A TEST FOR DIFFUSIBLE IONS.

I. THE IONIC NATURE OF TRYPSIN.

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The ionization of a substance which may be prepared in any reasonable degree of purity may be easily shown by the conductivity method. In the case of substances, however, which cannot be isolated, the proof of the existence of ions is difficult. The method of migration in an electric field is uncertain, owing to the fact that particles of practically any material show movement under these conditions and that such movement does not necessarily indicate the existence of ions.

The theory of membrane equilibrium advanced by Donnan, however, predicts conditions which offer a criterion for the ionic nature of a substance. Donnan showed by thermodynamic reasoning that, if a solution containing a mixture of diffusible and non-diffusible ions was separated by a membrane from another solution containing only diffusible ions, the concentration of diffusible ions would be different on the two sides of the membrane. At equilibrium the product of the concentration for any one pair of oppositely charged diffusible ions of the same valence on one side of the membrane must be equal to the product of the concentrations of the same pair on the opposite side of the membrane. It follows that the ratio of the concentration of any pair of negative ions inside to that outside must be equal to the ratio of the concentrations of any pair of positive ions of the same valence outside to that inside the membrane.

1 Donnan, F. G., Z. Elektrochem., 1911, xvii, 572.
Expressed mathematically, the equation is

\[
\frac{(A_o^{-n})^{1/m}}{(B_o^{+m})^{1/m}} = \frac{(A_i^{-n})^{1/m}}{(B_i^{+m})^{1/m}}
\]

or

\[
\frac{(A_o^{-n})^{1/m}}{(A_i^{-n})^{1/m}} = \frac{(B_i^{+m})^{1/m}}{(B_o^{+m})^{1/m}} = \frac{(C_i^{+l})^{1/l}}{(C_o^{+l})^{1/l}} = \cdots
\]

in which \(A_o\) is the concentration of an \(n\) valent negative ion inside the membrane, \(A_i\) is the concentration of the same ion outside. \(B, C, \ldots\), are any other diffusible ions present, having the valence \(m, l, \ldots\). It can further be shown that if the non-diffusible ion is positive, the concentration of diffusible positive ions will be greater outside the membrane, and that, as the total concentration of diffusible ions increases, the difference in concentration inside and outside decreases and approaches zero. The same equation had apparently been derived, also thermodynamically, by Gibbs. It has been experimentally verified by Procter and Wilson, and very thoroughly by Loeb, so that the equation rests on a substantial basis both theoretically and experimentally. In order to test the ionic nature of a substance therefore it is only necessary to set up such an equilibrium system, measure the concentrations of some ion such as hydrogen or chloride, and compare this ratio with the concentration ratio of the substance under investigation. The only difficulty lies in the fact that the equation predicts only the concentration of the ions and not the total concentration, so that if the substance is not completely ionized or is combined in non-ionic form in the solution, the determination of the total concentration will not lead to the correct ratio. In other words, if the experimental results do not agree with the ratio, the discrepancy may be due to complicating factors and no definite conclusion can be drawn, whereas if they do agree, the conclusion seems justified that the substance is ionic.

The above method has been applied to the distribution of trypsin and it has been found that trypsin behaves like a monovalent positive ion from pH 2 to 10.2. At this point it behaves as though it were unionized and on the alkaline side of 10.2 becomes a monovalent negative ion. The experiments in this strongly alkaline range, however, are not so satisfactory.

**Experimental Procedure.**

Owing to the unstable nature of trypsin, it is necessary to provide conditions under which equilibrium may be rapidly established. This can be done by using finely powdered gelatin. The fact that trypsin attacks gelatin might be expected to cause a complication. This was avoided as much as possible by working at 0°C, and no evidence was found to show that the action of the trypsin on the gelatin had any effect on the experiment. 2 gm. of dry isoelectric gelatin, passing an 80 sieve, were placed in 45 cc. of solution and kept at 0°C about 18 hours. This is more than sufficient for equilibrium to be reached. 1.0 cc. of a dilute trypsin solution was then added, the total volume made up to 50 cc., and the suspension allowed to stand 2 hours longer at 0°C. The gelatin was then quickly filtered off and the volume of filtrate measured. In preliminary experiments the hydrogen ion concentration of the solution and of the gelatin (after melting) were measured. This procedure is difficult, however, and it was found much more accurate and convenient to measure the chloride concentration of the filtrate. The concentration of the chloride ion in the gelatin was then found by difference. The ratios for the Cl concentration found in this way agreed with the H ratios as Loeb has found and in the experiments reported here the Cl ion has been taken as the indicator of the equilibrium. The Cl was determined by AgNO₃ titration. The trypsin concentration of the filtrate and gelatin was determined by the viscosity method described by Hussey and the writer. In order to make this determination, it was necessary to melt the gelatin and under some conditions there is danger of destruction of the trypsin by this process. A control experiment without gelatin was therefore made and the total amount of trypsin determined.

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from this solution. If no disturbing factor had occurred, therefore, the total amount of trypsin found in the experiment should agree with the amount found in the control.

*Preparation of Trypsin.*—A 2 per cent solution of Fairchild's trypsin was prepared and brought to pH 4.5 with HCl. A heavy precipitate of protein forms under these conditions. This was filtered off and the solution brought back to pH 7.4. It was kept at 0° for 2 or 3 days. At the end of this time the solution gave no precipitate with trichloroacetic acid and was therefore presumably free from protein. Some experiments were made with trypsin which had been dialyzed through collodion. There was no difference in the behavior of these solutions.

**Table I.**

*Time for Equilibrium.*

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Trypsin units</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td>0.2 cc. filtrate</td>
</tr>
<tr>
<td>0</td>
<td>3.7</td>
</tr>
<tr>
<td>0.02</td>
<td>1.2</td>
</tr>
<tr>
<td>0.30</td>
<td>0.47</td>
</tr>
<tr>
<td>1.10</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Time Required for Equilibrium.*—Since Donnan's equation only holds at equilibrium, it is necessary to be sure that sufficient time is allowed before analyzing the solution. Table I shows that equilibrium is reached in less than 1 hour. The solutions were allowed to stand 2 hours, however, and on the acid side, where the trypsin is more stable and no action on the gelatin occurs, they were kept at 0° for 18 hours after the addition of the enzyme.

*Experimental Results.*

The details of a single experiment are given in Table II. The ratio of the trypsin concentration inside to that outside the gelatin is...
evidently nearly equal to the chloride ratio outside to that inside. The trypsin must, therefore, be of the opposite sign from that of chloride and be monovalent. This experiment was then repeated at a series of different pH. On the acid side the pH was regulated simply by the addition of HCl, while on the alkaline side the chloride ion was furnished by the addition of \( \frac{w}{100} \) KCl and the pH regulated by NaOH. From pH 6 to 11 there was present in addition \( \frac{w}{100} \) sodium borate to act as a buffer. This depresses the equilibrium and accounts for the fact that the ratio does not increase beyond pH 6.

The results of these experiments are shown in Table III. Each figure is the average of two to ten experiments. The table shows

\[
\begin{array}{|c|c|}
\hline
\text{Total volume, cc} & 50 \\
\text{Total Cl, cc. of } \frac{w}{30} & 29 \\
\text{Total filtrate, cc} & 17 \\
\text{Cl/cc. filtrate, cc. of } \frac{w}{30} & 0.14 \\
\text{Total Cl filtrate, cc. of } \frac{w}{30} & 2.40 \\
\text{Total Cl gelatin, cc. of } \frac{w}{30} & 26.6 \\
\text{Volume gelatin, cc} & 33 \\
\text{Cl/cc. gelatin, cc. of } \frac{w}{30} & 0.81 \\
\end{array}
\]

that the trypsin ratio agrees with the chloride ratio throughout except near pH 4.7 and near 10.2. They further show the characteristic maximum at about pH 3.5 and the change in the value of the ratio from more than 1 on the alkaline side of the isoelectric point of the gelatin, to less than 1 on the acid side. From pH 4.5 to about 6.0 all the trypsin solutions used showed a slight precipitate, especially in the presence of gelatin. This precipitate contains trypsin and is probably the cause of the irregular results contained in this range of pH. The amount of trypsin in this precipitate becomes included in the trypsin found in the gelatin and makes the figure too high. The same effect was occasionally noted with very high salt concentrations.
When high concentrations of salt were used, so that the Cl-ion distribution was equal on both sides, it was occasionally found that the trypsin was more concentrated in the gelatin. In these experiments, as in those near the isoelectric point, the volume of gelatin is small (10 to 12 cc.). It is possible that a small amount of trypsin is combined with the gelatin and that this is the disturbing factor.

### Table III.

*Effect of pH on Distribution of Chloride Ions and Trypsin, Inside and Outside of Gelatin Particles.*

<table>
<thead>
<tr>
<th>pH</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
<th>4.0</th>
<th>4.7</th>
<th>5.5</th>
<th>6.0</th>
<th>6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl(^{-}) conc. liquid</td>
<td>0.60</td>
<td>0.40</td>
<td>0.24</td>
<td>0.13</td>
<td>0.38</td>
<td>1.0</td>
<td>1.3</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Cl(^{-}) conc. gelatin</td>
<td>1.5</td>
<td>1.5</td>
<td>11.0</td>
<td>4.0</td>
<td>1.6</td>
<td>6.0</td>
<td>1.4</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Trypsin conc. gelatin</td>
<td>0.50</td>
<td>0.30</td>
<td>0.23</td>
<td>0.17</td>
<td>0.35</td>
<td>1.0</td>
<td>1.3</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Trypsin conc. liquid</td>
<td>1.9</td>
<td>1.7</td>
<td>2.1</td>
<td>2.0</td>
<td>2.6</td>
<td>2.5</td>
<td>2.6</td>
<td>2.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

When the volume of gelatin is large, this factor would be negligible, but under such conditions as to destroy the Donnan equilibrium and prevent the swelling of the gelatin a small amount of combined trypsin would cause a larger error. This discrepancy near the isoelectric point of gelatin may be largely avoided by allowing the gelatin to stand for 48 hours at pH 3.5 and then adjusting the pH to 4.7.
The experiments as a whole in this range indicate that gelatin may combine with trypsin and that this effect is much greater when the gelatin is not completely permeated with water.

The table shows further that on the acid side of the isoelectric point the trypsin is more concentrated in the liquid while on the alkaline side it is more concentrated in the gelatin. This effect, however, would be common to any positive ion under the conditions of the experiment, and is not due to any peculiar relationship of the gelatin and trypsin. At pH 10.2 the trypsin is equally distributed in the gelatin and liquid and on the alkaline side of this point becomes distributed in the same way as the chloride instead of the reciprocal. This would be the result expected if the trypsin were amphoteric with an isoelectric point at 10.2 and became a negative ion on the alkaline side of this point. There is no evidence of an isoelectric point at or near pH 4 as found by Michaelis and Davidsohn. It seems possible that the migration of trypsin described by these writers was due to the carrying of the enzyme by some impurity in the solution as was apparently the case with pepsin.

Another characteristic of the Donnan equilibrium is the depressing effect of salts. The result of experiments in which the total salt concentration is varied is given in Table IV. The chloride and trypsin ratios are again in quite close agreement. It will be noted that on the alkaline side the concentration of the trypsin in the gelatin is decreased by the salt whereas on the acid side it is increased. This is typical of the Donnan equilibrium and is difficult to account for even qualitatively on any other basis. The volume of gelatin is decreased in both cases by the salt, so that if the trypsin were actually combined with the gelatin by any chemical mechanism or even by adsorption, the total amount combined would be constant (since the weight of gelatin is the same), and the concentration would therefore increase whenever the volume of gelatin decreased. This is not the case, however. On the alkaline side, decreasing the volume of gelatin also decreases the concentration of trypsin in the gelatin.

These and similar experiments were repeated a number of times with results that agreed more or less closely with the theory. It is impos-

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sible to secure accurate results in a single experiment of this type, owing to the large experimental errors. All the experiments may be

TABLE IV.
Effect of Increasing Salt Concentration.

pH 8.5
2 gm. of gelatin, 7 cc. of 0.1 N NaOH, 2 cc. of 0.1 N KCl + increasing concentrations of Na₂SO₄. Total volume 50 cc.

<table>
<thead>
<tr>
<th>Total salt concentration.</th>
<th>Ratio Cl liquid/Cl gelatin</th>
<th>Ratio Trypsin gelatin/Trypsin liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02</td>
<td>4.2</td>
<td>3.6</td>
</tr>
<tr>
<td>0.06</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>0.10</td>
<td>2.2</td>
<td>2.0</td>
</tr>
<tr>
<td>0.18</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>0.34</td>
<td>1.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

pH 3.5
2 gm. of gelatin, 10 cc. of 0.1 N HCl, and increasing amounts of KCl.

<table>
<thead>
<tr>
<th>Total Cl concentration.</th>
<th>Ratio Cl liquid/Cl gelatin</th>
<th>Ratio Trypsin gelatin/Trypsin liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.15</td>
<td>0.14</td>
</tr>
<tr>
<td>0.04</td>
<td>0.35</td>
<td>0.28</td>
</tr>
<tr>
<td>0.06</td>
<td>0.43</td>
<td>0.35</td>
</tr>
<tr>
<td>0.08</td>
<td>0.49</td>
<td>0.40</td>
</tr>
</tbody>
</table>

pH 3.5
Increasing NaNO₃.

<table>
<thead>
<tr>
<th>Conc. NaNO₃</th>
<th>Ratio Cl liquid/Cl gelatin</th>
<th>Ratio Trypsin gelatin/Trypsin liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.14</td>
<td>0.13</td>
</tr>
<tr>
<td>0.02</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>0.04</td>
<td>0.30</td>
<td>0.34</td>
</tr>
<tr>
<td>0.08</td>
<td>0.41</td>
<td>0.39</td>
</tr>
<tr>
<td>0.16</td>
<td>0.50</td>
<td>0.65</td>
</tr>
</tbody>
</table>

used, however, by comparing the trypsin ratio for any experiment with the corresponding chloride ratio. According to the theory these ratios should be equal, that is, the chloride ratio divided by the tryp-
The calculated ratio agrees with the experiment well within twice the probable error so that the result is statistically reliable. All the experiments were included in this average except those near pH 5.0 and 10.2, where there is evidently some complicating factor.

The experiments described above show clearly that the distribution of trypsin inside and outside of gelatin particles is regulated by the same forces that control the distribution of the hydrogen and chloride ions; in other words, by the Donnan equilibrium. Trypsin is therefore a diffusible ion and since the ratios are the reciprocal of the chloride ratios, it is a positive monovalent ion from pH 2 to 10.2, at which point it becomes isoelectric to become a negative ion in more strongly alkaline solution. It follows also that trypsin must be a fairly strong acid and base since the existence of any appreciable amount of unionized trypsin would disturb the ratios, owing to the equal distribution of the unionized portion. Since the method used determines the total amount of trypsin present, the presence of unionized trypsin would cause all the ratios to be nearer 1.0 than the chloride ratios; a result which is not obtained except at and near pH 10.2. This is presumably the isoelectric point.

These results further show that the removal of trypsin from solution by insoluble proteins is not evidence of the existence of a compound between the enzyme and the protein, as the writer formerly supposed, but on the contrary proves that there is no combination between the two.

The numerous experiments showing a relation between the swelling of insoluble proteins and their rate of digestion, which have frequently

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been cited as evidence for some relation between the rate of hydrolysis and the hydration of the protein are also explained. They are simply due to the fact that the greater the inequality of the ion concentration the greater the swelling and also the greater the concentration of the trypsin inside the protein particle. The more a protein swells, therefore, on the alkaline side of its isoelectric point, the greater will be the concentration of the enzyme inside the protein and the more rapidly it will digest. The mechanism evidently has nothing to do with the hydration of the protein and in any case is secondary to the chemical effect of the pH on the protein. In the case of pepsin, for instance, the optimum for the digestion of gelatin by pepsin is not at pH 3.5, where the maximum swelling occurs, but at 2.5. This conclusion is also borne out by the fact that the pH effect is presented when a solution of the protein is used. With insoluble proteins, however, the purely chemical effect of the pH may be modified by the distribution effect.

The fact that trypsin passes through a collodion membrane had been noted by Strada and also by Neill. The experiments have been repeated and confirmed by the writer.

The concentration of trypsin in gelatin on the alkaline side of pH 4.7 and in the liquid on the acid side of this point offers a method for the purification of the enzyme. If trypsin is added to a suspension of gelatin particles at pH 7.0, most of the trypsin will be concentrated inside the gelatin. The particles may then be washed and then brought to pH 3.5, where the trypsin will diffuse out and become more concentrated in the liquid. Any diffusible positive ions would be carried along, but proteins should be largely removed.

It had been found by the writer that trypsin enters into equilibrium with the products of hydrolysis and that these equilibria were rapidly and completely reversible. Such equilibria are much more common between ions than between unionized substances, so that the behavior of trypsin in this case also indicates its ionic nature. Most cases of homogeneous catalysis are also due to ions so that it would not be surprising if such powerful catalysts as the enzymes were ionic.

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Preliminary experiments indicated that pepsin is a monovalent negative ion.

SUMMARY.

1. The Donnan equilibrium furnishes a test for the ionic nature of any diffusible substance, since the ratio of the concentration of any ion on the two sides of a membrane must be equal to the ratio of the concentrations of any other ion of the same sign and valence, whereas a non-ionic substance would be equally distributed on both sides.

2. The distribution of trypsin inside and outside of gelatin particles has been compared to the distribution of hydrogen and chloride ions under the same conditions.

3. The ratio of the trypsin concentration in the gelatin to the concentration in the outside liquid is equal to the ratio of the hydrogen ion under the same conditions and to the reciprocal of the chloride ion ratio.

4. This result was obtained between pH 2.0 and 10.2. At pH 10.2 the trypsin is equally distributed and on the alkaline side of 10.2 the ratio is directly equal to the chloride ratio.

5. Trypsin is therefore a positive monovalent ion in solutions of pH 10 to 2. It is probably isoelectric at 10.2 and a monovalent negative ion on the alkaline side of 10.2.

6. Trypsin must also be a strong base since there is no evidence of any undissociated form on the acid side of pH 10.2.