the fact that the products of partial hydrolysis of the proteins form a series of increasing complexity—a result of the fact that some of the linkages are much more easily split than others. The same fact is brought out by following the course of the reaction in strong acid for example. If all the linkages were split at the same rate, the reaction would follow the course of a monomolecular one. This is not the case. The velocity decreases steadily as the reaction proceeds, showing that some of the linkages are split more easily than others.

The question arises then as to whether those linkages which are most easily split by acid, for instance, are also the most easily split by pepsin and trypsin. There is evidence already that this is not the case. Henriques and Gjaldbäk have shown by an ingenious modification of the formol titration that protein solutions having the same total content of titratable amino or carboxyl groups but which have been brought to this stage by different hydrolytic agents, may be distinguished from each other by their behavior when titrated with alkali to different end points, showing that, although the total

number of linkages split was the same in all the solutions, different ones had been split in each case. They found that the course of the reaction was similar with pepsin and acid, and with trypsin and alkali. The study of the hydrolytic products of trypsin and pepsin leads to the same conclusion. It is usually stated that trypsin hydrolysis leads to more amino-acid formation.

More quantitative evidence may be obtained, however, by a slightly different mode of procedure; namely, by adding the enzyme in question to a solution of the protein which has already been partially hydrolyzed by some other means and noting the subsequent increase in hydrolysis. If the two reactions follow the same course, i.e., if the same linkages are split in both cases, the final amount of hydrolysis will be the same irrespective of the stage at which the enzyme is added. If, however, the preceding hydrolysis has split the protein at a linkage which is not attacked by the enzyme, then the total amount of hydrolysis will be the greater the farther the hydrolysis had proceeded before the enzyme was added. That is, the total hydrolysis will be equal to the sum of the enzyme hydrolysis (on the unhydrolyzed protein) plus that which had taken place due to the other hydrolytic agent.

Since it is known that acids and alkali are capable of hydrolyzing the proteins to their constituent amino-acids, it is obvious that they must be able to split all the linkages so that a stage must eventually be reached in acid or alkali hydrolysis at which the addition of the enzymes will cause no further hydrolysis. The stage in the hydrolysis at which this occurs will evidently be a measure of the difference in the course of the two reactions. If the linkages which are most easily split by pepsin, for instance, are the ones which are most slowly attacked by acid, it will evidently be necessary to almost completely hydrolyze with acid the protein before reaching a stage at which the addition of pepsin will cause no further increase. As will be seen below, this is really the case.

The results are complicated by the fact that the ease of hydrolysis depends on other factors as well as on the particular linkage which is hydrolyzed. This was shown by Fischer who found that tetraglycyl glycine may be hydrolyzed by trypsin whereas triglycyl glycine is not

\[^{8}Cf.\, Fischer.\]
hydrolyzed. It is, therefore, not possible to conclude that the enzyme ceases to act, when a certain stage of the hydrolysis has been reached, because all the linkages which it is capable of hydrolyzing have already been split. The molecule may have been modified in some other way and so become resistant to the action of the enzyme although still containing a linkage which under certain conditions may be attacked. The same influence is undoubtedly also shown on the rate of hydrolysis by acids or alkali. There does not seem to be any evidence to distinguish qualitatively between the specificity of an enzyme and of hydrogen ions.

The procedure outlined above was followed in the experiments described in this paper. The gelatin was hydrolyzed to various stages by one of the hydrolyzing agents mentioned, pepsin or trypsin then added, and the increase in hydrolysis noted. The reaction was followed by means of a slight modification of the formol titration.

**EXPERIMENTAL.**

**Gelatin.**—Powdered gelatin, purified by washing at the isoelectric point, was used in all the experiments. The solutions were made up to contain 5 gm. of gelatin per 100 cc.

**Pepsin.**—The pepsin used was an active preparation of Fairchild Bros. (u. s. p. 1/19,000). It was prepared for use by dissolving 5 gm. in 100 cc. of water, adjusting to pH 2.5 with HCl and dialyzing under pressure against 0.01 N HCl for several days. The solution thus obtained had a very low titration which remained constant. In the concentration used the correction for the pepsin solution was negligible. The solution is referred to as 5 per cent pepsin.

**Trypsin.**—Fairchild's trypsin was used. It was prepared for use by dissolving 10 gm. in 100 cc. of water and dialyzing under pressure against running tap water at 6°C. for about 18 hours. The solution is very unstable and loses its activity in 3 or 4 days even at 2°C. The formol titration of this solution was negligible in the concentrations used and remained constant. This solution is referred to as 10 per cent dialyzed trypsin.

**Hydrogen ion determinations.**—The determinations were made by the E. M. method.

**Formol titration.**—The titration was carried out as described in a previous paper. The method consists essentially in freeing the solutions from CO₂ by adjusting the pH with acid, and boiling for a few seconds. The solution is then

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accurately adjusted to pH 7.0, using neutral red as an indicator. The formaldehyde is then added and the titration carried out as usual with 0.1 N NaOH, using thymol blue as indicator and an end-point of about 8.2. The titration was made on 10 cc. until the titration figure became more than 5 cc., after which 5 cc. were analyzed. The results have been calculated on the basis of 10 cc. of 5 per cent gelatin. The titration value for this concentration of gelatin was found to be 2.0 cc. The complete hydrolysis of gelatin increases the amino nitrogen (or formol titration) about 20 times. The percentage total hydrolysis may therefore be found from the figures by dividing by 40.0.

![Graph of formol titration per 10 cc. vs. days at 38°C](image)

Fig. 1. Hydrolysis of gelatin by pepsin. 500 cc. of solution containing 25 gm. of gelatin, 20 cc. of 5 per cent dialyzed pepsin, and 40 cc. of 1.0 N HCl placed at 38°C (pH = 2.2). 50 cc. samples pipetted out after 0, 1, and 4 days, and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin added to each sample and the samples placed at 38°C. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

Hydrolysis by Pepsin Alone.

The results of this experiment are given in Fig. 1. The figure shows that under the conditions of this experiment, the pepsin is able to double the formol titration of the gelatin (an increase of 2 cc. per 10 cc. of 5 per cent gelatin); i.e., the number of free carboxyl groups

is doubled. Since in completely hydrolyzed gelatin the free carboxyl
groups (or the amino nitrogen) is increased about twenty times,
the hydrolysis due to pepsin alone corresponds to about 5 per cent
of the total hydrolysis. It will be seen from the figure that this
result is independent of the amount of pepsin added and also of the
stage of hydrolysis at which the pepsin is added. It is not possible
to say, however, whether or not this really represents the complete
action of the pepsin, since the hydrolysis is still continuing slowly
and if the analyses were made at weekly intervals it would be found
that the values were increasing. This slow increase, however, ap-
proaches asymptotically the increase due to the acid alone, so that it is
impossible to say when the action of the pepsin stops. The real end-
point for pepsin digestion could be definitely fixed only by reaching the
same value from both sides. For the purpose of these experiments
it is permissible to take the end-point as that point at which the
addition of more pepsin causes no further hydrolysis in the course
of 3 or 4 days (the action of the acid alone in this time is within the
limits of error of the method). In the experiment just described this
value evidently corresponds to a titration of 4 cc. of 0.1 N NaOH per
10 cc. of 5 per cent gelatin.

Action of Pepsin on Gelatin Partially Hydrolyzed by Trypsin.

The results of this experiment are shown in Fig. 2. The experiment
shows that the increased hydrolysis due to the pepsin becomes less the
farther the hydrolysis due to the trypsin has been carried. The
linkages which are split by pepsin are also evidently attacked by
trypsin. At the same time, however, the trypsin is also hydrolyzing
some linkages which are not attacked by pepsin. This is shown by
the fact (Table I) that the addition of pepsin to a solution which has
been hydrolyzed by trypsin to a titration of 7.0 cc. causes a still
further increase, although, as we have seen, pepsin alone can only
carry the hydrolysis to 4.0 cc. The same result was obtained by
Henriques and Gjaldbäk with other proteins.
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Fig. 2. Action of pepsin on gelatin previously partially hydrolyzed by trypsin. 500 cc. of 5 per cent gelatin containing 25 cc. of 1.0 M Na₂CO₃ placed at 38°C. and 5 cc. of 10 per cent dialyzed trypsin solution added (pH = 9.5). 25 cc. pipetted out at intervals and 2 cc. of 4.2 M HCl added (pH = 1.8), and the samples kept at 3°C until all had been taken. 5 cc. of 5 per cent pepsin then added to each sample and the samples replaced at 38°C. Formol titration was run on 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE I.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Trypsin.

<table>
<thead>
<tr>
<th></th>
<th>Formol Titration per 10 cc. of Gelatin Solution.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before addition of pepsin</td>
<td>1.9</td>
</tr>
<tr>
<td>Increase in titration due to action of pepsin</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Action of Pepsin on Gelatin Partially Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 3 and Table II. The results are very similar to those obtained with trypsin and pepsin. The linkages attacked by pepsin are evidently quite rapidly attacked by the alkali although some are still left after the hydrolysis has reached titration value of at least 10 cc.

Action of Pepsin on Gelatin Partially Hydrolyzed by Acid.

These experiments are summarized in Fig. 4 and Table III. They show that the linkages attacked by pepsin are among the most resistant to the action of acid since the addition of the pepsin caused the normal increase of 2 cc. even after the acid hydrolysis had proceeded to a value of over 6.0 cc. There is no evidence of a true equilibrium in the presence of pepsin since no decrease is noted at any time even when the acid hydrolysis has been carried far beyond the end-point reached with pepsin acting on the unhydrolyzed protein. Such a reverse or synthetic action of pepsin has occasionally been recorded (the so called plastein formation7) in the case of other proteins. It is apparently always connected with the formation of a precipitate. This fact makes it appear possible that the decrease in the titration value is due to the formation of some insoluble compound from substances present in the pepsin and protein solutions. This decrease has not been noted in the case of gelatin solutions and these are the ones in which no precipitate forms. The experiments described here show that the hydrolysis by pepsin follows quite a different course from that followed by acid hydrolysis. The products formed must therefore be different. It seems unlikely that the pepsin could have any synthetic action on a solution containing substances which differ from those formed by the action of pepsin itself.

Hydrolysis by Trypsin.

The extent of the hydrolysis by trypsin alone is shown in Fig. 5. The hydrolysis continues until a titration value of about 15 cc. is reached. The same uncertainty as to whether this is the real end-point evidently exists here just as in the pepsin hydrolysis.

Fig. 3. Action of pepsin on gelatin partially hydrolyzed by alkali. 500 cc. of 10 per cent gelatin containing 8 cc. of 3 N NaOH kept at about 80°C. 50 cc. pipetted out at intervals and added to 50 cc. of 0.3 N HCl (pH = 2.0), these samples kept at 2° until all had been taken and 5 cc. of 5 per cent pepsin then added to each, and the samples placed at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

TABLE II.

Addition of Pepsin to Gelatin Partially Hydrolyzed by Alkali.

<table>
<thead>
<tr>
<th>Before addition of pepsin</th>
<th>1.9</th>
<th>3.0</th>
<th>3.9</th>
<th>5.7</th>
<th>7.3</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase due to action of pepsin</td>
<td>2.0</td>
<td>1.0</td>
<td>1.1</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Fig. 4. Action of pepsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin containing 12 cc. of 4.0 N HCl (pH 1.8) kept at 38°. pH kept constant by addition of more HCl from time to time. 50 cc. samples pipetted out at intervals of 2 days and kept at 3° until all had been taken. 5 cc. of 5 per cent pepsin then added and the samples replaced at 38°. Formol titration was run on 5 or 10 cc. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.

### TABLE III.

<table>
<thead>
<tr>
<th>Addition of Pepsin to Gelatin Partially Hydrolyzed by Acid.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Before addition of pepsin</td>
</tr>
<tr>
<td>Increase due to pepsin</td>
</tr>
</tbody>
</table>

Formol Titrations per 10 cc. of Solution.
Fig. 5. Hydrolysis of gelatin by trypsin alone. 8 cc. of 10 per cent trypsin were added to 200 cc. of 5 per cent gelatin containing 10 cc. of 1.0 M Na₂CO₃, and the solution kept at 22°. 25 cc. samples pipetted out at intervals and kept at 3°C, until all had been collected. 1 cc. of 10 per cent trypsin was then added to each sample, the bottles placed at 22°, and formol titration was run on 5 cc., as shown in the figure. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.
Action of Trypsin on Gelatin Partially Hydrolyzed by Pepsin.

The results of this experiment are shown in Fig. 6. The final point reached by the trypsin hydrolysis is independent of the amount of hydrolysis previously accomplished by the pepsin. The linkages hydrolyzed by pepsin are therefore evidently also hydrolyzed by trypsin so that it is immaterial whether the pepsin acts on the protein or not, as far as the final stage reached is concerned. It is possible that the rate of hydrolysis may be greater if the pepsin acts first since Fig. 2 showed that those linkages attacked by pepsin are among the most resistant to the action of trypsin. The experiment confirms the result shown in Fig. 2, that trypsin hydrolyzes the same linkages as does pepsin but also attacks others which are split slowly if at all by pepsin.

TABLE IV.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Alkali.

<table>
<thead>
<tr>
<th>Formal Titration per 10 cc. of Solution.</th>
<th>Before addition of trypsin</th>
<th>Increase due to trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>8.6</td>
<td>4.8</td>
</tr>
</tbody>
</table>

TABLE V.

Addition of Trypsin to Gelatin Partially Hydrolyzed by Acid.

<table>
<thead>
<tr>
<th>Formal Titration per 10 cc. of Gelatin.</th>
<th>Before addition of trypsin</th>
<th>Increase due to trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>8.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Action of Trypsin on Gelatin Previously Hydrolyzed by Alkali.

The results of this experiment are given in Fig. 7 and Table IV. Alkali hydrolysis evidently follows very nearly the same course as does trypsin hydrolysis since the increased hydrolysis due to the addition of trypsin becomes rapidly less and stops when the alkali hydrolysis has reached about 13 cc. This corresponds to the point reached by trypsin alone. The linkages split must therefore be the same in both cases. In this case also no evidence of a reversal of the action was observed although the experiment was carried much further than is shown in the figure.
Fig. 6. Action of trypsin on gelatin partially hydrolyzed by pepsin. 500 cc. of 5 per cent gelatin solution brought to pH 2.2 with HCl, 20 cc. of 5 per cent pepsin solution added and the solution kept at 38°C. 25 cc. samples pipetted out at intervals, 5 cc. of 1.0 M Na₂CO₃ added and the samples kept at 3°C until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added and the samples placed at 22°C. Formol titration was run on 5 cc. samples. The values plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.
Fig. 7. Action of trypsin on gelatin partially hydrolyzed by alkali. 500 cc. of 5 per cent gelatin solution containing 50 cc. of 1.0 M Na₂CO₃ kept at 90°C. 25 cc. samples pipetted out at intervals, and kept at 3° until all had been collected. 1 cc. of 10 per cent dialyzed trypsin then added to each sample and the solutions kept at 22°. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.
FIG. 8. Action of trypsin on gelatin partially hydrolyzed by acid. 500 cc. of 5 per cent gelatin brought to pH 1.8 with HCl and the solution kept at 90°C. 25 cc. samples pipetted out at intervals and 5 cc. of 1.0 M Na₂CO₃ added to each. Samples kept at 3°C until all had been collected. They were then put at 22°C and 1 cc. of 10 per cent dialyzed trypsin added to each sample. Formol titration was run on 5 cc. The figures plotted have been calculated on the basis of 10 cc. of 5 per cent gelatin.
Action of Trypsin on Gelatin previously Hydrolyzed by Acid.

The results of this experiment are shown in Fig. 8, and Table V. The course of the acid hydrolysis is quite different from that due to the trypsin since the addition of trypsin to the hydrolyzed gelatin is able to cause a still further increase even after the total number of linkages already split by the acid is greater than that which could be split by trypsin alone.

SUMMARY.

A comparison has been made of the relative velocity of hydrolysis of the various peptid linkings of the gelatin molecule when hydrolyzed by acid, alkali, pepsin or trypsin. It has been found that:

1. Those linkages which are most rapidly split by pepsin or trypsin are among the more resistant to acid hydrolysis.

2. Those linkages which are hydrolyzed by pepsin are also hydrolyzed by trypsin.

3. Trypsin hydrolyzes linkages which are not attacked by pepsin.

4. Of the linkages which are hydrolyzed by both enzymes, those which are most rapidly hydrolyzed by pepsin are only slowly attacked by trypsin.

5. Those linkages which are attacked by trypsin or pepsin are among the ones first (most rapidly) hydrolyzed by alkali.

In general it may be said that the course of the early stages of hydrolysis of gelatin is similar with alkali, trypsin, or pepsin and quite different with acid.