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AMPHOTERIC COLLOIDS.

III. CHEMICAL BASIS OF THE INFLUENCE OF ACID UPON THE PHYSICAL PROPERTIES OF GELATIN.

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I.

Many of the authors who have worked on the physical chemistry of proteins, like Hardy, Pauli, Michaelis, Robertson,¹ and others, have pointed out that the different properties of proteins, *e.g.* swelling, viscosity, are affected by electrolytes in a parallel way, a fact which suggests that these variations are due to the same variable. The nature of this variable is not known and the majority of authors believe it to be connected with the colloidal character of the proteins, while others are inclined to assume a purely chemical or stoichiometrical relation. The reason for this doubt lies in the fact stated appropriately by Pauli² in discussing the influence of acid and alkali upon the osmotic pressure of gelatin.

Pauli and Handowski have pointed out that in these experiments too the essential feature is the formation of ionic protein. But a satisfactory explanation of this increase is still lacking, because we have no measurements of the molecular concentrations with the aid of other methods, which prove that we are dealing with a true osmotic pressure in the sense of van't Hoff.

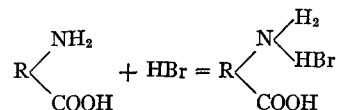
Pauli assumes that the ionized protein undergoes a stronger "hydration" than non-ionized protein and that this hydration explains the swelling of gelatin, as well as the apparent osmotic pressure, the latter being only a phenomenon similar to swelling.

¹ The reader is referred for the literature on the subject to Robertson, T. B., *The physical chemistry of the proteins*, New York, 1918.

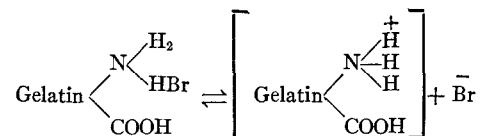
² Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 245.

It seemed to the writer that experiments on gelatin might permit us to satisfy the demand of Pauli; namely, to supply the molecular measurements necessary to show that the osmotic pressure and the other properties of gelatin solutions vary in proportion to the amount of acid combining with a given amount of gelatin. In two previous papers the writer has already shown that this is true for the influence of neutral salts on these properties of gelatin.³ Procter's⁴ experiments also indicate a purely stoichiometric basis for the influence of acids on the swelling of gelatin.

According to Werner⁵ amphoteric electrolytes are characterized by their ability to add H ions or OH ions and not by their ability to give off H and OH ions. It is generally assumed, and probably correctly, that when an acid like HBr combines with an amino-acid or a protein, the reaction occurs in an NH₂ group of the amino-acid or protein. According to Werner when NH₃ and HBr combine, the positively charged hydrogen ion of HBr is attached by a secondary valency to the N whose three negative charges now hold four positively charged H ions. No other positive ion except H can act in this way. The Br is able to dissociate as freely in the NH₄Br as in the free acid. The same assumption is to be made for the way an acid, *e.g.* HBr, combines with amino-acids or proteins.



Such a molecule, *e.g.* gelatin bromide, dissociates into a positively charged gelatin ion and a negatively charged Br ion, the H ion of the HBr now forming a part of the complex and positively charged gelatin ion.



³ Loeb, J., *J. Gen. Physiol.*, 1918, i, 39, 237.

⁴ Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv.

⁵ Werner, A., *Neuere Anschauungen auf dem Gebiete der anorganischen Chemie*, Braunschweig, 2nd edition, 1909.

Such a gelatin salt can only exchange the Br with the anion of a neutral salt and it is impossible for a complete molecule of a neutral salt like KCl to combine with the NH_2 group as has been assumed. The writer's experiments on the action of neutral salts on gelatin treated previously with acid are in harmony with the ideas of Werner and opposed to the assumption of a pentavalent N atom in the protein molecule capable of adding a whole molecule of a neutral salt. We do not know yet whether only one or more NH_2 groups in the gelatin molecule are able to bind a molecule of HBr.

Gelatin is an amphoteric electrolyte which at the isoelectric point (which for gelatin lies at $\text{pH} = 4.7$) is practically insoluble. When we prepare a gelatin solution and give it a hydrogen ion concentration of $2 \cdot 10^{-5}$ (*i.e.* $\text{pH} = 4.7$), the solution in less than 24 hours becomes opaque on cooling in as low a concentration as 0.25 per cent and probably at any concentration; except that the opacity due to the insolubility becomes too slight in very low concentrations to be noticeable. This explains why gelatin at the isoelectric point has practically no osmotic pressure, no swelling, a minimal conductivity, viscosity, etc.

When we treat isoelectric gelatin with a limited quantity of HBr of a low concentration a certain amount of gelatin is transformed into gelatin bromide, which is soluble and dissociates electrolytically. The higher the concentration of acid used the more gelatin bromide is formed and the more molecules of gelatin go into solution, until at a certain point all the insoluble gelatin molecules are converted into soluble gelatin bromide molecules. Since a 1 per cent gelatin bromide solution should possess the same degree of electrolytic dissociation as the HBr combined with it and since a 1 per cent gelatin bromide solution on account of the high molecular weight of gelatin must be considered as a very dilute solution, we shall commit no great error in assuming a complete electrolytic dissociation of the gelatin bromide. If it is true that the increase in osmotic pressure of gelatin under the influence of HBr is merely due to an increase in the number of soluble gelatin molecules, it must be possible to show that the osmotic pressure in this case increases approximately with the number of gelatin bromide molecules formed. This we intend to prove in the present paper.

While Pauli is right in stating that gelatin treated by acid is more highly ionized than common gelatin, I cannot agree with him that it is this ionization which causes the difference in the osmotic pressure of the gelatin at the isoelectric point and gelatin treated with acid. The increase in osmotic pressure is as our numerical results will show merely the expression of the increase in the number of particles in solution and there is no need or room for the assumption that the hydration or any other quality except the number of particles in solution has anything to do with this increase in pressure.

Since viscosity and swelling vary practically parallel with the osmotic pressure, these phenomena must also be a function of the number of particles or ions in solution.

Hardy⁶ has pointed out that the ionization of a protein increases its viscosity, and the increase of viscosity of gelatin with its ionization might then account for the parallelism between the curves for the bromine number and for the viscosity of the gelatin solution.

As far as a theory of swelling is concerned, the only one possessing any quantitative basis at present is Procter's.⁴

If we can, therefore, prove that under the influence of HBr the osmotic pressure of gelatin changes in proportion with the gelatin bromide formed, we have no further reason to question the purely chemical or stoichiometrical basis of the influence of acid upon all the physical properties of gelatin.

II. Measurements without Washing.

1 gm. of finely powdered gelatin is put for 30 minutes at 15°C. into each of a series of beakers containing 100 cc. of HBr of a different concentration, varying from $M/8$ to $M/8192$. As a control 1 gm. of gelatin is put for 30 minutes at 15°C. into 100 cc. of distilled water. The powdered gelatin is then poured into a cylindrical funnel and the acid allowed to drain off. The diameter of all the funnels being the same, the height of the gelatin gives a measure for the relative amount of swelling. Each gram of gelatin is then put into 100 cc. of HBr of the same concentration with which it had been treated before and is liquefied by heating to 50°C. Immediately after melting the time

⁶ Hardy, W. B., *J. Physiol.*, 1905-06, xxxiii, 251.

of outflow through an Ostwald viscometer is ascertained at a temperature of 24°C. By keeping temperature and time required for melting and time between completing this process and the viscosity measurement constant in each case comparable results are obtained.⁷ The time of outflow of distilled water through the viscometer was 55 seconds. The two curves of Fig. 1 give the values for swelling and viscosity, with the logarithms of the concentration of acid used as abscissæ. Under each acid is found the pH for the gelatin solution ascertained after the viscosity determination.

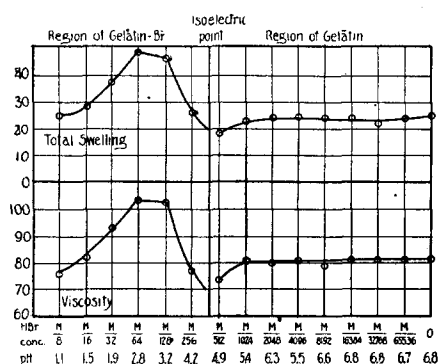


FIG. 1. Curves for viscosity and swelling of gelatin in the presence of various concentrations of HBr. Abscissæ, logarithms of concentration of HBr used. Under each concentration is found the pH of the solution. Isoelectric point and minimal values for curves at pH about 4.7. Both curves parallel, showing a maximum at pH between 2.8 and 3.2, and a drop when pH < 2.8.

The rest of the liquefied solution of gelatin in acid was then put into bags of collodion,⁸ to ascertain the osmotic pressure of the gelatin solution, the pressure being expressed in mm. of height of the column of 1 per cent gelatin solution in the glass tube inserted through the tightly fitting rubber stopper closing the bag of collodion. The

⁷ Loeb, *J. Biol. Chem.*, 1918, xxxiv, 395.

⁸ Lillie, R. S., *Am. J. Physiol.*, 1907-08, xx, 127. Loeb, *J. Biol. Chem.*, 1918, xxxv, 497. It is hardly necessary to state that these bags are freely permeable for HBr and that HBr produces no osmotic pressure when put in such bags. There is a slight rise of the column of liquid in the manometer at the beginning of the experiment which disappears completely in a few hours, while the rise due to the gelatin, for which the membrane is impermeable, is permanent.

outside liquid was in each case HBr of the same concentration as that in which the gelatin was dissolved. The whole experiment was carried on in a water bath of a temperature of 24°C. The curve at the top in Fig. 2 is the curve for the osmotic pressure observed after about 20 hours, at which time equilibrium was established.

The conductivity of the gelatin solution was then ascertained, as well as the amount of Br found in 25 cc. of 1 per cent gelatin solution. The curves are given in Fig. 2. A glance at Fig. 2 will show that the curve for osmotic pressure is *not* parallel to the curves for conductivity and for the Br number. It is, therefore, impossible to arrive from experiments of this type at a decision whether the influence of HBr (or other electrolytes) upon gelatin is of a stoichiometrical or of a colloidal character. Yet those familiar with the literature of this subject will remember that the conclusions of most colloid chemists are based on experiments in which the action of the electrolyte upon the protein was measured in the presence of an excess of electrolyte. The second fact which deserves attention becomes clear by a comparison of Figs. 1 and 2; namely, that the minima which appear in the two sets of curves lie at different acid concentrations: in Fig. 1 between $M/256$ and $M/512$, in Fig. 2 (for osmotic pressure) between $M/2048$ and $M/4096$. Comparing, however, the pH in the two sets of curves we notice that the minimum is at the same pH, namely about 4.7, which is the isoelectric point for gelatin. A good deal of the work on which the colloidal theory of the behavior of proteins rests was done without any measurements of pH and by plotting the effect against the concentration of the acid. It is no wonder that work with two such shortcomings in its method did not furnish any proof for the stoichiometrical character of the action of electrolytes on the physical properties of amphoteric colloids.

III. Effect of Washing.

Our method consists in removing the excess of HBr (or of any other electrolyte) after it has had a chance to act on the gelatin. 1 gm. of powdered gelatin is put again into each of a series of beakers for 30 minutes at about 15° or 20°C., each beaker containing 100 cc. of HBr of a different concentration. Then the gelatin is poured on a

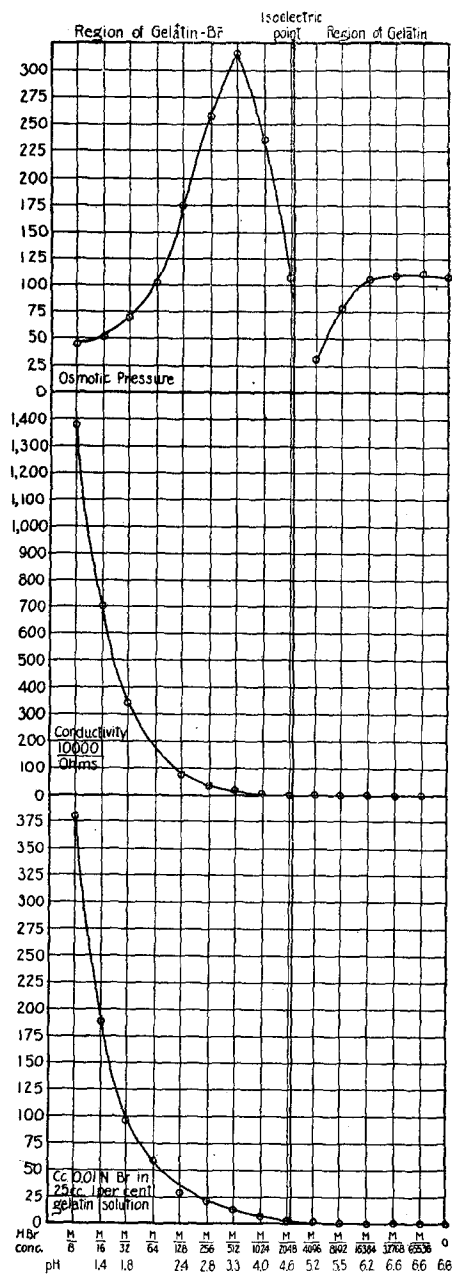
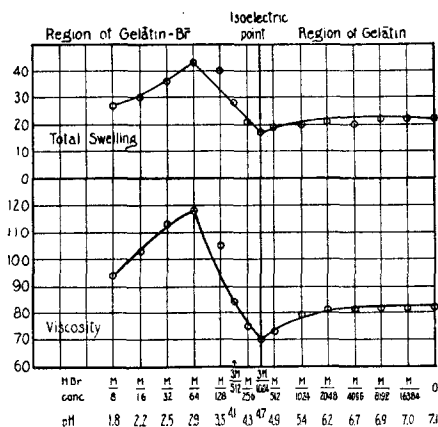


FIG. 2. Curves for bromine number (cc. 0.01 N Br in 25 cc. of 1 per cent gelatin solution), conductivity, and osmotic pressure of gelatin in presence of various concentrations of HBr. Abscissæ as in Fig. 1. No parallelism between curve for osmotic pressure and curves for bromine number and conductivity. Curve for osmotic pressure has minimum at pH about 4.6, maximum at pH = 3.3, and then drops. No conclusion can be drawn from such experiments as to the "colloidal" or true character of osmotic pressure. Osmotic pressure measured in height of column of 1 per cent gelatin solution.

filter and the acid allowed to drain off. The swelling is measured as described. From now on the method of procedure is different from that in the previously mentioned experiment. Instead of melting the gelatin in 100 cc. of the acid solution with which it had been treated, we melt it in 100 cc. of distilled water. The rest of the determinations—viscosity, osmotic pressure, conductivity, and titration for Br—are all made with such gelatin. Moreover, the osmotic pres-



FIGS. 3 and 4. Curves for viscosity and swelling (Fig. 3); bromine number, conductivity, and osmotic pressure (Fig. 4) of 1 per cent gelatin solution treated previously with different concentrations of HBr (abscissæ) the acid having been allowed to drain off. A 1 per cent solution of the gelatin in distilled water is then prepared, and the osmotic pressure of this gelatin is measured against distilled water, and conductivity and Br number are determined after 20 hours dialysis against distilled water. The curves for osmotic pressure, swelling, viscosity, and conductivity are parallel to the curve for bromine number from pH = 4.7 to pH = 2.9 or 3.3 respectively. The gelatin is free from bromine for pH \geq 4.7.

sure was measured against H_2O , thereby allowing more of the free acid not combined with the gelatin which had not drained off to diffuse out during the process. The result of this experiment is represented in Figs. 3 and 4. Fig. 4 contains the measurements for osmotic pressure and Br number, and the curves are almost parallel (with the exception of the value for the osmotic pressure for gelatin treated with $m/8$ acid). This parallelism is the missing link which allows us to decide in favor of the purely chemical and against the colloidal conception

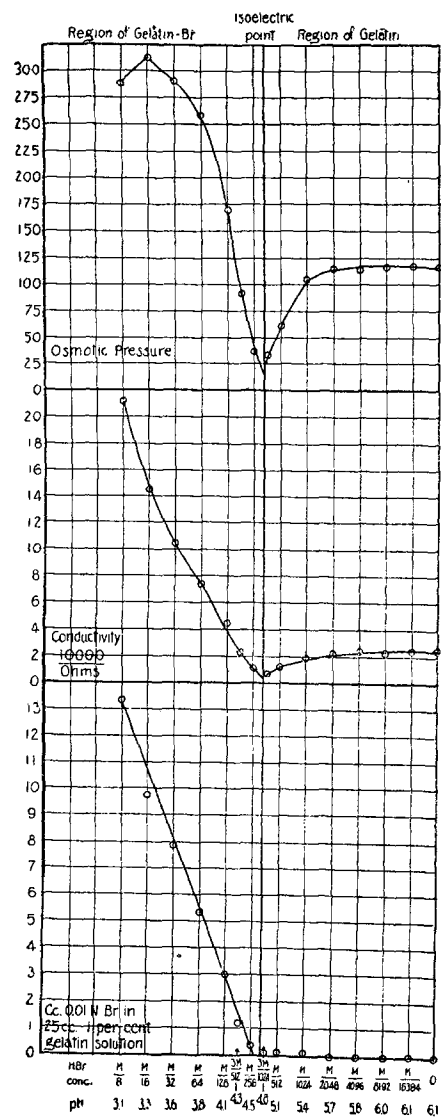


FIG. 4. See explanation under Fig. 3.

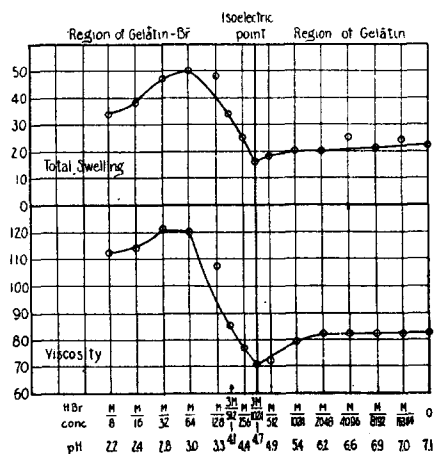
of the influence of electrolytes upon the osmotic pressure. Since the bromine number of gelatin increases parallel with the osmotic pressure (and also the viscosity and the swelling) of gelatin, these properties must depend upon the same variable; namely, the number of gelatin bromide molecules formed.

We are giving the values for the bromine number and for conductivities as actually found by analysis and measurements of resistance. These values demand, however, a correction owing to the fact that in all cases a certain amount of free HBr was present. The actually measured quantity for the bromine number is in each case the sum of the Br contained in the form of gelatin bromide and of the Br contained in the form of free HBr. The latter can be calculated from the pH. This value for the quantity of free HBr should be deducted from the Br numbers given in the curves. Since the correction thus required would be slight within the limits of pH from 4.7 to 3.6, we have omitted it in this paper. We may state, however, that the parallelism between the corrected curves for the Br number and those for osmotic pressure is even more perfect than that between our uncorrected curves for bromine number and the curves for osmotic pressure. The correction necessary for the conductivity curves can be found by measuring the resistance of a HBr solution for each pH in the same measuring cell (with fixed electrodes) which served for the measurements of the resistance of the gelatin bromine solutions. These corrections are greater than those required for the bromine number, especially for $\text{pH} < 3.9$. In order to obtain reliable values for conductivity we must use purified gelatin. Experiments of this kind will be reported in a subsequent paper. The conductivity measurements will not be considered in this paper.

All three curves for osmotic pressure, viscosity, and bromine number show a drop again after having reached a maximum. This drop exists in a still more pronounced way in the curves for viscosity and swelling than in that for osmotic pressure, because the gelatin contained more HBr before than after dialysis. This drop is of great theoretical significance because it shows free HBr is present in excess of the binding capacity of gelatin for HBr. The free HBr represses the ionization of gelatin bromide on account of the common Br ion and

this causes the drop in the curves for the osmotic pressure of the gelatin, since the free HBr, being able to diffuse through the collodion membrane, cannot cause any increase in osmotic pressure. The drop begins usually when pH becomes < 3.3 and the drop is the more considerable the more pH falls below this level.

The correctness of this view is proved by the fact that if we wash away the traces of free acid left in the capillary spaces between the particles of gelatin after the process of draining, by perfusing the



FIGS. 5 and 6. Same curves as in Figs. 3 and 4 except that the gelatin after the acid had been allowed to drain off was washed once with 25 cc. of H_2O . Parallelism between curves for Br number, conductivity, osmotic pressure (Fig. 6), viscosity, and swelling from pH = 4.7 to pH = 3.0. No Br found for pH ≤ 4.7 .

gelatin on the filter with 25 cc. of H_2O , and if we allow the water to drain off also before we make up the gelatin into a 1 per cent solution in distilled water, the drop will disappear, as is obvious from Figs. 5 and 6. In Fig. 6 the drop has disappeared, the pH going only to 3.3, and in these curves there is as complete a parallelism between the bromine number and the osmotic pressure of the gelatin solution as the strict validity of the theory of van't Hoff demands. We still observe the drop for the curves for viscosity and swelling, but the pH in these cases falls below 3.3; namely, to 2.2 (Fig. 5).

If instead of giving one washing we give four washings with 25 cc. of H_2O after the acid has drained off, we avoid the low values of pH completely and the drop in the curves for swelling disappears (Fig.

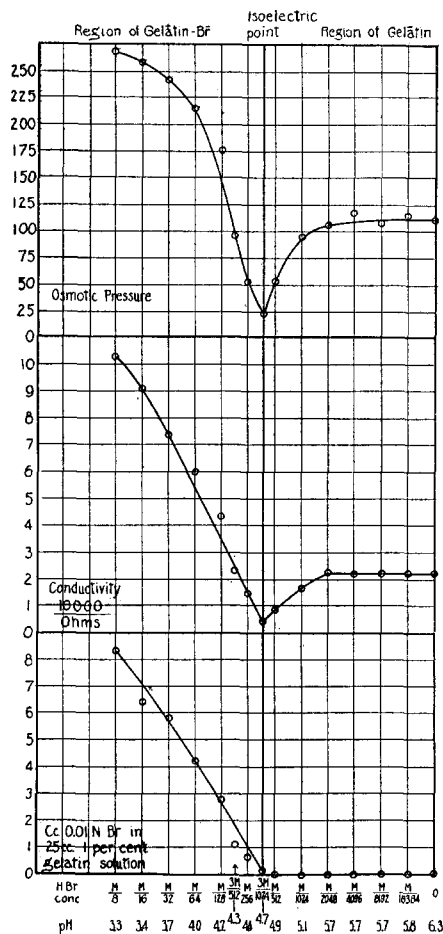


FIG. 6. See explanation under Fig. 5.

7). The parallelism between the curves for bromine number, for osmotic pressure and swelling is now practically complete.

The curves show distinctly that the independent variable is the bromine number. Thus in Fig. 7 this number was slightly in ex-

cess in the gelatin treated with $M/128$ and $M/64$ HBr. Corresponding abnormal values are found in the curves for conductivity, osmotic pressure, and swelling. The same is shown in Fig. 8. It

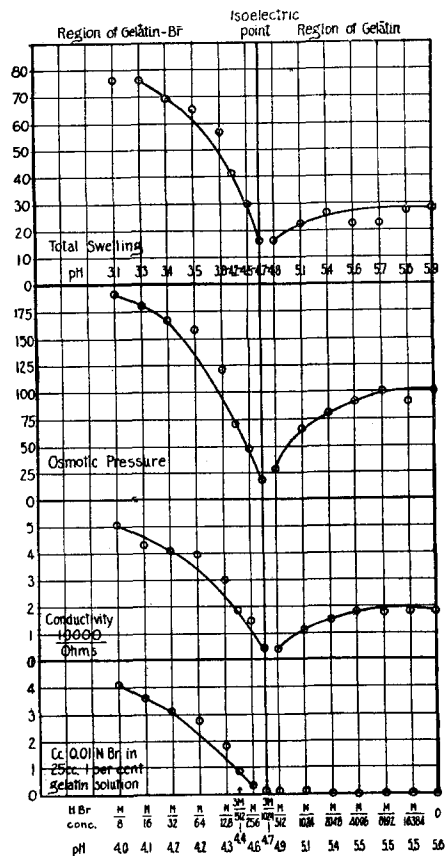


FIG. 7. Same curves as in preceding figures except that gelatin, after the acid treatment, had been washed four times with H_2O . Explanation as in preceding figures. Notice that curves are parallel and the gelatin is free from Br for $pH \geq 4.7$.

is, therefore, the Br number which determines the curves, *i.e.* the amount of gelatin bromide formed. Fig. 8 gives the curves for eight washings. Again the parallelism between the curves for the Br number and the other physical properties is obvious.

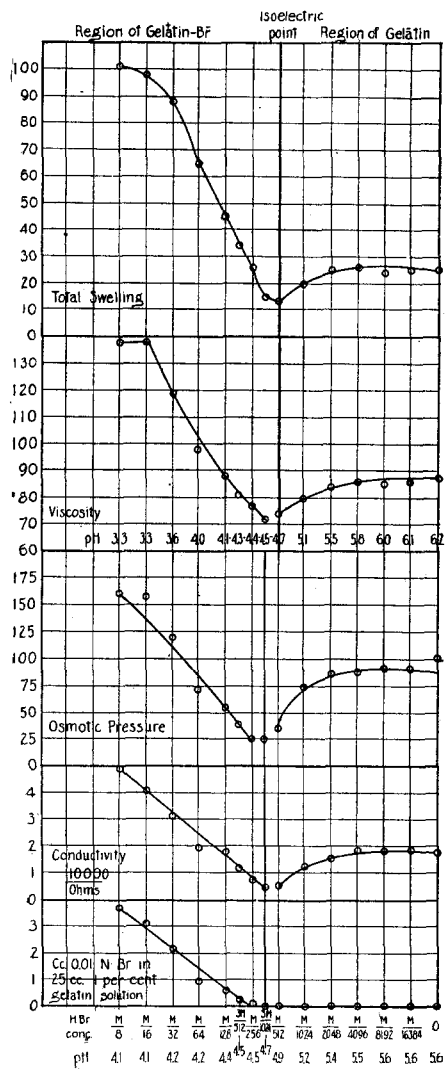


FIG. 8. Same curves as in preceding figures except that gelatin had been washed eight times with H₂O. Curves parallel and gelatin free from Br for pH $\bar{\bar{5}}$ 4.7.

These experiments furnish the proof that the effect of acid (HBr) upon the physical properties of gelatin is the unequivocal function of the amount of gelatin bromide formed; the probable reason for this being that pure gelatin (as it exists at the isoelectric point) is practically insoluble (and undissociated), while gelatin bromide is soluble.

We must now furnish the proof that not only is there a parallelism between the curve for the bromine number on the one hand and the curves for the physical properties of gelatin treated with HBr, but

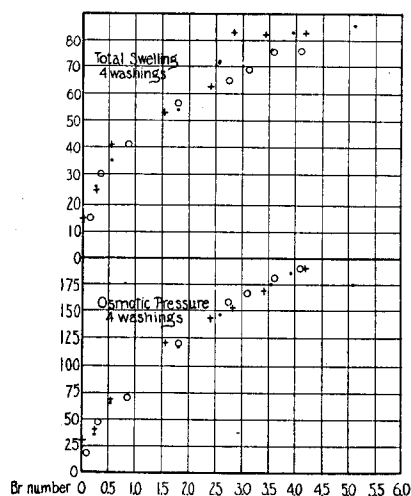


FIG. 9. The abscissæ represent the bromine number, the ordinates the values for osmotic pressure found in three different experiments with 1 per cent gelatin solution previously treated with HBr and washed four times with water. Notice agreement between values.

that to each definite Br number belongs a definite and absolute value for osmotic pressure, conductivity, swelling, and viscosity. We can do this by plotting the results of different experiments with the values for Br numbers as abscissæ and the values for osmotic pressure, swelling, etc., as ordinates. In this case the values obtained for osmotic pressure in the different experiments should differ only within the limits of the accuracy of our measurements.

In Fig. 9 the curves for three different experiments with four washings each are plotted for osmotic pressure and for swelling. The

variations lie within the limit of error. In the experiments plotted in Fig. 10 the number of washings of gelatin varied. In spite of the difference in the treatment we notice that for the same Br number practically the same value of osmotic pressure was found in all experiments. Since the curves for the other properties are parallel to the curve for osmotic pressure, it is not necessary to reproduce all the curves.

We therefore reach the conclusion that the variation of the physical properties of gelatin under the influence of HBr is an unequivocal function of the number of gelatin bromide molecules formed and that colloidal speculations not based on the laws of classical chemistry are neither needed nor warranted.

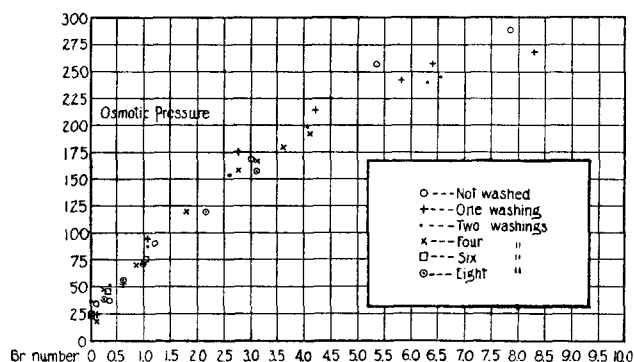


FIG. 10. Abscissæ represent bromine number, ordinates, values for osmotic pressure found in different experiments with 1 per cent gelatin solution previously treated with HBr and washed a different number of times.

IV. Titration of Gelatin with NaOH.

The following facts constitute an important link in the proof for a chemical theory of the action of electrolytes (in our case HBr) upon the physical properties of gelatin.

We notice that in all the curves the gelatin was found to be absolutely free from Br at the isoelectric point as well as on the alkaline side from the isoelectric point; *i.e.*, it was free from Br whenever $\text{pH} \approx 4.7$. Yet this gelatin had been treated with HBr. The fact that HBr cannot combine or remain in combination with gelatin for

$\text{pH} \approx 4.7$ is a further proof of the correctness of the purely chemical theory according to which for $\text{pH} > 4.7$ ionized gelatin can only exist as an anion, not capable of holding HBr in combination.

When we titrate 25 cc. of 1 per cent gelatin solution at the isoelectric point with 0.01 N NaOH, we find that it acts as an acid, requiring between 5.25 and 5.5 cc. 0.01 N NaOH for neutralization against phenolphthalein. (The number of cc. 0.01 N NaOH required to neutralize 25 cc. of 1 per cent gelatin we will call the NaOH number of gelatin.) When the pH of common gelatin, not treated with acid, is greater than 4.7 the NaOH number becomes less than 5.25 cc., probably on account of the fact that part of the gelatin exists as a metal gelatinate (probably chiefly Ca gelatinate) owing to ionogenic impurities remaining from the process of manufacture. Our previous papers have shown that at the isoelectric point gelatin is compelled to give off these ionogenic impurities.

On the more acid side from the isoelectric point the gelatin contains Br and the Br number increases with decreasing pH. With the exception of a small fraction this Br is held in combination with the gelatin as can be shown on the basis of titration with NaOH of the gelatin treated previously with HBr and possessing a $\text{pH} < 4.7$. In such a titration the gelatin solution whose $\text{pH} < 4.7$ is gradually rendered more alkaline through the addition of NaOH until finally its pH becomes equal to 4.7, and when that happens all the HBr held in combination with gelatin must be set free. As a consequence in a titration of gelatin bromide with NaOH two acids must be saturated with NaOH, the pure gelatin, and the HBr set free when during the process of titration the gelatin reaches its isoelectric point. It follows from this that the NaOH number found in this case must equal the sum of the Br number of the gelatin plus the NaOH number for gelatin at the isoelectric point; regardless of how the gelatin had been treated before and regardless of the pH for which this rule is tried out.

If we denote a given pH with n , the NaOH number at this $\text{pH} = n$ as " $(\text{NaOH})_n$," the NaOH number at the isoelectric point with "NaOH (isoelectric)," and the Br number at $\text{pH} = n$ with " Br_n ," then the following equation will hold: $(\text{NaOH})_n = \text{NaOH (isoelectric)} + \text{Br}_n$. In Table I, I have selected at random four experiments in which the

NaOH number and the Br number for different values of pH are given. It is easy to show that the equation holds within the limits of accuracy of our experiments. Thus the NaOH number for the isoelectric point is practically a constant in all experiments, namely between 5.25 and 5.5; and this value represents the binding capacity of "pure" gelatin for NaOH; or in other words, the binding capacity of 0.25 gm. of gelatin freed from ionogenic impurities is between 5.25 and 5.5 cc. 0.01 N NaOH, with phenolphthalein as indicator. Thus

TABLE I.

Experiment I. No washings, but made up in H ₂ O and dialyzed against H ₂ O.														
pH.....	3.1	3.3	3.6	3.8	4.1	4.3	4.5	4.8	5.1	5.4	5.7	5.8	6.0	6.1
Br number.	13.35	9.75	7.85	5.35	3.0	1.2	0.35	0.1	0.1	0.1	0	0	0	0
NaOH "	18.00	15.00	13.50	11.50	9.0	8.0	7.00	5.0	4.0	2.5	1.75	1.5	1.5	1.5
Experiment II. One washing.														
pH.....	3.3	3.4	3.7	4.0	4.2	4.3	4.4	4.7	4.9	5.1	5.7	5.7	5.8	6.3
Br number.	8.3	6.4	5.8	4.2	2.75	1.05	0.6	0.1	0	0	0	0	0	0
NaOH "	12.5	12.0	11.0	10.0	9.50	7.5	7.0	5.5	4.5	3.0	2.0	1.5	1.5	1.5
Experiment III. Four washings.														
pH.....	3.8	4.0	4.1	4.2	4.3	4.5	4.8	5.0	5.3	5.6	5.7	5.8	5.9	
Br number....	4.15	3.45	2.85	2.4	0.55	0.25	0	0	0	0	0	0	0	
NaOH "	9.00	8.50	8.00	8.0	7.0	6.00	5.5	4.0	2.5	2.25	1.5	1.5	1.5	
Experiment IV. Eight washings.														
pH.....	4.1	4.1	4.2	4.2	4.4	4.5	4.5	4.7	4.9	5.2	5.4	5.5	5.6	
Br number....	3.7	3.1	2.15	0.95	0.6	0.25	0.1	0	0	0	0	0	0	
NaOH "	8.0	8.0	7.0	6.5	6.5	6.00	5.5	5.25	4.5	3.25	2.5	2.5	2.0	

in Experiment III, for pH = 4.1, $(\text{NaOH})_n = 8.0$, NaOH (isoelectric) = 5.5, and $\text{Br}_n = 2.85$. $5.5 + 2.85 = 8.35$, while $(\text{NaOH})_n$ actually found is 8.0. Table II gives a comparison of the agreement in all the experiments.

In Table II we call the sum of NaOH (isoelectric) + Br_n the calculated and $(\text{NaOH})_n$ the observed value for a given pH. The table shows that the calculated and observed values agree within the limits of the degree of accuracy of the experiments.

We have now to make sure that the HBr measured in these titrations

is practically the HBr which was in combination with the gelatin, and not free HBr left in the capillary spaces between the particles of powdered gelatin after the treatment of the latter with HBr. The reader will remember that in Experiment I (Table I) the acid was allowed to drain off and that the gelatin solution was afterwards dialyzed for about 20 hours against distilled water. In the other experiments, in addition to this the last traces of acid were removed by one or more additional washings with distilled water. It is a striking fact that for $\text{pH} \leq 4.7$ the gelatin was always free from Br although it had been treated with HBr. This coincidence of the point where Br begins to appear in the gelatin with the value $\text{pH} > 4.7$ where it theo-

TABLE II.

Experiment I.			Experiment II (two washings).			Experiment III (four washings).			Experiment IV (eight washings).		
pH	(NaOH) _n		pH	(NaOH) _n		pH	(NaOH) _n		pH	(NaOH) _n	
	Calcu- lated.	Found.		Calcu- lated.	Found.		Calcu- lated.	Found.		Calcu- lated.	Found.
4.7	5.5		4.7	5.5		4.7	5.5		4.7	5.5	
4.5	5.85	7.0	4.4	6.1	7.0	4.5	5.75	6.0	4.5	5.35	5.5
4.3	6.7	8.0	4.2	8.25	9.5	4.2	7.9	8.0	4.5	5.5	6.0
4.1	8.5	9.0	4.0	9.7	10.0	4.1	8.3	8.0	4.4	5.85	6.5
3.8	10.8	11.5	3.7	11.3	11.0	4.0	9.0	8.5	4.2	6.2	6.5
3.6	13.3	13.5	3.4	11.9	12.0	3.8	9.6	9.0	4.2	7.4	7.0
3.3	15.2	15.0	3.3	13.8	12.5				4.1	8.3	8.0
3.1	18.8	18.0							4.1	8.9	8.0

retically should begin to appear speaks already against the assumption that the Br number is the expression of free HBr not drained or dialyzed or washed off.

The direct proof lies, however, in a comparison between the pH and the Br number. We select at random in Experiment III (Table I) $\text{pH} = 4.0$. The Br number found in 25 cc. of gelatin solution is for this pH 3.45 cc. 0.01 N Br. In the form of free acid this Br number would represent a hydrogen ion concentration of $\frac{3.45}{2,500}$ N, which is approximately $\frac{1}{700}$ N, while the actual normality of the gelatin solution was $\frac{N}{10,000}$; *i.e.*, less than one-tenth of $\frac{N}{700}$ (about 7 per cent). Hence more than 90 per cent of the HBr existed in chemical com-

bination with the gelatin and the small amount of free acid found was probably due to hydrolytic dissociation of gelatin bromide or to a trace of HBr not removed. This quantity of free acid is the correction of the value for the bromine number referred to in an earlier part of this paper. When, however, the free HBr is not washed off, as was the case in the experiment represented in Fig. 1, or when the free acid is only incompletely removed, the value $(\text{NaOH})_n$ will represent, of course, more or less free acid and in this case the discrepancy between $(\text{NaOH})_n$ and the pH found will be correspondingly smaller (see Fig. 1). This, however, manifests itself by the fact that a further rise in the Br number is no longer accompanied by a corresponding rise or is accompanied by a drop in the curves for osmotic pressure, swelling, and viscosity.

We may, therefore, consider it as proved that the bromine numbers given in this paper represent practically the HBr held in chemical combination by the gelatin with the exception of the small amount to be deducted owing to the presence of free HBr which can be calculated from the pH.

V. Theoretical Remarks.

Our experiments show that the influence of hydrobromic acid upon the physical properties of gelatin has a purely chemical or stoichiometrical basis. Gelatin and probably all proteins and amphoteric colloids behave as if they were merely amphoteric electrolytes capable of adding a H or OH ion.

Whether a protein adds an acid or a base depends on the hydrogen ion concentration; when the hydrogen ion concentration exceeds a critical point (which for gelatin is $C_H = 2.10^{-5}$), the gelatin will add acid; when it is lower it will add base. This critical hydrogen ion concentration is the isoelectric point. When gelatin is at the isoelectric point, it is free from ionogenic impurities and this "pure" gelatin is practically insoluble and hence can have no osmotic pressure, and, moreover, all the properties which depend upon its solubility are a minimum. When it is transformed into a salt by the addition of an acid (or a base) it becomes soluble, provided it is in combination with a monovalent ion, like Br or Na, etc. When pure gelatin is

transformed into a salt with monovalent anion or cation, all those properties which depend upon the number of gelatin molecules in solution increase with the proportion of gelatin salt formed, the maximum being reached when all the insoluble gelatin is transformed into soluble gelatin salt. This explains the parallelism between the curves for the bromine number of gelatin treated with HBr and the curves for the osmotic pressure of the solution. These molecular data must explain also the parallelism between the curves for viscosity and swelling with that of the bromine number.

The view taken in this paper that the osmotic pressure of protein solutions obeys the laws of classical physical chemistry is shared by one of the greatest authorities in this field, namely Sørensen.⁹ Sørensen worked on egg albumin of a well defined composition which necessitated the investigation of the osmotic pressure of gelatin in the presence of ammonium sulfate. In spite of the great theoretical and experimental difficulties, which only a master like Sørensen could succeed in overcoming, he arrived at constant values for the osmotic pressure and the molecular weight of egg albumin. He states:

"The properties of colloidal solutions can be most efficiently inquired into by application, as far as possible, of the same views and methods as those generally applied to true solutions. . . .¹⁰

"Colloidal chemistry in the shape which has been given it by its energetic champion Wo. Ostwald offers, no doubt, to protein study a system organized with great talent, but exact experimental investigation has not been able to keep up with the systematic treatment, and therefore the value of the contents does not always correspond with the perfection of the system itself. Thus I disagree with Ostwald, who . . . warns us against a comparison of the circumstances in colloidal and real solutions. In the case of albumin solutions, and doubtless also several other typical emulsoid systems, such a comparison between the properties of the colloidal solution and those of a real solution is of the greatest significance for the right understanding of the character of the colloidal solution. Indeed, I think it is even possible to go one step further and to say that the study of real solutions may derive considerable advantage from the results which an exact research of well-defined protein solutions can give, the colloidal character of these permitting the use of research-methods—I refer especially to the use of

⁹ Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

¹⁰ Sørensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 369.

semi-permeable membranes—, which, when dealing with real solutions, can only be used in exceptional cases and under especial circumstances.”¹¹

SUMMARY.

1. The method of removing the excess of hydrobromic acid after it has had a chance to react chemically with gelatin has permitted us to measure the amount of Br in combination with the gelatin. It is shown that the curves representing the amount of bromine bound by the gelatin are approximately parallel with the curves for the osmotic pressure, the viscosity, and swelling of the gelatin solution. This proves that the curves for osmotic pressure are an unequivocal function of the number of gelatin bromide molecules formed under the influence of the acid. The cc. of 0.01 N Br in combination with 0.25 gm. of gelatin we call the bromine number.

2. The explanation of this influence of the acid on the physical properties of gelatin is based on the fact that gelatin is an amphoteric electrolyte, which at its isoelectric point is but sparingly soluble in water, while its transformation into a salt with a univalent anion like gelatin Br makes it soluble. The curve for the bromine number thus becomes at the same time the numerical expression for the number of gelatin molecules rendered soluble, and hence the curve for osmotic pressure must of necessity be parallel to the curve for the bromine number.

3. Volumetric analysis shows that gelatin treated previously with HBr is free from Br at the isoelectric point as well as on the more alkaline side from the isoelectric point ($\text{pH} \approx 4.7$) of gelatin. This is in harmony with the fact that gelatin (like any other amphoteric electrolyte) can dissociate on the alkaline side of its isoelectric point only as an anion. On the more acid side from the isoelectric point gelatin is found to be in combination with Br and the Br number rises with the pH.

4. When we titrate gelatin, treated previously with HBr but possessing a $\text{pH} = 4.7$, with NaOH we find that 25 cc. of a 1 per cent solution of isoelectric gelatin require about 5.25 to 5.5 cc. of 0.01 N NaOH for neutralization (with phenolphthalein as an indicator).

¹¹ Sørensen, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 5-6.

This value which was found invariably is therefore a constant which we designate as "NaOH (isoelectric)." When we titrate 0.25 gm. of gelatin previously treated with HBr but possessing a $\text{pH} < 4.7$ more than 5.5 cc. of 0.01 N NaOH are required for neutralization. We will designate this value of NaOH as " $(\text{NaOH})_n$," where n represents the value of pH. If we designate the bromine number for the same pH as " Br_n " then we can show that the following equation is generally true: $(\text{NaOH})_n = \text{NaOH (isoelectric)} + \text{Br}_n$. In other words, titration with NaOH of gelatin (previously treated with HBr) and being on the acid side of its isoelectric point results in the neutralization of the pure gelatin (NaOH isoelectric) with NaOH and besides in the neutralization of the HBr in combination with the gelatin. This HBr is set free as soon as through the addition of the NaOH the pH of the gelatin solution becomes equal to 4.7.

5. A comparison between the pH values and the bromine numbers found shows that over 90 per cent of the bromine or HBr found was in our experiments in combination with the gelatin.