THE IONIZATION OF PROTEIN CHLORIDES.

BY DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

INTRODUCTION.

When isoelectric protein is dissolved in dilute hydrochloric acid, the concentration of hydrogen ion in the hydrochloric acid is diminished. This fact has led to the belief that the acid combines chemically with the protein, the reaction being similar to the combination of acid with an amino-acid or any other amphoteric electrolyte. If this idea is correct it should follow that the protein chloride so formed should itself be ionized, yielding a positive ion composed of protein with H⁺ added, the corresponding negative ion being chloride ion. The degree of ionization of the protein chloride should be of the same order as that of ammonium chloride; that is, protein chloride should be a highly ionized salt. Accordingly it would be expected that the concentration of chloride ion in hydrochloric acid should be diminished very little, if at all, by the addition of protein.

This idea was tested by Loeb, who found that the chloride ion concentration of hydrochloric acid, as measured by the calomel electrode, was not appreciably altered by the presence of 1 per cent gelatin. Similar results had been obtained with gelatin by Manabe and Matula, who found, however, that in the case of dialyzed ox serum and serum albumin there appeared to be considerable combination of chloride ion with the protein. Robertson has proposed a theory of protein ionization which is based on the idea that compounds

2 Manabe, K., and Matula, J., Biochem. Z., 1913, lxi, 369.
of protein with acid or alkali do not yield on electrolytic dissociation
the simple anion of the acid or cation of the base in question. In
order to determine which of these views might be of general applica-
tion, measurements have been made of the chloride ion concentration
in solutions of the chlorides of several proteins.

II.

EXPERIMENTAL.

The proteins used were gelatin, egg albumin, casein, edestin, and
serum globulin. The gelatin was rendered isoelectric as described by
Loeb.\(^4\) The egg albumin was purified by the method of Sörensen.\(^5\)
The casein was prepared from milk by the method of Van Slyke and
Baker,\(^6\) washed free from HCl, and dried with acetone. The edestin
was prepared from ground hemp-seed by the method of Osborne,\(^7\)
and the serum globulin was obtained from ox serum by the procedure
given by Robertson.\(^8\) The edestin and serum globulin were not dried,
but were washed free from salt by decantation with water of a pH
close to that of their isoelectric points. With each protein a series of
solutions was made up so that 100 cc. contained in each case 1 gm. of
protein, as determined by dry weight determinations. Each solution
contained also hydrochloric acid, varying in concentration from 0.001
to 0.100 molar.

The chloride ion concentrations were determined by means of the
silver-silver chloride electrode. The electrodes were prepared as
described by MacInnes and Parker,\(^9\) except that a short spiral of
platinum wire was used instead of platinum gauze, being plated
electrolytically first with silver and then with silver chloride. These
electrodes were fitted into vessels of the type devised by Clark for
hydrogen electrode work, as modified by Cullen\(^10\) by the introduction

\(^4\) Loeb, J.,\(^1\) p. 35; \textit{J. Gen. Physiol.}, 1918–19, i, 39; \textit{J. Am. Chem. Soc.}, 1922,
xliv, 213.

penhagen, 1915–17, xii, 12; \textit{Z. physiol. Chem.}, 1918, ciui, 16.


\(^7\) Osborne, T. B., in Abderhalden, E., \textit{Handbuch der biochemischen Arbeits-
methoden}, Berlin, 1910, ii, 289.

\(^8\) Robertson, T. B.,\(^2\) p. 40.


of a thermometer. The vessels were connected by a salt bridge of saturated potassium chloride to a saturated potassium chloride calomel cell. It was at first thought that reliable results could not be secured in this way owing to possible diffusion of KCl into the cell while the stop-cock was opened to make a reading, and to avoid this some measurements were carried out with a salt bridge of saturated ammonium nitrate. These measurements gave higher values for pCl (pCl = \(-\log [Cl^-]\)) than were obtained with the KCl bridge. That this difference was not due to diffusion of KCl into the cell is indicated by the fact that the pCl values for HCl solutions agree closely with the pH values. With NH₄NO₃ and HCl alone, equally good readings were obtained. The use of NH₄NO₃ was abandoned in favor of KCl because it was found that the pCl values obtained with gelatin chloride did not check those of Manabe and Matula² and of Loeb¹ but were higher, as well as less closely reproducible than the results obtained with KCl. Apparently this difference is due to a contact potential difference at the junction of the NH₄NO₃ solution and the protein chloride solution, which does not exist at the junction with saturated KCl. The apparatus was kept in an air bath at 25° ± 0.2°, the temperature being checked by the thermometer in each cell.

The measurements of E. M. F. were made with a Leeds and Northrup portable potentiometer, reading to 0.5 millivolt. The sensitivity of this instrument was found to be ample in solutions containing more than 0.002 N HCl. It was found possible to bring the solutions to equilibrium with the electrodes by the same procedure as that used in hydrogen electrode work. Each cell, with its electrode in place, was washed with the solution, half filled, and rocked for 3 minutes. A reading was taken, and the cell was emptied, refilled with the same solution, and rocked again. This was repeated until the readings obtained with two successive fillings of the cell agreed to within 0.5 millivolt, which was usually found to be the case on the second and third fillings of the cell. That this procedure gave the true equilibrium values for the E. M. F. is borne out by the agreement of the values for pCl and pH obtained with HCl solutions alone. This was confirmed by some preliminary measurements in which the electrodes were allowed to stand over night in the solutions before readings were taken. The pCl values obtained in this way with pure HCl solutions
and with gelatin chloride solutions were nearly the same as those obtained by the more rapid procedure.

Another conceivable source of error might be a change in the solubility of AgCl due to the presence of the protein. Measurements of pCl in solutions containing only protein and water did indicate a somewhat greater concentration of chloride ion than that calculated from the known solubility of AgCl in water. A correction could be made for this, as was done by Oryng and Pauli in the case of calomel, by subtracting the chloride ion concentration so observed from that observed with protein chloride and water. When this was done, however, it was found that the correction was negligible except in the two most dilute solutions (0.001 and 0.002 N HCl). Since with these solutions the readings were not very reliable, and since in all other cases the correction amounted to less than 0.01 pCl, the use of the correction was considered unnecessary.

The determinations of hydrogen ion concentration were made with the same apparatus, except that platinized platinum electrodes were inserted in place of the silver chloride electrodes. The procedure was identical except that a stream of hydrogen was passed through the empty cell for 10 seconds just before the solution was put in.

The values for pH and pCl are based on 0.100 M HCl as a standard, its pH or pCl being taken as 1.035 at 25°. The electrodes were standardized against this HCl before and after each day's use. Accordingly, the values for pH and pCl were calculated by the use of the Nernst formula in the form:

$$\text{pH (or pCl)} = \frac{E_x - E_{HCl}}{0.0591} + 1.035$$

where $E_x$ and $E_{HCl}$ represent the E. m. f. in volts observed with the solution under investigation and the 0.100 M HCl respectively.

III.

DISCUSSION.

The values for pCl obtained with pure HCl and with the protein chloride solutions are given in Table I. The reliability of the method is indicated by the agreement between the values for pH and pCl in the case of HCl free from protein. In the case of gelatin and egg

11 Oryng, T., and Pauli, W., Biochem. Z., 1915, lx, 368.
albumin the pCl values are nearly the same as those for pure HCl. This indicates that the chlorides of these proteins are practically completely ionized in the solutions studied. With casein, edestin, and serum globulin the values are somewhat higher, indicating that the ionization of the chlorides of these proteins is not quite complete.

In order to compare the concentration of ionized protein chloride with the total concentration of protein chloride formed in any given solution, the amounts of combined hydrogen ion were determined. This was done by means of measurements of pH of the protein chloride solutions and of pure HCl solutions. A large scale graph was made of the pH of HCl solutions as a function of the concentration of HCl. From this curve were read off the amounts of free HCl corresponding to the pH actually measured in each protein chloride solution. These amounts of free HCl were subtracted from the total HCl present in each protein chloride solution, the difference representing the H⁺ combined with the protein or the total equivalent concentration of

### TABLE I.

**Measurements of Chloride Ion Concentration in Protein Chloride Solutions.**

<table>
<thead>
<tr>
<th>Concentration of HCl</th>
<th>−log HCl</th>
<th>pH of HCl</th>
<th>pCl of HCl</th>
<th>pCl of 1 per cent protein chloride solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Egg albumin</td>
</tr>
<tr>
<td>0.001</td>
<td>3.00</td>
<td>3.00</td>
<td>2.97</td>
<td>2.90</td>
</tr>
<tr>
<td>0.002</td>
<td>2.70</td>
<td>2.70</td>
<td>2.69</td>
<td>(2.67)</td>
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<tr>
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<td>2.52</td>
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<td>2.51</td>
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</tr>
<tr>
<td>0.004</td>
<td>2.40</td>
<td>2.40</td>
<td>2.41</td>
<td>2.41</td>
</tr>
<tr>
<td>0.005</td>
<td>2.30</td>
<td>2.30</td>
<td>2.28</td>
<td>2.31</td>
</tr>
<tr>
<td>0.006</td>
<td>2.22</td>
<td>2.22</td>
<td>2.20</td>
<td>2.24</td>
</tr>
<tr>
<td>0.007</td>
<td>2.15</td>
<td>2.15</td>
<td>2.15</td>
<td>2.18</td>
</tr>
<tr>
<td>0.008</td>
<td>2.10</td>
<td>2.10</td>
<td>2.10</td>
<td>2.12</td>
</tr>
<tr>
<td>0.010</td>
<td>2.00</td>
<td>2.00</td>
<td>1.98</td>
<td>2.02</td>
</tr>
<tr>
<td>0.015</td>
<td>1.82</td>
<td>1.83</td>
<td>1.79</td>
<td>1.85</td>
</tr>
<tr>
<td>0.020</td>
<td>1.70</td>
<td>1.71</td>
<td>1.68</td>
<td>1.73</td>
</tr>
<tr>
<td>0.030</td>
<td>1.52</td>
<td>1.54</td>
<td>1.52</td>
<td>1.54</td>
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<tr>
<td>0.040</td>
<td>1.40</td>
<td>1.42</td>
<td>1.40</td>
<td>1.42</td>
</tr>
<tr>
<td>0.050</td>
<td>1.30</td>
<td>1.33</td>
<td>1.31</td>
<td>1.33</td>
</tr>
<tr>
<td>0.100</td>
<td>1.00</td>
<td>1.035</td>
<td>1.035</td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Values in parentheses were uncertain on account of the low sensitivity of the galvanometer used.
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protein chloride formed. Table II gives the pH values and the amounts of combined hydrogen ion.

The equivalent concentration of ionized protein chloride was obtained by taking the difference between the measured concentrations of Cl\(^-\) and H\(^+\) in each solution. The validity of this method of cal-

TABLE II.  
Total and Ionized Protein Chloride, Concentrations in Milli-Equivalents per Liter.

<table>
<thead>
<tr>
<th>Concentration of HCl</th>
<th>Gelatin chloride</th>
<th>Egg albumin chloride</th>
<th>Casein chloride</th>
<th>Edestin chloride</th>
<th>Serum globulin chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Total</td>
<td>Ionized</td>
<td>pH</td>
<td>Total</td>
<td>Ionized</td>
</tr>
<tr>
<td>0</td>
<td>4.80</td>
<td>0</td>
<td>5.63</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>4.44</td>
<td>1.0</td>
<td>5.44</td>
<td>1.8</td>
<td>1.24</td>
</tr>
<tr>
<td>2</td>
<td>2.25</td>
<td>5.44</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>3.00</td>
<td>2.9</td>
<td>2.94</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>3.60</td>
<td>3.80</td>
<td>3.73</td>
<td>0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>3.64</td>
<td>4.8</td>
<td>4.73</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td>6</td>
<td>3.43</td>
<td>5.05</td>
<td>4.35</td>
<td>4.0</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>3.25</td>
<td>6.46</td>
<td>6.12</td>
<td>4.7</td>
<td>4.6</td>
</tr>
<tr>
<td>8</td>
<td>3.09</td>
<td>7.26</td>
<td>6.82</td>
<td>5.2</td>
<td>5.3</td>
</tr>
<tr>
<td>10</td>
<td>2.73</td>
<td>8.17</td>
<td>7.72</td>
<td>6.0</td>
<td>6.1</td>
</tr>
<tr>
<td>12</td>
<td>2.19</td>
<td>8.67</td>
<td>7.62</td>
<td>7.2</td>
<td>7.6</td>
</tr>
<tr>
<td>15</td>
<td>2.07</td>
<td>9.27</td>
<td>9.19</td>
<td>8.5</td>
<td>7.1</td>
</tr>
<tr>
<td>30</td>
<td>2.0</td>
<td>9.57</td>
<td>9.17</td>
<td>9.2</td>
<td>7.1</td>
</tr>
<tr>
<td>40</td>
<td>1.52</td>
<td>8.47</td>
<td>8.14</td>
<td>8.7</td>
<td>7.9</td>
</tr>
<tr>
<td>50</td>
<td>1.41</td>
<td>8.97</td>
<td>9.14</td>
<td>8.7</td>
<td>7.5</td>
</tr>
<tr>
<td>100</td>
<td>1.08</td>
<td>10.1</td>
<td>0</td>
<td>1.08</td>
<td>8.7</td>
</tr>
</tbody>
</table>

* 1 gm. casein is completely soluble in 100 cc. of HCl only between 0.005 and 0.040 N.

culation follows from the fact that the solution must be electrically neutral. Representing protein by P, the possible equilibria in the solution may be indicated as follows:

\[
P + H_2O \rightleftharpoons HPOH \rightleftharpoons HP^+ + OH^-
\]

\[
HCl \rightleftharpoons Cl^- + H^+
\]

\[
\downarrow \quad \downarrow
\]

HFCI H_2O
The acidic ionization $\text{HPOH} \rightleftharpoons \text{H}^+ + \text{POH}^-$ may safely be neglected in acid solutions; therefore $[\text{HP}^+] + [\text{H}^+] = [\text{Cl}^-] + [\text{OH}^-]$. In acid solutions $[\text{OH}^-]$ is negligible in comparison with the other terms; therefore $[\text{HP}^+] = [\text{Cl}^-] - [\text{H}^+]$. If the protein acts as a poly-acid base the same reasoning applies if concentrations are expressed in equivalents. The equivalent concentrations of ionized protein so obtained are given in Table II.

The order of the degree of ionization of the protein chlorides is shown by the curves in Figs. 1 to 5, in which the equivalent concentrations of protein chloride found (or $[\text{H}^+]$ combined) and of ionized protein chloride (or $[\text{Cl}^-] - [\text{H}^+]$) are plotted as functions of the pH. The ratio of the ordinates of the two curves in each figure, at the same pH, indicates the fraction of protein chloride ionized. It is evident that this ratio is close to 100 per cent except in the most acid solutions. Only in the most acid solutions is there some indication of a repression of the ionization of the protein chloride by the high concentration of Cl$^-$ from the HCl added.

The irregularity of the results in the more acid solutions is partly explained by the lower curve in Fig. 1, which represents the error in concentration due to an error of 0.01 in pH. When it is considered...
that each of the upper curves represents the difference between two concentrations, and that there may be an error of 0.02 in the values for pH or pCl, the scattering of the points is not surprising.

The curve for the HCl combined with gelatin agrees closely with that previously found at 33°, and the values for the pH of casein solutions agree with those given by Loeb. The pH values of the egg albumin solutions agree fairly well with Loeb's values if correction is made for the HCl required (namely, \( 1.35 \times 10^{-3} \) mols per liter) to bring the solution used in these experiments to the isoelectric point, which is,

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14 Loeb, J., p. 52; *J. Gen. Physiol.*, 1920–21, iii, 547.
15 Loeb, J., p. 43; *J. Gen. Physiol.*, 1920–21, iii, 92.
Fig. 4. Ionization of 1 per cent edestin chloride.

Fig. 5. Ionization of 1 per cent serum globulin chloride.
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according to Sørensen, at pH 4.80. This correction was made in obtaining the curves given in Fig. 3. The curves for the H⁺ combined with edestin and serum globulin, however, are both higher than those previously found at 33°. This discrepancy may be due to the fact that different preparations of the proteins were used, and the suspensions of the present preparations in water were more alkaline than those of the former preparations. The isoelectric points of edestin and serum globulin are not known with certainty, so that at present the true starting points for the curves are not known. A correction for the true isoelectric point, however, would not alter the relative positions of the curves for total and ionized protein chloride, but would simply shift both curves along the axis of ordinates.

Since these experiments show that the ionization of these protein chlorides is nearly complete, they do not support the idea of Robertson that a protein chloride does not yield chloride ions on dissociation. If his idea were correct, the values of pH and pCl for each solution should be identical, which is far from true.

The results of this work agree with those of Pauli and his coworkers in so far as they confirm the idea that the ionization of protein chlorides is like that of ammonium chloride. The present results differ, however, from those quoted by Pauli with regard to any connection between the maximal ionization of the protein chloride and the maximum observed in measurements of the colloidal properties of the solutions. Pauli gives data which indicate that in the case of the dialyzed serum or serum albumin used in his experiments the maximum ionization and maximum viscosity occurred at the same pH. He uses this coincidence as support for his idea that the depressing effect of an excess of acid on the viscosity and other properties of protein chloride solutions is due to a repression of the ionization of the protein chloride. Facts opposed to this view have been presented by Loeb. The present results show that in the case of the five protein chlorides studied the maximum ionization occurs at a very different


pH from the maxima for various colloidal properties. This is brought out clearly by Table III, which is an amplification of the table given by Loeb,\textsuperscript{19} including more recent work.\textsuperscript{16,18} In so far as the table is complete the figures are quite at variance with Pauli's explanation of colloidal behavior.

The theory of colloidal behavior proposed by Loeb is based on the idea that proteins are amphoteric electrolytes and on Donnan's theory of membrane equilibrium. The former idea suggested that protein chlorides should be highly ionized salts, and this has been confirmed by the present work. The equations for Donnan's theory of membrane equilibrium were deduced on the assumption of complete ionization of the electrolytes present, and the present work lends support to the validity of this assumption with regard to protein chlorides. The present results, therefore, while not in agreement with the other theories mentioned, are in complete agreement with the theory of colloidal behavior which has been applied to the proteins by Loeb.

### IV.

#### SUMMARY.

1. By the use of the silver-silver chloride electrode, measurements have been made of the chloride ion concentrations of 1 per cent solutions of five proteins, containing from 0.001 N to 0.1 N hydrochloric

\textsuperscript{19}Loeb, J.,\textsuperscript{1} p. 115.

acid. The hydrogen ion concentrations of the same solutions have been measured by the use of the hydrogen electrode.

2. The measurements indicate that the chlorides of gelatin, egg albumin, casein, edestin, and serum globulin are highly ionized electrolytes, ionizing to yield chloride ion and a positive protein-hydrogen ion. Their ionization is therefore similar to that of ammonium chloride.

3. The results do not support the idea that a protein chloride does not yield chloride ion on dissociation. They are not in agreement with the idea that the depressing effect of an excess of HCl on the viscosity and other colloidal properties of a protein chloride solution is due to a repression of the ionization of the protein chloride. The results are, however, in complete accord with the theory of colloidal behavior advocated by Loeb.

The writer wishes to express his gratitude to Dr. Jacques Loeb for his supervision of this work.