VALENCY RULE AND ALLEGED HOFMEISTER SERIES
IN THE COLLOIDAL BEHAVIOR OF PROTEINS.

II. THE INFLUENCE OF SALTS.

By Jacques Loeb and M. Kunitz.

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

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Difference between the Action of Acids and of Salts on Colloidal Behavior.

In order to understand the action of salts on colloidal behavior, it is necessary to understand the difference between the action of acids and of salts. When acid is added to a solution of isoelectric protein, the membrane potentials, osmotic pressure, and, in the case of some proteins (e.g., gelatin), viscosity, are first increased until a maximum is reached; and when more acid is added, the values representing these properties drop again. The action of acid on the swelling of gelatin is the same, since the swelling due to acid is merely an osmotic phenomenon. This rise and fall of the curves for these four properties finds its explanation through the following facts. The addition of acid to isoelectric protein has two opposite effects; first, a salt formation between protein and acid, which increases the concentration of ionized protein, and this causes a rise in the value of the four properties mentioned with the addition of acid; and second, the depressing influence of the anion of the acid on the value of the four properties. Both effects increase with the increase in the concentration of acid, but not at the same rate. When little acid is added to isoelectric protein, the salt formation and ionization of the protein increases at first more rapidly than the depressing effect of the anion of the acid until a point is reached where the further addition of acid increases the ionization little, and finally not at all, while the depressing effect of the anion of the acid continues to increase. This peculiar action of
the acid finds its explanation in the theory of membrane equilibria, as shown in preceding papers and a book.\textsuperscript{1}

If this theory of membrane equilibria is correct, we should expect that, when the protein salt is a salt of the type of gelatin chloride, where the gelatin ion is positively charged, only the anion of an acid (or a salt) should influence the four properties dependent on the membrane equilibrium, namely membrane potentials, osmotic pressure, swelling, and that type of viscosity which is due to the swelling of protein particles; and furthermore, that only the valency of the anion but not its chemical nature should have an effect (unless these properties are affected in an indirect way by a change in the chemical or physical constitution of the protein by a specific acid). The validity of the valency rule has already been discussed for acids and will be discussed more fully here for salts.

The difference between the action of salts and acids on the four properties of protein is this, that while acids (and alkalies) cause ionization of isoelectric protein, salts have no such effect. When a salt is added to a solution of a protein salt, \textit{e.g.} gelatin chloride, of a given pH, only the depressing action of the anion of the salt on these properties is noticed, but no augmenting effect of the cation of the salt. This was proved by Loeb by the following two sets of experiments. When all the protein is ionized, \textit{e.g.} when gelatin chloride of pH 2.5 is chosen, HCl and NaCl act quantitatively alike, since now no further ionization can be produced by the acid. When, however, the pH of the gelatin chloride is higher (but below pH 4.7), the addition of little acid will cause an increase in the values of the four properties which will not be produced by the salt.\textsuperscript{1}

The second proof consisted in the fact that if the pH of the protein solution is kept constant, salts of the type of NaCl, CaCl\textsubscript{2}, and LaCl\textsubscript{3} will have an equal depressing effect on the properties of protein-acid salts for the same concentration of the Cl ions of the three salts, at the same pH of the protein solution or protein gel. This was proven for membrane potentials and osmotic pressure.\textsuperscript{2}

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\textsuperscript{1}Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

If the explanation of these effects of salts on the basis of the Donnan equation is correct, it follows furthermore that only the anion of a salt should have a depressing effect on the four properties of protein-acid salts, and only the cation of a salt should have a depressing effect on the properties of metal proteinates. Loeb has already shown that this is correct and it is intended to add in this paper some further experimental evidence. This was considered advisable on account of the fact that some authors continue to maintain that for the action of salts on proteins the Hofmeister series are valid. Those who believe in the validity of the Hofmeister series for the action of salts on the four above mentioned properties of proteins arrange the effect of salts on the swelling of gelatin in the following way:

\[
\text{SO}_4, \text{tartrate}, \text{citrate} < \text{acetate} < \text{Cl} < \text{Br}, \text{NO}_3 < \text{I} < \text{CNS}
\]

where the swelling is said to be a maximum in CNS and a minimum in \(\text{SO}_4\). "Above a certain concentration the sulfates, tartrates, and citrates cause a shrinkage of the gel of gelatin, and acetate acts in the same sense but less strongly, while the other anions cause an increasing swelling of the gel of the same concentration." Loeb had shown that these and similar statements were simply due to the failure of the older authors to measure the pH of the gel and that if this quantity is properly measured with the hydrogen electrode, it is found that there exists only an effect of the valency of the anion of the salt on swelling and no effect of the chemical nature of the anion (provided the pH of the protein solution or gel is on the acid side of the isoelectric point).\(^1\)

As far as the actions of the cations of a salt are concerned, the following statement is made. "The differences in the effects of cations are less marked; it might be possible to propose the series \(\text{Li} < \text{Na} < \text{K}, \text{NH}_3\); then follow the alkali earths with Mg in a position between."

It is also stated that these anion and cation series are correct at a neutral reaction. Since the isoelectric point of gelatin is at pH 4.7, at neutral reaction, i.e. pH near 7.0, gelatin exists as metal gelatinate, the properties of which are not affected at all by the anions of a salt.

and are affected strongly by the cation of a salt. This alone shows that the anion series at pH 7.0 must be the result of a methodical error. As far as osmotic pressure is concerned, Lillie\textsuperscript{4} states that salts depress the osmotic pressure of neutral solutions of egg albumin in the following order

\[
SO_4 > Cl > Br > I > NO_3 > CNS
\]

At neutral reaction of the albumin solution the anions of a salt can have no influence on the osmotic pressure of a protein solution. Neither Lillie nor any of the other authors had measured the pH of their solutions or gels, and the Hofmeister series on osmotic pressure and swelling are the result of this methodical error, as a consequence of which these authors mistook effects due to variation of the pH for effects due to the nature of the anion. If we wish to study the effect of anions on the four physical properties of proteins—membrane potentials, osmotic pressure, swelling, and viscosity—we must use protein-acid salts, the pH of which is in the case of gelatin less than 4.7, and we must make sure that the pH of the protein solution or protein gel is not altered by the addition of salt. This can be done only by controlling the pH with the hydrogen electrode, as will be shown in the following experiments.

\textit{Membrane Potentials.}

For these experiments three stock solutions all of pH 3.8 were prepared: first, solutions of gelatin chloride of pH 3.8 containing 2 gm. dry weight of originally isoelectric gelatin and 8 cc. of 0.1 n HCl in 100 cc. solution; second, \(\text{M}/2\) solutions of different salts brought to a pH of 3.8 by the addition of HCl; and third, distilled water brought also to the pH of 3.8 by the addition of HCl. By successive dilution of the \(\text{M}/2\) salt solutions of pH 3.8 with distilled water of pH 3.8, series of salt solutions of different degree of concentration, but all of pH 3.8, were prepared. 50 cc. of the 2 per cent solutions of gelatin chloride of pH 3.8 and 50 cc. of the salt solutions of pH 3.8 were then mixed and 1 per cent solutions of gelatin chloride in salt solutions of different concentrations, but all of pH 3.8, were obtained.

\textsuperscript{4}Lillie, R. S., \textit{Am. J. Physiol.}, 1907-08, xx, 127.
Collodion bags of about 50 cc. volume were filled with such solutions (closed with stoppers perforated by manometer tubes as described), and the collodion bags were submerged in beakers containing each 350 cc. of the same salt solution as that inside the collodion bags and all of pH 3.8, but without protein. The experiments lasted for 18 to 24 hours at 24°C. At that time osmotic equilibrium was reached, the osmotic pressure was read, and the membrane potentials between the protein solution inside the collodion bag and the outside aqueous solution free from protein were measured with a pair of indifferent calomel electrodes (in saturated KCl) as described.

Fig. 1 represents the effect of six different salts with monovalent anions, NaCl, NaBr, NaI, NaNO₃, NaCNS, and Na acetate, and one salt with divalent anion, Na₂SO₄. These salts were selected to find out whether the above mentioned Hofmeister series are real or whether the valency rule holds. The abscissae are the concentrations of the salts and the ordinates are the observed membrane potentials in millivolts. When no salt was added, i.e. when the concentration was zero, the values vary within about 3 to 4 millivolts due to the limits of experimental accuracy. This variation was, of course, also found when salts were added. The curves in Fig. 1 show that the variations between the effects of the six different salts with monovalent anion are chance variations such as are observed when no salt is added, i.e. at concentration 0 in Fig. 1, and that if this is taken into consideration it is found that the effects of the six salts on membrane potentials lie all on one curve. The curve for Na₂SO₄ is, however, considerably lower than the curve for the six salts with monovalent anions; namely, a little less than two-thirds. The curves in Fig. 1, therefore, show that the six salts, NaCl, NaBr, NaI, NaNO₃, NaCNS, and Na acetate, have (within the limits of accuracy of measurements) the same effect on the membrane potentials, while the depressing effect of SO₄ is about two-thirds or still less. These experiments prove the valency rule but contradict the Hofmeister ion series.

In order to bring 100 cc. of M/2 stock solutions of the different salts to a pH of 3.8, the solutions had to contain different amounts of 0.1 N HCl, as Table I shows.

Attention is called to the enormous amount of HCl required to maintain the pH of 3.8 in the presence of M/2 Na acetate. Yet the
older colloid chemists compared the effect of Na acetate with that of NaCl without correcting for the difference of pH.

Fig. 1. All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na$_2$SO$_4$ is much greater.

This fact has to be kept in mind when the effect of buffer salts with divalent anions is examined. The following number of cc. of
0.1 N HCl (or their equivalent) was required in 100 cc. of m/2 solutions of such salts to produce a pH of 3.8.

<table>
<thead>
<tr>
<th>Salt</th>
<th>0.1 N HCl required</th>
</tr>
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<tbody>
<tr>
<td>Na₂ oxalate</td>
<td>55.0 cc. 0.1 N HCl</td>
</tr>
<tr>
<td>Na₂ tartrate</td>
<td>150.0 cc. 0.1 N HCl</td>
</tr>
<tr>
<td>Na₂ succinate</td>
<td>430.0 cc. 0.1 N HCl</td>
</tr>
</tbody>
</table>

Now in these solutions, NaCl and weak dibasic acids are formed, the latter dissociating chiefly, though not exclusively, as monobasic acids. By adding such buffer salts to a solution of gelatin chloride of pH 3.8 and maintaining the pH by adding HCl, part of the bivalent anions of the salts are transformed into monovalent anions. Hence

The depressing effect of the three above mentioned salts should lie between that of NaCl and that of Na₂SO₄, but that of Na₂ oxalate or Na₂ tartrate nearer to Na₂SO₄ than that of Na₂ succinate. Fig. 2 shows that this is correct.

The older authors ignored the pH and compared the effect of e.g. Na₂ tartrate with that of NaCl, disregarding the change in pH. Their error was not one of colloid chemistry, but one of physical chemistry. When the pH of the gelatin solution is kept constant, the influence of salts on membrane potentials of a gelatin chloride solution is determined only by the valency of the anion of the salt but not by its chemical nature.
In the preceding experiments on membrane potentials the influence of the salts on osmotic pressure of the protein solutions at pH 3.8 was also measured. Fig. 3 gives the result. The ordinates are the observed osmotic pressures in terms of a column of water. The de-

**Osmotic Pressure.**

Fig. 2. Effect of strong and weak dibasic salts on the membrane potentials of gelatin chloride solutions at pH 3.8.
pressing effects of the six salts with monovalent anion (NaCl, NaBr, NaI, NaNO₃, NaCNS, Na acetate) on the osmotic pressure of the gelatin chloride solutions of pH 3.8 (containing 1 gm. dry weight of originally isoelectric gelatin in 100 cc.) lie, within the limits of experimental accuracy, on one curve, which is entirely different from the curve for the effect of Na₂SO₄. The osmotic pressures are a little

![Graph showing osmotic pressure vs. concentration for various salts.](image)

Fig. 3. All salts with monovalent anions depress the osmotic pressure of gelatin chloride solutions of pH 3.8 to the same extent (within the limits of experimental accuracy). Na₂SO₄ depresses considerably more.
over twice as high when the anion of the salt is monovalent than when it is divalent. The variations in the effects of the six salts with monovalent anion are chance variations, since they are also found when no salt is added, i.e. at concentration 0 in Fig. 3.

This shows that all the salts with monovalent anions have the same effect on the osmotic pressure when the pH is kept constant and that the Hofmeister anion series is based on error. The anion series has
to be replaced by the valency rule. This statement is supported by Fig. 4 which gives the influence of Na₂ oxalate, Na₂ tartrate, and Na₂ succinate upon the osmotic pressure. The effect of these salts lies between that of Na₂SO₄ and NaCl as the valency rule demands.

Hence we come to the conclusion that when the pH of the solution is kept constant, the influence of the anion of different salts on the osmotic pressure of a gelatin chloride solution is determined by the valency, but not by the chemical nature of the anion. It was shown in former publications that the cation of the salt has no effect on the osmotic pressure of gelatin chloride or edestin chloride solutions. Both the Hofmeister cation and anion series for the influence of salts on the osmotic pressures of protein solutions are fictitious and the consequence of a methodical error.

Swelling.

The general method described in the first part for measuring the influence of acids on swelling was also used for measuring the influence of salts on swelling. It was intended to use gels which at equilibrium had a pH of 3.8. It had been found in previous experiments that 1 gm. of powdered isoelectric gelatin has at equilibrium a pH of 3.82 when it is put into 150 cc. of water containing 4.5 cc. 0.1 N HCl.

The following stock material was prepared: first, doses of wet powdered isoelectric gelatin of 8 gm. each containing about 1 gm. dry weight of isoelectric gelatin; second, a stock solution of HCl containing 3 cc. of 0.1 N HCl per 100 cc. H₂O (since the isoelectric gel of gelatin had in such a solution at equilibrium a pH of 3.82); and third, ⅔ solutions of various salts made up with HCl to the same pH as the stock solution of HCl. The stock solution of HCl was used for the dilutions of the ⅔ salt solutions.

Doses of 8 gm. of the wet isoelectric gelatin (as described in Part I) were added to 150 cc. of various salt solutions made up as described, and allowed to stand for 2 hours in the solutions at 15°C. The swelling of the gelatin was then measured by weight, as described in Part I.

In the figures the abscissae are the concentrations of salts, and the ordinates are the weights of the gelatin. Without salts the weight varied generally at the end of the experiment around 27 gm., while it was depressed by salts to about 10 gm.
It had already been shown in preceding papers\(^5\) that when different chlorides, e.g. NaCl, CaCl\(_2\), or LaCl\(_3\), are added to a solution of gelatin chloride, the depressing effect of the three salts on osmotic pressure and membrane potentials is the same for equal concentrations of Cl ions of the salt, provided that the pH is kept constant. This agrees with the theory that the effect of salts is a consequence of membrane equilibria. It seemed of importance to see whether the same is true for the influence of salts on swelling. The influence of LiCl, NaCl, KCl, CaCl\(_2\), and LaCl\(_3\) was tested and the results are given in Fig. 5. The abscissae are the molar concentrations of the Cl ions of these salts, while the ordinates are the amount of swelling measured by the weight of the gelatin. It is obvious that the effects of these five salts on swelling lie all on one curve, proving that the effect of salts on swelling of gelatin chloride is determined only by the anion of the salt and that the cation has no effect whatever on the swelling of gelatin chloride.

The next fact to be ascertained was whether or not only the valency of the anion of the salt is of influence or whether the anion series generally quoted in colloidal literature is valid, according to which the swelling is a maximum in NaCNS, and a minimum in Na acetate (leaving the divalent anions out of consideration for the present).
Seven salts with monovalent anions were tried; namely, NaCl, NaBr, NaI, NaNO₃, NaCNS, Na acetate, and Na lactate. The results are given in Fig. 6. It is obvious that the effects of all of these seven salts lie on one curve, and that the variations are essentially the chance variations due to the limits of experimental accuracy. This is proven by the fact that the same variations are observed when the concentration of salt is zero; i.e., when no salt is added. There is not the slightest indication of the Hofmeister anion series. Slight influences of the salts on the cohesion of the gel of gelatin may exist, but they are too small to play a rôle.

While salts with monovalent anions have the same depressing effect for the same concentration of anions, salts with bivalent anions have a much greater depressing effect on swelling than salts with monovalent anions. This is illustrated in Fig. 7 showing the difference in the effect of equal molar concentrations of NaCl and Na₂SO₄ on swelling. NaCl does not depress swelling in concentrations of 1/1,024 or below, and the depressing effect of NaCl on the swelling of gelatin chlo-

**Fig. 6.** All salts with monovalent anions depress the swelling of a gelatin chloride gel to the same extent (within the limits of experimental accuracy) at pH 3.8.
ride of pH 3.8 commences to be noticeable at a concentration of \( \text{m}/512 \). This is true for all salts with monovalent anions as Fig. 6 shows. \( \text{Na}_2\text{SO}_4 \) begins, however, to depress at a concentration between \( \text{m}/4,000 \) and \( \text{m}/2,000 \), and the curve for the \( \text{SO}_4 \) effect drops much more rapidly to the minimum than in the case of \( \text{NaCl} \).

The effects of salts on the viscosity of gelatin solutions have already been discussed so fully in previous publications by Loeb that it does not seem necessary to add to the facts already published.

![Graph](image)

**Fig. 7.** \( \text{Na}_2\text{SO}_4 \) depresses the swelling of a gelatin chloride gel considerably more than \( \text{NaCl} \).

Summarizing all the results, we can say that the membrane potentials, osmotic pressure of gelatin chloride solutions or the swelling of gelatin chloride gels, and that type of viscosity of gelatin chloride solutions which depends on the swelling of submicroscopic solid particles in the solutions are only affected by the anion but not by the cation of a salt; that all anions of the same valency have the same depressing effect on these four properties of gelatin chloride, and that the depressing effect is greater for the divalent than for monovalent anions.

The so-called Hofmeister series of the effects of cations as well as of anions on these four properties are fictitious and the result of the
fact that the previous authors have failed to measure the pH of their solutions or of their gels. If this error is avoided and if the pH is kept constant, it is obvious that the Hofmeister series has to be replaced by the valency rule as was to be expected from the membrane equilibria theory of colloidal behavior. This is true only for those four properties of proteins which depend upon membrane equilibria; namely, membrane potentials, osmotic pressure, swelling, and that type of viscosity which depends on swelling. Such properties of proteins as do not depend on the Donnan equilibrium, e.g. solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.

**SUMMARY.**

It is shown by the older experiments by Loeb and by the experiments reported in this paper that the effect of salts on the membrane potentials, osmotic pressure, swelling of gelatin chloride, and that type of viscosity which is due to the swelling of protein particles, depends only on the valency but not on the chemical nature of the anion of the salt, and that the cation of the salt has no effect on these properties, if the pH of the protein solution or protein gel is not altered by the salt. The so called Hofmeister series of salt effects on these four properties are purely fictitious and due to the failure of the former authors to measure the hydrogen ion concentration of their protein solutions or gels and to compare the effects of salts at the same pH of the protein solution or the protein gel. These results confirm the older experiments of Loeb and together they furnish a further proof for the correctness of the idea that the influence of electrolytes on the four properties of proteins is determined by membrane equilibria. Such properties of proteins which do not depend on membrane equilibria, such as solubility or cohesion, may be affected not only by the valency but also by the chemical nature of the ions of a salt.