THE COMBINATION OF DEAMINIZED GELATIN WITH HYDROCHLORIC ACID.

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I.

INTRODUCTION.

When a protein such as gelatin is treated with nitrous acid there is an evolution of nitrogen, presumably from the reaction of the nitrous acid with NH$_2$ groups in the protein. This reaction is the basis of the well known Van Slyke's method for the quantitative determination of free amino groups. The resulting deaminized proteins, in which each NH$_2$ group may be assumed to have been replaced by OH, were studied by Skraup, who investigated in some detail the products of their hydrolysis. Deaminized gelatin was prepared according to Skraup's method by Blasel and Matula, who showed by hydrogen electrode measurements that the deaminized protein was still capable of combining with hydrochloric acid.

The present work was undertaken to compare the maximal combining capacity for hydrochloric acid of gelatin with that of deaminized gelatin. If the combination of these proteins with hydrochloric acid is true chemical combination, and if the process of deaminization consists simply in replacing NH$_2$ groups by OH, then it would be expected that the difference in their combining capacities should be chemically equivalent to the NH$_2$ groups removed. That this expectation has been confirmed is shown by the following experimental results.

1 Van Slyke, D. D., J. Biol. Chem., 1911, ix, 185; 1912, xii, 275.
It was found by Van Slyke\(^1\) that the deaminizing reaction took place rapidly and quantitatively if the amino substance was treated with a great excess of a solution containing equivalent amounts of sodium nitrite and acetic acid. The preparation of Skraup was obtained under similar conditions, but after allowing the mixture to stand over night he heated the acid solution for 2 hours on a water bath and then precipitated the deaminized gelatin repeatedly by saturation with ammonium sulfate.

In order to determine whether this treatment caused any further reaction than that occurring in the Van Slyke apparatus, measurements were made of the amino nitrogen in gelatin, by Van Slyke's\(^1\) method, and of the total nitrogen in gelatin and in deaminized gelatin, by the Kjeldahl method.\(^4\) Three lots of deaminized gelatin were used. No. 1 was prepared from Cooper's commercial gelatin according to Skraup, except that it was precipitated only once by ammonium sulfate, while No. 2 was part of the same lot which was not heated on the water bath and not precipitated, but was dialyzed under pressure in collodion cylinders after standing over night in the nitrous acid solution. The dialysis was started in running tap water; after about 4 days the contents of the bags were brought to pH 3.6 with hydrochloric acid, and then dialyzed for a week against repeated changes of hydrochloric acid of pH 3.6; finally the bags were kept for 3 days in repeated changes of distilled water. This reduced the specific conductivity of the resulting 3 per cent solutions to less than \(10^{-4}\) reciprocal ohms. The concentrations of these solutions were determined by drying 25 cc. samples to constant weight at 110\(^\circ\)C., and 25 cc. samples were likewise used for the Kjeldahl determinations. The gelatin analyzed was a solution of isoelectric gelatin which had been prepared from Cooper's gelatin as described by Loeb.\(^5\) Preparation 3 of deaminized gelatin was made by dialyzing the residues


from the Van Slyke determinations, for which the initial material was isoelectric gelatin.

By the Van Slyke method the following figures were found for the percentage of amino nitrogen, using samples containing from 0.8 to 1.2 gm. of dry gelatin: 0.525, 0.505, 0.582, 0.662, 0.540, 0.514; average, 0.555 per cent. This agrees with the results of Van Slyke and Birchard, 6 who found 3.12 per cent of the total nitrogen in gelatin to be amino nitrogen; taking the total nitrogen as 17.96 per cent (see below), their figure for amino nitrogen becomes 0.560 per cent of the gelatin. Similar figures were obtained by Northrop, 7 who found the normality of 1 per cent gelatin to be 0.0036 or 0.0038 with respect to NH₂ groups; accordingly his value for the percentage of amino nitrogen in dry gelatin is 10 × 0.0037 × 14.01 = 0.519 per cent.

By the Kjeldahl method the following figures for total nitrogen were obtained.

<table>
<thead>
<tr>
<th>Gelatin</th>
<th>18.02</th>
<th>17.94</th>
<th>17.97</th>
<th>17.89</th>
<th>average, 17.96 per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deaminized gelatin No. 1</td>
<td>16.98</td>
<td>16.94</td>
<td>16.99</td>
<td>16.97</td>
<td>average, 16.97</td>
</tr>
<tr>
<td>Deaminized gelatin No. 2</td>
<td>17.46</td>
<td>17.35</td>
<td>17.41</td>
<td>17.37</td>
<td>average, 17.40</td>
</tr>
</tbody>
</table>

It is evident that the difference between the figure for gelatin and that for deaminized gelatin No. 2 is almost exactly equal to the percentage of amino nitrogen removed in the Van Slyke analysis. In the case of deaminized gelatin No. 1, which was prepared according to Skraup, with heating, the loss of nitrogen is considerably greater, indicating that a more extensive reaction has taken place than the simple removal of free amino groups. This was corroborated by the fact that in the dialysis of No. 1 much more material was lost by diffusion through the collodion than was the case with No. 2.

These figures may be summarized as follows:

1 gm. gelatin = 0.00555 gm. or 0.000396 equivalents amino N.

1 " " = 0.1796 " " 0.01282 " " total " "

1 " deaminized gelatin No. 2 = 0.1740 " " 0.01242 " " " "

Loss in deamination = 0.0056 " " 0.00040 " " N.

Equal weights of gelatin and deaminized gelatin are taken as chemically equivalent, because the formula weight of the group OH, 17, is so close

DEAMINIZED GELATIN

to that of the NH₂ group, 16, and because the groups affected are such a small part of the weight of the protein. These results indicate that if the preparation of deaminized gelatin is carried out without heating, the reaction is simply a replacement of NH₂ by OH, as in the case of ordinary aliphatic amines.

This simple conception of the reaction occurring in the preparation of deaminized gelatin was confirmed by analyzing the product by the Van Slyke method; the percentage of amino nitrogen found was 0.025 for Preparation 1 and 0.029 for Preparation 2. It is believed that these figures should both be zero, since the volumes of nitrogen obtained, after correction for the blank from the reagents, were only 0.16 and 0.15 cc. The absence of amino nitrogen in several other preparations of deaminized gelatin, prepared both with and without heating, was further confirmed by the formol titration method of Sörensen. In no case was there any difference between the titrations with and without formaldehyde, if proper correction was made for the acidity of the formaldehyde solution itself. It may be stated that this is quite at variance with the results reported by Herzig and Lieb, who found over twice as much amino nitrogen in gelatin as any of the authors quoted above, and found similar high figures for amino nitrogen in deaminized gelatin, even after it had been twice deaminized.

III.

Combination of Deaminized Gelatin with HCl.

In order to calculate the combining capacity of deaminized gelatin for acid, it was necessary to know the isoelectric point of the material. In the work of Blasel and Matula this was not considered, and their curve therefore does not represent the true amounts of combined acid.

The isoelectric point of a protein has been shown by Loeb to coincide with a minimum of osmotic pressure. Accordingly measurements were made of the osmotic pressure at 24°C. and of the pH of the

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8 Sörensen, S. P. L., Compt. rend. trav. Lab. Carlsberg, 1907, vii, 1; Biochem. Z., 1908, vii, 64.
protein solutions at equilibrium, in the manner described by Loeb. The results obtained with 1 per cent solutions of Preparation 1, and other preparations which had been heated even more than 2 hours on the water bath, showed a minimum of osmotic pressure at pH 3.5 to 3.8. Preparation 2, however, exhibited a minimum of osmotic pressure at pH 4.0, as is shown by Fig. 1.

This location of the isoelectric point was confirmed in another way in the case of Preparation 3, which was also prepared without heating. The protein was coated on particles of collodion by mixing 50 cc. of a

Fig. 1. Effect of pH on osmotic pressure of 1 per cent deaminized gelatin.
0.1 per cent solution with 50 cc. of a suspension of collodion particles, prepared as described by Loeb, and allowing the mixture to stand over night. The mixture was then centrifuged, and the sediment was again put into suspension by trituration in a mortar with about 15 cc. of distilled water. A series of acetate buffers was prepared as described by Michaelis, with respect to sodium acetate, and 5 drops of the concentrated suspension were added to 25 cc. of each buffer. Measurements were made of the velocity of migration of the particles in an electric field, using the microscopic apparatus of Northrop. For these measurements the writer is indebted to Mr. M. Kunitz, who found that the direction of migration changed its sign between pH 3.7 and 4.0. 10 cc. samples of the various suspensions were allowed to stand over night in test-tubes, and it was found that the suspension at pH 3.9 settled clear, those differing by about pH 0.1 or more being cloudy. In this work the pH determinations were made with the hydrogen electrode at 33°C. in Clark cells with a saturated KCl junction, and were based on a pH value of 1.037 for 0.1000 M HCl.

The determination of the combining capacity of deaminized gelatin was carried out by a method similar to that already employed in the case of gelatin. This consists in measuring the pH in protein solutions containing various amounts of acid, and subtracting from the total acid concentration the amount of acid required to give the same pH to an equal volume of water. The difference represents the amount of acid combined with the protein, and by dividing by the protein concentration figures may be obtained for the amounts of acid combined with 1 gm. of protein at different pH values. It was found in the case of gelatin that the amounts so obtained for a given pH were independent of the protein concentration, and that between pH 1 and 2 the amount of hydrochloric acid combined with 1 gm. of gelatin was constant.

In the case of deaminized gelatin the method was modified by performing the titration with a single sample of protein, instead of making up a fresh sample to the same volume for each pH determination. A vessel of the type described by Bovie was used, with an electrode of platinized platinum wire and bubbling hydrogen. Contact was made with a saturated potassium chloride bridge through a tube not quite tightly closed by a ground glass stopper. Junctions of this type were tested by Lamb and Larson and recommended by LaMer and Baker. The salt bridge was connected to a saturated potassium chloride calomel cell, and the whole apparatus was kept in a water bath at $33^\circ \pm 0.1$. 50 cc. of the protein solution were placed in the cell, and after the electrode had come to equilibrium the titrations were made by running in standard HCl solution from a burette. After each addition of acid, readings of the E.M.F. were taken at intervals until they became constant for 2 minutes. The E.M.F. measurements were made with a Leeds and Northrup portable potentiometer reading to 0.5 millivolt.

The calculation of the combined hydrochloric acid was performed as follows:

Let $a = \text{No. of cc. of HCl added to } e \text{ cc. of protein solution.}$

$b = \text{concentration of HCl in burette, mols per liter.}$

$c = \text{initial concentration of protein solution, gm. per liter.}$

$d = \text{concentration of HCl having the same pH in water alone, mols per liter.}$

$e = \text{initial volume of protein solution, cc.}$

Then $a + e = \text{volume of mixture, cc.}$

\[
\frac{ab}{a+e} = \text{concentration of HCl in mixture, mols per liter.}
\]

\[
\frac{ab}{a+e} - d = \frac{ab - ad - ed}{a+e} = \text{combined HCl, mols per liter.}
\]

\[
\frac{ec}{a+e} = \text{concentration of protein in mixture, gm. per liter.}
\]

\[
\frac{ab - ad - ed}{ec} = \text{mols of HCl combined with 1 gm. of protein.}
\]

The values of $d$ were obtained from the curve constructed in connection with the previous work on gelatin; this curve was checked by titrating 50 cc. of water with 0.201 m HCl in the new apparatus. In case the

original protein solution is not at the isoelectric point, the values for
the combined acid must be corrected by adding or subtracting a con-
stant amount to make the point of zero combination coincide with the
isoelectric point. The only assumptions involved in the calculation
are those used previously; namely, that the same concentration of
uncombined HCl is required to give the same pH to equal volumes of
water and of protein chloride solution, and that there is no acid com-
bined with the protein at its isoelectric point.

TABLE I.
Titration of Deaminized Gelatin with Hydrochloric Acid.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl</td>
<td>pH</td>
<td>HCl</td>
<td>pH</td>
</tr>
<tr>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
</tr>
<tr>
<td>0.00</td>
<td>3.88</td>
<td>0.00</td>
<td>4.02</td>
</tr>
<tr>
<td>0.50</td>
<td>3.18</td>
<td>0.94</td>
<td>3.66</td>
</tr>
<tr>
<td>0.99</td>
<td>2.78</td>
<td>2.81</td>
<td>2.94</td>
</tr>
<tr>
<td>1.99</td>
<td>2.29</td>
<td>4.58</td>
<td>2.32</td>
</tr>
<tr>
<td>3.17</td>
<td>2.03</td>
<td>6.50</td>
<td>2.00</td>
</tr>
<tr>
<td>4.51</td>
<td>1.85</td>
<td>8.40</td>
<td>1.75</td>
</tr>
</tbody>
</table>

A. Preparation 3. Initial volume, e = 50 cc. Concentration of protein,
c = 6.99 gm. per liter. Concentration of HCl, d = 0.201 M.
B and C. Preparation 2. Initial volume, e = 50 cc. Concentration of protein,
c = 29.69 gm. per liter. Concentration of HCl, d = 0.201 M.
D. Preparation 2. In this case the solutions were made up to constant vol-
ume before measuring pH. The figures are cc. of 0.1003 M HCl contained in 35 cc.
of solution containing also 0.743 gm. of deaminized gelatin.

The results of the titration experiments are given in Table I. From
these results the amounts of hydrochloric acid combined with 1 gm.
of protein were calculated in the way just described; the quantities so
obtained are plotted in Fig. 2. The correction mentioned was neces-
sary only in the case of Experiment A; in the other cases the solution
happened to be at the isoelectric point when the dialysis was stopped.
The curve indicates that deaminized gelatin has a maximum combin-
ing capacity of 0.00044 mols of HCl per gm. of protein.
IV. CONCLUSIONS.

In a previous paper it was stated that 1 gm. of gelatin could combine with 0.00092 mols of HCl. This figure should be corrected by subtracting the amount of acid necessary to shift the zero point from pH 4.78 to 4.70, the isoelectric point of gelatin. This correction makes the value for the maximum combined HCl 0.00089 mols per gm. of gelatin, and changes the combining weight of gelatin to about 1,120.

The number of equivalents of amino nitrogen removed in deaminizing 1 gm. of gelatin was found to be 0.00040, and this was checked by the loss in total nitrogen. The difference between the maximum combining capacities for HCl of 1 gm. of gelatin and 1 gm. of deaminized gelatin is 0.00089 - 0.00044 = 0.00045. Considering the limitations of the methods which had to be employed to get these figures, the agreement is good.

This means, then, that the loss in combining capacity for HCl suffered by 1 gm. of gelatin in being deaminized is chemically equivalent to the number of amino groups removed. The combining
capacity for HCl still retained by deaminized gelatin is presumably to be ascribed to the NH groups which are not attacked by HNO₃. For each atom of nitrogen lost in the deaminizing reaction, the protein loses the capacity to combine with one hydrogen ion. Therefore the present work constitutes a new indication of the truly chemical nature of the combination between protein and acid.

V.

SUMMARY.

1. The analysis of isoelectric gelatin by the Van Slyke method indicates 0.00040 equivalents of amino N per gm. gelatin.
2. If deaminized gelatin is prepared without heating, the product contains less nitrogen than the original gelatin by an amount equal to 0.00040 equivalents N per gm. protein.
3. Deaminized gelatin, prepared either with or without heating, contains no amino nitrogen detectable with certainty by either the Van Slyke or the formol titration method.
4. The isoelectric point of deaminized gelatin prepared without heating is at pH 4.0.
5. The maximum combining capacity of this protein for HCl is 0.00044 equivalents per gm.
6. The maximum combining capacity of gelatin for HCl should be corrected to 0.00089 equivalents per gm.
7. The difference between these maximum combining capacities, 0.00045, is nearly equivalent to the loss in amino or total nitrogen occurring in the deaminizing reaction.
8. This equivalence constitutes a new indication that the combination of protein with acid is chemical combination.

The writer is indebted to Dr. Jacques Loeb for suggesting this work.