ION SERIES AND THE PHYSICAL PROPERTIES OF PROTEINS. I.

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I. INTRODUCTION.

It had been shown in a preceding paper that the relative influence of ions on the rate of diffusion of water through collodion membranes can be expressed by arranging the ions in two definite series, as follows:

1. Rb < K < Na < Li < divalent < trivalent cations.
2. Cl < Br < I < divalent < trivalent anions.

These results are intelligible on the assumption that we are dealing in this case with electrostatic effects of ions, the electrostatic effects of the cations being due to the excess charges of the positive nucleus and the electrostatic effects of the anions being due to the charges of the captured valency electrons. In this case the relative efficiency of monatomic and monovalent cations should increase inversely with the radius of the ion; and the relative efficiency of the monovalent, monatomic anions should increase directly with the radius of the ion. In the cation series the Li ion should be next to the divalent cations, while in the anion series the iodion should be next to the divalent anions.

This order differs from that usually given for the action of ions on the physical properties of proteins. Hofmeister and afterwards Pauli determined the relative order of efficiency of ions for the precipitation of proteins. This order is according to Pauli:

For cations, \(\text{Mg} < \text{NH}_4 < \text{K} < \text{Na} < \text{Li}\).

For anions, \(\text{CNS} < \text{I} < \text{Br} < \text{Cl} < \text{acetate} < \text{tartrate} < \text{citrate} < \text{phosphate} < \text{sulfate} < \text{F}^{-}\).

The reader will notice that in the cation series the divalent cation \(\text{Mg}\) is next to \(\text{NH}_4\) and \(\text{K}\), while we should expect it to follow \(\text{Li}\) in order of efficiency. On the other hand, if the efficiency of the monovalent, monatomic cations increases in the order of \(\text{K} < \text{Na} < \text{Li}\) as Hofmeister and Pauli observed we should expect the efficiency of the anions to increase in the order of \(\text{Cl} < \text{Br} < \text{I}\) instead of in the reverse order. We must conclude that the phenomena of precipitation of proteins by ions are either not determined by the electrostatic forces of the ion or that other variables enter which are not yet known.

This latter possibility exists since the concentration of electrolytes required for precipitation is very high and the writer has shown that the influence of ions causing the anomalous diffusion of water through membranes already ceases to be noticeable in comparatively low concentrations of electrolytes.\(^4\)

It therefore seems advisable to restrict our attention to such influences of ions where they act in low concentrations. This is possible when we compare the effects of low concentrations of different acids and alkalies on the physical properties of proteins. The first one who undertook such a study on a larger scale was Pauli. He recognized that in order to investigate the effect of acids and alkalies on proteins it was necessary to free them first from salts. When Pauli and Handovski\(^5\) added to blood albumin dialyzed for 6 weeks various quantities of different acids they found that all the acids increased the viscosity of the blood albumin but in a different degree. Their results are expressed in curves from which we gather that the relative order of efficiency of various acids tried by them was

\[\text{HCl} > \text{monochloracetic} > \text{oxalic} > \text{dichloracetic} > \text{citric} > \text{acetic} > \text{sulfuric} > \text{trichloracetic acid}\]

where \(\text{HCl}\) raises the viscosity most, and trichloracetic least. The viscosity was measured by the time of outflow through a viscometer. Pauli assumes, as Laqueur and Sackur\(^6\) had done previously in their

experiments on casein solutions, that the increase in the viscosity of
the protein caused by the addition of acid (or of alkali) is due to an
increase in the ionization of proteins, as a consequence of the salt for-
formation between protein and acid or alkali, the protein salt being capable
of a greater degree of ionization than the protein not treated with acid
or alkali. In order to explain the relative differences in the effect of
various acids on the viscosity, Pauli assumes that the protein salts
formed with different acids differ in their degree of electrolytic dis-
sociation. He states; *e.g., that* "the strong trichloracetic acid and
the slightly weaker sulfuric acid furnish few protein ions." 7 He also
states that the relative efficiency of different anions on the other
properties of proteins, as osmotic pressure, precipitation by alcohol,
is the same as that found for viscosity.

In his experiments on the influence of acids and alkalies on the
osmotic pressure of gelatin solutions the writer had arrived at the con-
clusion that the results he obtained cannot be expressed in terms of
ion series. 8 These experiments showed only an influence of the val-
cency of the anion or cation in combination with the gelatin, but no
other influence. Solutions of Li, Na, K, and NH₄ gelatinate of the
same pH and the same concentration of originally isoelectric gelatin
had the same osmotic pressure. The same was true for Ca and Ba
gelatinate; but the osmotic pressure of Ba and Ca gelatinate was only
one-half or less than that of Li, Na, K, or NH₄ gelatinate of the same
pH and the same concentration of originally isoelectric gelatin. The
influence of acids on the physical properties of proteins was still more
interesting. Solutions of gelatin chloride, bromide, nitrate, acetate,
phosphate, citrate, tartrate, succinate, of the same pH and the same
concentration of originally isoelectric gelatin, had approximately the
same osmotic pressure, while the osmotic pressure of solutions of
gelatin sulfate was only half or less than half of that of gelatin
chloride, etc. The osmotic pressure of solutions of gelatin oxalate
was almost but as a rule not quite as high as that of gelatin chloride. 8

This peculiarity found its explanation in an investigation of the
combining ratios of these acids with gelatin. The writer was able to

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show that the strong dibasic acid H₂SO₄ combines in equivalent proportions with gelatin while the weaker acids, e.g. phosphoric or oxalic, combine in molecular proportions. At a given pH, e.g. pH = 3.5, 1 gm. of originally isoelectric gelatin is in combination with three times as much 0.1 N acid when the acid is H₃PO₄ than when it is HCl; and with almost twice as much 0.1 N acid when the acid is oxalic than when it is HCl, while the ratio of HCl and H₂SO₄ is 1. It follows from this that the strong dibasic acid H₂SO₄ forms a salt with gelatin in which the anion is divalent, namely SO₄²⁻, while the weak tribasic acid H₃PO₄ forms a salt with gelatin in which the anion is monovalent; namely, H₂PO₄⁻ (instead of the trivalent anion PO₄³⁻). Likewise, citric, succinic, and tartaric acids form gelatin salts in which the anion is always monovalent; namely, H-succinate, H-tartrate, etc. In the case of oxalic acid this is also the case though a slight amount of gelatin salt with divalent anion is probably formed and this might account for the fact that the osmotic pressure of gelatin oxalate is generally slightly less than that of gelatin chloride.

It was also found that Ca(OH)₂ and Ba(OH)₂ combine with gelatin in equivalent and not in molecular proportion thus showing that the cation of Ca gelatinate and Ba gelatinate is divalent.

All these facts together show that solutions of salts of gelatin with a bivalent ion have an osmotic pressure of one-half or less of that of solutions of salts of gelatin with monovalent ions at the same pH and the same concentration of originally isoelectric gelatin.

This then proves that while the valency of the ion in combination with the gelatin has a strong influence on the osmotic pressure of the gelatin solution differences in the nature of ions of the same valency have either no effect on the osmotic pressure or if they have such an effect it is too slight to be noticeable by our method of experimentation. We can certainly say that it is impossible to express our observations in terms of the Hofmeister series. In the Hofmeister series phosphates and chlorides stand at almost opposite ends of the anion series while in our experiments the effects of H₃PO₄ and HCl are identical if we compare the effects at the same pH of the gelatin solutions. The reason that Pauli arrived at a different conclusion lies

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probably in the fact that he compared the effects of the addition of equal quantities of acid to a protein while we compared the effects of different acids at equal hydrogen ion concentrations of the solutions.

II. Action of Weak and Strong Monobasic Acids.

We will first indicate why it is necessary to choose equal hydrogen ion concentrations as a standard of comparison instead of using equal quantities of acid. We always use as standard material isoelectric protein, to which we add enough acid or alkali to bring it to the desired hydrogen ion concentration. In the case of gelatin we proceed usually in this way; we add to 1 gm. of powdered gelatin brought to the isoelectric point (pH = 4.7) small quantities of acid to bring the samples to the desired hydrogen ion concentration, then melt the gelatin, and bring the volume of the solution to 100 cc. by adding H₂O. The pH is then determined. It is hardly necessary to state that it requires greater quantities of weak than of strong acid to bring the gelatin to the same pH on the acid side of the isoelectric point. Fig. 1 gives the quantities of 0.1 N acetic, monochloracetic, dichloracetic, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin in a 1 per cent solution to the same pH. The abscissæ in Fig. 1 are the pH of the gelatin solution resulting from the addition of acid and the ordinates are the numbers of cc. of 0.1 N acid which must be contained in 100 cc. of a 1 per cent solution of originally isoelectric gelatin to produce the pH. The curve indicates that the quantity of acid required is the less the stronger the acid. If we now measure the osmotic pressure of the solutions of the four gelatin acetates (all 1 per cent in regard to the originally isoelectric gelatin) and if we plot the curves with the values for the osmotic pressure as ordinates and the pH as abscissæ, the curves for all four gelatin-acetate salts are practically identical (Fig. 2). All four curves have a minimum at the isoelectric point pH = 4.7; they all rise identically with a diminution in pH (i.e. a rise in hydrogen ion concentration); all reach their maximum at a pH of about 3.5, and all drop almost as steeply with a further diminution of pH as they rise on the ascending side of the curve. The drop will not be discussed in this paper.
The slight differences in the maximal height of the four curves are within the limits of the accuracy of these experiments, and the same or even greater variations may be observed when the same experiment is repeated several times with the same acid. The maximal values of osmotic pressure reached in these experiments at pH 3.5 are the same as those reached with gelatin salts of any other monovalent anion, Cl, Br, NO₃, phosphate, citrate, succinate, etc.

![Graph](image)

**Fig. 1.** The ordinates represent the number of cc. of 0.1 N acetic, mono-, di-, and trichloracetic acids required to bring 1 gm. of isoelectric gelatin to the pH indicated by the abscissa. Enough H₂O was added to bring the gelatin-acid solution to a volume of 100 cc.

It follows from this that the conclusions which are based upon a comparison of the quantities of the acid added instead of upon the pH of the protein solutions cannot be correct.

The reason why we get identical curves when we plot the osmotic pressures as ordinates over the pH as abscissae seems to be as follows. When we add a definite small quantity of acid to 1 gm. of isoelectric gelatin, melt, and bring the volume of the solution to 100 cc. by adding H₂O, part of the acid will combine with gelatin and part will remain...
free in the solution. The more acid we add, the greater the amount of isoelectric gelatin transformed into gelatin-acid salt. We assume that there is a definite equilibrium between the hydrogen ion concentration of the solution, the amount of gelatin-acid salt formed, and isoelectric gelatin. If this view is correct, it follows that at the same

![Graph showing osmotic pressure vs pH](image)

**FIG. 2.** The ordinates indicate the osmotic pressure (in mm. of the height of a column of the gelatin solution) of 1 per cent solutions of originally isoelectric gelatin which have been brought to different pH by the addition of the acids used in Fig. 1. The abscissae are the pH. The curves are practically identical, the slight differences at the summit being inside the range of the variation found for the same acids.
pH and with the same concentration of originally isoelectric gelatin all mixtures of acid and gelatin solution must have the same concentration of gelatin-acid salt; and if this is the case they must all have the same osmotic pressure if the valency of the anion is the same, since the osmotic pressure is almost entirely determined by the gelatin-acid salt, that of the isoelectric gelatin being very low. This conclusion is in harmony with the view expressed by Laqueur and Sackur, Pauli, and others. The writer differs from Pauli only in regard to the statement that the different acids, e.g. HCl, H₃PO₄, tartaric, acetic, and trichloracetic, have different effects on the physical properties of proteins.

III. Combining Ratios of Acids and Alkalies with Crystalline Egg Albumin and the Osmotic Pressure of the Albumin Solutions.

Crystalline egg albumin was prepared under Dr. Northrop's supervision according to Sörensen's method, and crystallized three times. The only difference in procedure was in the dialysis. Instead of putting the water under negative pressure as was done by Sörensen, pressure was put on the egg albumin by attaching a long glass tube full of water to the dialyzing bag so that the solution was under about 150 cm. water pressure during dialysis. This was necessary to avoid too great an increase in volume. The same stock solution of albumin served for all the experiments and was diluted before the experiment to a 1 per cent solution. The concentration of ammonium sulfate left in the solution was between M/1,000 and M/2,000. The pH of the stock solution was about 5.20. By adding about 1 cc. of 0.1 N HCl to 100 cc. of a 1 per cent solution of this albumin the solution was brought to the isoelectric point of the egg albumin, which is according to Sörensen at pH = 4.8.

The 1 per cent solutions were made up with different quantities of acid (or alkali) and the pH of the albumin solution was determined electrometrically. In Fig. 3 are plotted the curves in which the pH are the abscissæ and the cc. of 0.1 N acid required to obtain the various

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11 The colorimetric determination which gives fairly good results in the case of gelatin is unreliable in the case of egg albumin.
pH as ordinates. The curves represent these values for four acids, HCl, H₂SO₄, H₃PO₄, and oxalic acid. Beginning with the lowest curve we notice that the curve is the same for 0.1 N HCl and 0.1 N H₂SO₄, since both are strong acids; or, in other words, H₂SO₄ combines

![Graph showing the number of cc. of 0.1N acid required to bring 1 gm. of isoelectric crystalline egg albumin to a pH indicated on the axis of abscissae. The graph compares HCl, H₂SO₄, oxalic, and phosphoric acids.](image)

**Fig. 3.** The ordinates represent the number of cc. of 0.1 N HCl, H₂SO₄, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissae. Enough H₂O was added to bring the solutions of albumin and acid to a volume of 100 cc. For the same pH the ordinates for HCl, H₂SO₄, and phosphoric acid are approximately as 1:1:3. The ratio of HCl:oxalic acid is a little less than 1:2.
in equivalent proportions with egg albumin. The curve for H₃PO₄ is the highest curve and if we compare the values for H₃PO₄ with those for HCl (or H₂SO₄) we notice that for each pH the ordinate for H₃PO₄ is as nearly three times as high as that for HCl as the accuracy of our experiments permits. This means that under the conditions of our experiments phosphoric acid combines with albumin in molecular proportions and that the anion of albumin phosphate is the monovalent anion H₂PO₄.

![Graph showing osmotic pressure of different albumin-acid salts.](image)

**Fig. 4.** Osmotic pressure of different albumin-acid salts. The ordinates indicate the osmotic pressure (in mm. of 1 per cent albumin solution); the abscissa are the pH. All solutions are 1 per cent in regard to isoelectric albumin. The curves for albumin chloride and albumin phosphate are identical.

The values for oxalic acid are for pH above 3.2 not quite twice as high as those for HCl, indicating that for these values of pH oxalic acid combines to a greater extent in molecular and only to a smaller extent in equivalent proportions with albumin.

If egg albumin behaves like gelatin we should expect that the curves of osmotic pressure for albumin phosphate and albumin chloride when plotted as a function of the pH should be identical, since the anion is in both cases monovalent; that the curve for albumin sulfate should
be considerably lower since the anion SO$_4$ in combination with albumin is bivalent; while the curve for the osmotic pressure of albumin oxalate should be between the curves for albumin chloride and albumin sulfate, but much nearer the chloride than the sulfate curve. Fig. 5 gives the curves for the combining ratios of acetic acid and dichloracetic acid with isoelectric albumin, showing the same differ-

**Fig. 5.** Curves expressing the cc. of 0.1 N acetic and dichloracetic acids required to bring 1 gm. of isoelectric albumin to different pH.

4 giving the curves of the osmotic pressures of the four albumin salts shows that this expectation is fulfilled.

Fig. 5 gives the curves for the combining ratios of acetic acid and dichloracetic acid with isoelectric albumin, showing the same differ-
ence as the corresponding curves for gelatin in Fig. 1. Fig. 6 gives the curves for the influence of the two acids upon the osmotic pressure of 1 per cent solutions of originally isoelectric albumin. The two curves are identical and are also identical with those of albumin chloride and albumin phosphate in Fig. 4, thus confirming our theory.

In Fig. 7 are given the curves for combining ratios of NaOH, Ca(OH)$_2$, and NH$_4$OH with isoelectric albumin. The curve for Ca(OH)$_2$ is identical with that for the strong base NaOH, indicating that Ca(OH)$_2$ combines with egg albumin in equivalent proportions.

![Fig. 6. Curves of osmotic pressure of solutions of albumin acetate and albumin dichloracetate. The curves for both acids are identical.](image)

Fig. 8 shows that the curve for the osmotic pressure of Ca albuminate is only one-half as high as that of Na albuminate as was to be expected. The curve for NH$_4$ albuminate is identical with that for Na albuminate, which was to be expected since the NH$_4$ is monovalent.

The results with albumin are therefore identical with those obtained in the case of the corresponding gelatin salts. The result that gelatin phosphate and albumin phosphate behave like gelatin chloride and albumin chloride may be considered as a crucial test against the colloidal conception of ion effects on proteins and in favor of a purely chemical theory.
FIG. 7. Curves representing the number of cc. of 0.1 N NH₄OH, NaOH, and Ca(OH)₂ required to bring 1 gm. of isoelectric, crystalline egg albumin to different pH. The curves for NaOH and Ca(OH)₂ are identical.
Fig. 8. Curves of the osmotic pressure of NH₄, Na, and Ca albuminate at different pH. The curves for NH₄ and Na albuminate are practically identical.
I. Combining Ratios of Acids and Bases with Gelatin and the Viscosity of Gelatin Salts.

Since Pauli's ion series was based primarily on the influence of acids or their anions on the viscosity of protein solutions it seemed necessary to find out whether or not viscosity measurements confirm the conclusions at which we arrived on the basis of osmotic pressure experiments on gelatin and egg albumin. A few remarks concerning our method are required.

The gelatin is first rendered isoelectric in the following way. 25 gm. of powdered gelatin of pH about 7.0 are put into 1 liter of m/128 acetic acid for 30 minutes at 10°C, after which time the acetic acid is renewed and left in contact with gelatin again for 30 minutes at 10°C. The acid is then decanted and replaced with distilled water of about 5°C. The mixture is filtered in a Buchner funnel through muslin, employing slight suction. The gelatin is then washed about six times with 100 cc. of distilled water of 5°C each, and is made into a 5 per cent solution which serves as a stock solution. The pH of this solution is about 4.7, or, in other words, the gelatin is isoelectric.

Some of the stock solution is heated to 45°C and made up to a 2 per cent solution in quantity sufficient for a day's experiments. This 2 per cent solution is kept during the day at 24°C. To 50 cc. of this solution is added the desired acid or alkali in sufficient quantity and then the volume is raised to 100 cc. by the addition of enough distilled water. This 1 per cent solution is then rapidly brought to a temperature of 45°C, kept there for 1 minute, and is then rapidly cooled to 24°C. The solution is stirred constantly during the heating and cooling. The viscosity is measured immediately after the solution is cooled to 24°C. The measurements were all made at 24°C by using the time of outflow through a viscometer. The time of outflow of distilled water through an Ostwald viscometer at 24°C was exactly 1 minute. Each measurement of viscosity was repeated with the same gelatin solution and the beginning and the end of a series consisted in the measurement of viscosity of isoelectric gelatin. These latter measurements agreed in all experiments within 1 second varying only between 80 and 81 seconds, thus guaranteeing the reproducible character of the experiments.
The results can be given briefly. Fig. 9 gives the curves for the cc. of 0.1 N HNO₃, H₂SO₄, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH. The curve shows again that for each pH the number of cc. of 0.1 N acid required are for HNO₃, H₂SO₄, oxalic, and phosphoric acids approximately as 1:1:2:3.

![Fig. 9](image_url)

**Fig. 9.** Curves for the number of cc. of 0.1 N HNO₃, H₂SO₄, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). Curves similar to those for egg albumin (Fig. 3). For the same pH the ratio of HNO₃, H₂SO₄, oxalic, and phosphoric acids required is approximately as 1:1:2:3.
Fig. 10 gives the curves for the viscosity of 1 per cent solutions of gelatin chloride, sulfate, oxalate, and phosphate. The abscissae are the pH, the ordinates the ratio of the time of outflow of the gelatin solutions divided by the time of outflow of pure water. For the sake
of brevity we will call this quotient the specific viscosity of the gelatin solution. The curves for the four acids all rise steeply from the isoelectric point with increasing hydrogen ion concentration until they reach a maximum at pH about 3.0. The curves then drop again.

Fig. 11. Curves of specific viscosity of gelatin succinate, tartrate, and citrate. The curves are practically identical with those for the viscosity of gelatin chloride and phosphate.

The curves for the three acids, gelatin chloride, oxalate, and phosphate, are practically identical while the curve for gelatin sulfate is considerably lower.
Fig. 11 gives the curves for the viscosity of gelatin citrate, tartrate, and succinate. The three curves are practically identical and also identical with the curves for gelatin chloride and gelatin phosphate in Fig. 10.

![Graph showing viscosity curves for gelatin acetate, mono-, di-, and trichloracetate. Curves identical with those for gelatin chloride and phosphate.]

Fig. 12 gives the curves for the viscosity of 1 per cent solutions of originally isoelectric gelatin to which acetic and mono-, di-, and trichloracetic acids have been added. The curves are again identical
Fig. 13. Curves for the number of cc. of 0.1 N NaOH, KOH, Ba(OH)₂, and Ca(OH)₂ required to bring 1 gm. of isoelectric gelatin to different pH (in 100 cc. of solution). All four curves are identical.

Fig. 14. Curves for specific viscosity of Na and Ca gelatinate for different pH.
with those for gelatin chloride, phosphate, etc. These curves are interesting in connection with Pauli's statement that trichloracetic acid causes the same low values of viscosity as sulfuric acid. This was the case neither in our experiments on the osmotic pressure nor in those on the viscosity of gelatin solutions.

Fig. 13 gives the cc. of 0.1 N NaOH, KOH, Ca(OH)$_2$, and Ba(OH)$_2$ required to bring 1 gm. of originally isoelectric gelatin in 100 cc. solution to a given pH. One curve suffices for the four alkalies thus proving that Ca and Ba combine with gelatin as bivalent ions. We should expect the curve for the viscosity of Ca gelatinate to be considerably lower than that of Na gelatinate. Fig. 14 shows that this is true.

SUMMARY.

1. This paper contains experiments on the influence of acids and alkalies on the osmotic pressure of solutions of crystalline egg albumin and of gelatin, and on the viscosity of solutions of gelatin.

2. It was found in all cases that there is no difference in the effects of HCl, HBr, HNO$_3$, acetic, mono-, di-, and trichloracetic, succinic, tartaric, citric, and phosphoric acids upon these physical properties when the solutions of the protein with these different acids have the same pH and the same concentration of originally isoelectric protein.

3. It was possible to show that in all the protein-acid salts named the anion in combination with the protein is monovalent.

4. The strong dibasic acid H$_2$SO$_4$ forms protein-acid salts with a divalent anion SO$_4$ and the solutions of protein sulfate have an osmotic pressure and a viscosity of only half or less than that of a protein chloride solution of the same pH and the same concentration of originally isoelectric protein. Oxalic acid behaves essentially like a weak dibasic acid though it seems that a small part of the acid combines with the protein in the form of divalent anions.

5. It was found that the osmotic pressure and viscosity of solutions of Li, Na, K, and NH$_4$ salts of a protein are the same at the same pH and the same concentration of originally isoelectric protein.

6. Ca(OH)$_2$ and Ba(OH)$_2$ form salts with proteins in which the cation is divalent and the osmotic pressure and viscosity of solutions of these two metal proteinates are only one-half or less than half of
that of Na proteinate of the same pH and the same concentration of originally isoelectric gelatin.

7. These results exclude the possibility of expressing the effect of different acids and alkalies on the osmotic pressure of solutions of gelatin and egg albumin and on the viscosity of solutions of gelatin in the form of ion series. The different results of former workers were probably chiefly due to the fact that the effects of acids and alkalies on these proteins were compared for the same quantity of acid and alkali instead of for the same pH.