

THE OSMOTIC PRESSURE OF CONCENTRATED SOLUTIONS  
OF GELATIN IN EQUILIBRIUM WITH SOLUTIONS  
OF MAGNESIUM CHLORIDE

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I

INTRODUCTION

The osmotic pressures of dilute solutions of gelatin in equilibrium with dilute solutions of electrolytes have been discussed in detail by Loeb,<sup>1</sup> from the point of view of Donnan's theory of membrane equilibrium. In the present work, the equilibrium of concentrated solutions of gelatin and of magnesium chloride has been investigated and an attempt has been made to estimate the effects of some of the factors responsible for deviations from the laws for dilute solutions formulated by Loeb.

Theoretically, the effects of the unequal distribution of ions across a membrane should be small, and the osmotic pressure should be proportional to the "corrected" concentration in mols per litre of solvent, if the protein is equilibrated with pure water or a solution of a neutral salt in the region of the isoelectric point. Observations on gelatin dissolved in water,<sup>2</sup> sodium salicylate,<sup>3</sup> molar calcium chloride<sup>4</sup> as well as the observations recorded below agree with the equation  $p(V - b) = RT$  rather than the ideal solution law.

In this work, the thermodynamic formulae which have been pub-

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<sup>1</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, McGraw-Hill Book Co., Inc., New York, 1922.

<sup>2</sup> Kunitz, M., *J. Gen. Physiol.*, 1926-27, **10**, 811.

<sup>3</sup> Horne, E. V., *Biochem. J.*, 1924, **18**, 1107.

<sup>4</sup> Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925-28, **8**, 317.

lished recently (Adair<sup>5</sup>) have made it possible to test the validity of the hypothesis that the effects of the unequal distribution of ions are relatively small, and an attempt has been made to determine the relationship between the high osmotic pressures of concentrated solutions and the hydration of the protein.

## II

*Experimental Methods*

The manufacture of osmometers with rigid collodion membranes and methods for eliminating errors in osmometric measurements have been described in a previous paper.<sup>6</sup> The membranes used in this work were filled with gelatin-magnesium chloride solutions prepared from the approximately ash free gelatin supplied by the Eastman Kodak Company, and equilibrated for about 3 weeks in a room at approximately 22°C., with solutions containing from 4.0 to 9.0 equivalents of magnesium chloride per litre. In previous investigations (Adair<sup>5,6,7</sup>), buffer mixtures have been used rather than pure solutions of a single salt, but for the purposes of this work, it appears justifiable to simplify the system by the omission of the buffer mixtures because the observations of Horne<sup>3</sup> show that wide variations in hydrogen ion concentration have no measurable effect on the osmotic pressure of gelatin dissolved in concentrated salt solutions. On account of the viscosity of the solutions and other sources of error, the approximation to equilibrium is less exact than that obtained in previous work on haemoglobin,<sup>6,7,8</sup> but it is unlikely that the pressures recorded in Table I differ by more than 5 or 10 per cent from the thermodynamic equilibrium values, because the manometer readings returned to within a few per cent of their initial values if they were reset at different levels, and the pressures remained constant for 10 days or more in successful experiments.

At the end of the experiment, the density of the solution was measured and 10 cc. of the solution was diluted for the determination of the chloride content by a modification of Volhard's method in which the protein was destroyed by ashing with sodium peroxide. In a number of experiments the potential difference across the membranes was measured using saturated calomel electrodes. The protein concentration  $C$  in grams of dry protein per 100 cc. of protein solution has been calculated from Kjeldahl nitrogen determinations, assuming that 1 gm. of protein contains 0.179 gm. of nitrogen.

Provisional estimates of the corrected concentration  $c$  in grams of

<sup>5</sup> Adair, G. S., *Proc. Roy. Soc., A*, 1929, 126, 16.

<sup>6</sup> Adair, G. S., *Proc. Roy. Soc., A*, 1925, 108, 627.

<sup>7</sup> Adair, G. S., *Proc. Roy. Soc., A*, 1928, 120, 573.

<sup>8</sup> Adair, G. S., *Proc. Camb. Phil. Soc., Biol., Sci.*, 1923-25, 1, 75.

dry protein per 100 cc. of solvent have been made by the application of Formula 1.

$$c = \frac{C}{1 - .01 \alpha C} \quad (1)$$

$\alpha$  = approximately 0.7 cc. + 0.5 cc. = 1.2 cc., where 0.7 cc. is approximately equal to the volume of 1 gm. of dry gelatin, corrected for the contraction in volume on solution, and 0.5 cc. is a provisional estimate of the hydration (Moran<sup>9</sup>).

A number of different methods for the estimation of hydration are discussed below.

### III

#### *The Relationship between the Osmotic Pressure and the Concentration of Gelatin*

The observations recorded in Table I and shown graphically in Fig. 1 show that in the case of gelatin equilibrated with 4 normal and 9 normal solutions of magnesium chloride, the osmotic pressure increases more rapidly than the concentration, even if it is corrected for the volume of the protein hydrate by the application of Formula 1. Over the range of pressures greater than 12 mm. but less than 120 mm., the observations can be represented by the simple formula with two empirical constants which has been applied to haemoglobin<sup>7,8</sup>):

$$p = \frac{K_m C}{1 - .01 K_b C} = \frac{K_m c}{1 - .01 K'_b c} \quad (2)$$

$C$  = grams protein per 100 cc. solution.

$c$  = grams protein per 100 cc. solvent.

In the case of gelatin at 22°C.,  $K_m = 6.54$ ,  $K_b = 10.0$  and  $K'_b = 8.8$ . In the case of haemoglobin at 0°C.,  $K_m$  is 2.72 and  $K_b$  is 2.42. The molecular weight of the protein, or the average size of the particles if the protein is a mixture, can be calculated from the coefficient  $K_m$  if the formula applies to dilute solutions, as shown in a previous paper;<sup>7</sup> unfortunately the majority of our experiments with dilute solutions

<sup>9</sup> Moran, T., *Proc. Roy. Soc., A*, 1926, 112, 30.

of gelatin failed to satisfy the strict criteria for osmotic equilibrium given in a previous paper,<sup>6</sup> and until further data are available it is not advisable to express any opinion concerning the molecular weight of gelatin or its relationship with  $K_m$ . Earlier work on the molecular weight of gelatin has been discussed by Jordan Lloyd,<sup>10</sup> and additional evidence has been given by Eggert and Reitsstötter,<sup>11</sup> Adair,<sup>8</sup> and Kunitz.<sup>2</sup>

TABLE I  
*Osmotic Pressures of Gelatin Solutions in Equilibrium with Magnesium Chloride Solutions at 22°C.*

[Cl]'' Gram equiv. MgCl <sub>2</sub> per litre of outer fluid	[Cl]' Gram equiv. Cl per litre of gelatin solution	C Grams of dry gelatin per 100 cc. solution	c Grams of dry gelatin per 100 cc. solvent (pro- visional estimates)	p Observed osmotic pressure in mm. of mercury	$\frac{p}{c}$
3.91	3.93	5.23	5.6	54.9	9.8
3.94	3.71	7.20	7.9	116.9	14.8
4.03	3.76	7.28	8.0	123.5	15.4
6.14	5.68	6.95	7.6	124.3	16.4
6.30	5.86	7.18	7.9	130.7	16.6
9.16	9.12	1.10	1.11	5.4	4.9
8.5		2.07	2.1	17.6	8.3
8.5		2.22	2.3	17.8	7.8
8.57	8.18	5.27	5.6	73.0	13.0
8.96	8.53	5.75	6.2	86.2	14.0
9.03	8.56	6.38	6.9	118.9	17.2
9.10	8.50	6.55	7.1	124.2	17.5
8.95	8.55	9.37	10.6	278.1	26.4
8.99	8.45	9.96	11.3	306.1	27.1

The constant  $K_b$  is a volume correction term comparable with "b" in van der Waals' equation  $p(v-b) = RT$ . It is interesting to observe that gelatin resembles haemoglobin in that  $K_b$  diminishes at pressures exceeding 120 mm. The data in Table I indicate that  $K_b = 8.33$  cc. at a pressure of 278 mm. and 7.92 at 306.1 mm. Prof. Wolfgang

<sup>10</sup> Jordan Lloyd, D., *Biochem. J.*, 1920, 14, 147.

<sup>11</sup> Eggert, J., and Reitsstötter, J., *Z. physik. Chem.*, 1926, 123, 363.

Ostwald (personal communication) has found a comparable diminution in van der Waals' "b" term for a number of solutions, crystalloidal as well as colloidal. Ostwald and Müндler<sup>12</sup> have obtained a formula which is applicable to a wider range of pressures than the simpler equation of van der Waals.

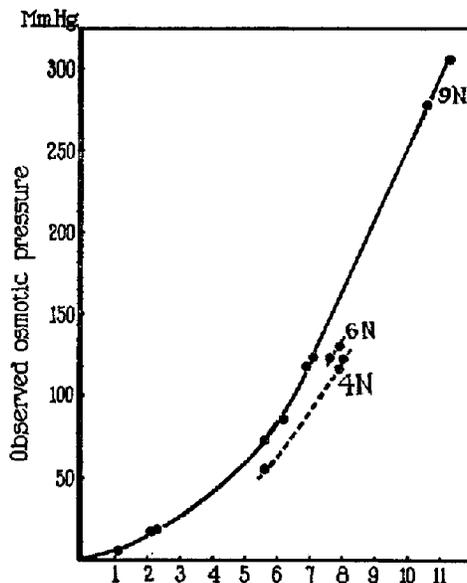


FIG. 1. Concentration of gelatin in grams per 100 cc. of solvent calculated by Formula 1.

#### IV

#### *The Relationship between the High Osmotic Pressures of Concentrated Solutions of Gelatin and the Membrane Equilibrium of Diffusible Ions*

There are at least three different factors which might account for the relatively high pressures observed in concentrated solutions of gelatin, namely, an unequal distribution of diffusible ions, the hydration of the protein, and the effect represented by the term "b" in van

<sup>12</sup> Ostwald, Wo., and Müндler, K., *Kolloid Z.*, 1919, 24, 7.

der Waals' equation, and other deviations from the ideal solution laws.

Sørensen<sup>13</sup> has pointed out that if Donnan's formulae apply to concentrated solutions of electrolytes in equilibrium with approximately isoelectric proteins, the pressure due to the unequal distribution of ions should be small. He drew attention to the uncertainty of some of the assumptions on which this conclusion is based. A formula based on different assumptions which allows for deviations from the ideal solution laws is given below (Adair<sup>5</sup>).

$$p_i = RT \int_0^u m_p n_p du \quad (3)$$

$p_i$  = the pressure due to the unequal distribution of diffusible ions.

$m_p$  = gram mols of protein per litre of solvent.

$n_p$  = the mean valence of the protein ions (due to the ionization of the protein or the adsorption of other ions).

$u$  =  $E \times F/RT$ , where  $E$  is the membrane potential measured under conditions where the concentration of the protein is varied and the composition, temperature and pressure of the solution of crystalloids outside the membrane are constant.

It appears probable that when the membrane potential is less than 1 millivolt,  $m_p n_p$  is approximately equal to  $uJ$ , where  $J$  is the sum of the concentrations of the ions in the outer fluid, each multiplied by the square of its valence. In a solution of  $MgCl_2$  containing 9 equivalents per litre,  $J = 9 + 4.5 \times 2^2 = 27$ .

In most of the experiments referred to in Table I, the membrane potentials were so small that they could not be measured with the instruments available, but in the two experiments with approximately 9.3 per cent and 9.9 per cent of gelatin the value of  $E$  appeared to be about  $-0.05$  millivolt. By substitution in Formula 1, it was found that the term  $p_i$  did not exceed 2 mm. within the range of concentrations referred to in Table I, and therefore the high osmotic pressures cannot be attributed to an unequal adsorption of ions.

This conclusion does not prove that the osmotic pressure of the protein is unaffected by an adsorption of magnesium and chloride ions in equivalent proportions, because the forces of attraction or repul-

<sup>13</sup> Sørensen, S. P. L., *Compt. rend. Laboratoire Carlsberg*, 1917, 12, 295.

sion between protein molecules might be altered by the adsorption of a neutral salt. Thermodynamical calculations of the effects of adsorption have been made by Polanyi<sup>14</sup> and by Christiansen.<sup>15</sup> The similarity of Kunitz' curves<sup>2</sup> for gelatin dissolved in water and the data for gelatin dissolved in concentrated solutions of magnesium chloride given above seems to indicate that the deviation from the ideal solution laws is mainly due to factors other than adsorption.

## v

*The Hydration of the Protein*

The relationship between the high osmotic pressures of concentrated solutions of proteins and their hydration may be considered from two different points of view, represented by Formulae 4 and 5:

$$p(V - v_p) = RT \quad (4)$$

$$p(V - \beta v_p) = RT \quad (5)$$

$p$  = the osmotic pressure of a solution of an isoelectric protein.

$V$  = the volume of solution per mol of protein.

$v_p$  = the volume of 1 mol of protein hydrate.

$\beta$  = a coefficient greater than unity, which may diminish as the protein concentration is increased.

Formula 4 is a restatement of the equation derived by Callendar<sup>16</sup> and other workers on the assumption that hydration affects osmotic pressure by altering the molar fraction of the solvent. The second formula is obtained on the assumption that there may be an analogy between the volume correction term and the "b" of van der Waals' equation for gases. It is known that van der Waals' "b" is larger than the volume of the molecules.

The arguments in favour of the simpler Formula 4 have been presented by Kunitz.<sup>2</sup> He finds that if the volume of the protein hydrate  $\phi$  is calculated from the relative viscosity  $\eta$  by his empirical formula  $\eta = (1 + 0.5\phi)/(1 - \phi)$ ,<sup>4</sup> Formula 4 is applicable to aqueous solutions of gelatin at 35°C. According to the viscosity formula, the hydration

<sup>14</sup> Polanyi, M., *Z. physik. Chem.*, 1925, 114, 387.

<sup>15</sup> Christiansen, J. A., *Compt. rend. Laboratoire Carlsberg*, 1928, 17, No. 6.

<sup>16</sup> Callendar, H. L., *Proc. Roy. Soc. A*, 1907-08, 80, 466.

varies from 7 cc. of water per gram of gelatin in a 1 per cent solution to 4.5 cc. of water in a 9.5 per cent solution.

The arguments against the simple Formula 4 are given below:

1. Kunitz' empirical formula for the viscosity of solutions is based on observations on sugar, and it is by no means certain that the effective volume of sugar in solution is the same as its volume in the dry state. Porter<sup>17</sup> and Callendar<sup>18</sup> have come to the conclusion that sugar is hydrated in solution.

2. The effects of the shape of the molecules on the viscosity has not been estimated. If the gelatin molecule consists of long branching chains of amino acids, its effect in increasing the viscosity may be much greater than that which would be caused by spherical molecules of the same volume.

3. The apparent hydration of gelatin calculated by the viscosity formula or by Formula 4 tends to diminish as the protein concentration is increased, although the change in the activity of water caused by the protein is relatively small. The formulae of Lewis and Randall show that an osmotic pressure of 306.1 mm. is correlated with a diminution of 0.038 per cent in the activity of water. If the degree of hydration is determined by the adsorption equation of Freundlich or the formula based on chemical considerations due to Langmuir,<sup>18</sup> the alteration in the degree of hydration should be less than 0.038 per cent, whereas the changes in the apparent hydration calculated from viscosity measurements may be 50 per cent or more.

4. The apparent hydration estimated by the viscosity formula or by the simple osmotic pressure Formula 4 may be from 5 cc. to 10 cc. of water per gram of dry gelatin, whereas the hydration estimated by Moran<sup>9</sup> from the concentration of a gelatin gel in equilibrium with ice at  $-20^{\circ}\text{C}$ . or lower is about 0.53 cc. per gram of dry protein. Moran's conclusion that the hydration of gelatin is relatively small is in agreement with the chloride distribution measurements recorded in Table I. As a first approximation, the chloride distribution may be correlated with the volume of the isoelectric protein hydrate by an equation of the form stated below.

<sup>17</sup> Porter, A. W., *Trans. Faraday Soc.*, 1917, **13**, 123.

<sup>18</sup> Langmuir, I., *J. Am. Chem. Soc.*, 1916, **38**, 2221.

$$\alpha = \frac{1}{x} \left( 1 - \frac{[\text{Cl}]'}{[\text{Cl}]''} \right) + \frac{b}{[\text{Cl}]''} \quad (6)$$

$\alpha = v_p/M$ , where  $v_p$  = the volume in cubic centimeters of 1 mol of protein hydrate.

$M$  = grams of dry protein per mol of protein hydrate.

$x$  = grams of dry protein per cubic centimeter of solution.

$[\text{Cl}]'$  = gram mols of chloride ion per litre of protein solution.

$[\text{Cl}]''$  = gram mols of chloride ion per litre of "outer fluid."

$b$  = gram mols of chloride ions combined with 1000 grams of protein.

If we assume that  $b$  is not greater than 1.2 mols, the maximum chloride combining capacity given by Cohn,<sup>19</sup> the value of  $\alpha$  given by Formula 6 is from 0.8 to 1.3 cc. The preliminary measurements recorded in Table I are not accurate enough for an exact estimation of  $\alpha$ , but it is obvious that they are consistent with the low values obtained by Moran rather than the high values calculated by Kunitz from viscosity measurements.

Further work is necessary before we can arrive at a final decision concerning the hydration of gelatin, but on the whole the evidence now available indicates that the term  $\phi$  in Kunitz' viscosity formula is different from and probably greater than the volume of the protein hydrate.

Since the effects of the unequal distribution of ions and the effects of hydration estimated by the methods which seem most reliable are insufficient to account for the high osmotic pressures of concentrated solutions of gelatin, it seems necessary to assume that there are inter-ionic or inter-molecular forces which become important in concentrated solutions. From the theoretical point of view this conclusion is not improbable because the correction term " $b$ " in van der Waals' equation is larger than the volume of the gas molecules. A similar observation has been made in the case of solutions of haemoglobin, where the term " $b$ " in the osmotic equation,  $p(V - b) = RT$ , appears to be considerably larger than the volume of the protein hydrate.

Experimental evidence bearing on this problem has been published in a previous paper,<sup>7</sup> and further evidence is given in the following section.

<sup>19</sup> Cohn, E. J., *Physiol. Rev.*, 1925, 5, 349.

## VI

*The Relationship between Hydration and the Density of a Protein*

If a protein is hydrated, the difference between the density ( $D'$ ) of the protein solution and the density ( $D''$ ) of the solution of crystalloids

TABLE II  
*Densities of Haemoglobin Solutions at Approximately 17°C.*

Expt. No.	Outer fluid. Composition	Outer fluid. Density	Inner fluid. Density	$\alpha$	$\frac{D' - D''}{\alpha}$
246	NaCl† 0.15 N	1.005	1.056	0.204	0.250
245	NaCl 0.5 N	1.020	1.065	0.195	0.232
259	NaCl 2.0 N	1.080	1.116	0.197	0.183
243	NaCl 4.0 N	1.152	1.172	0.191	0.107
251	KCl 0.025 N	1.003	1.050	0.190	0.250
250	KCl 0.1 N	1.013	1.060	0.194	0.242
260	KCl 2.0 N	1.019	1.124	0.196	0.168
	Dextrose 1.0 N	1.072	1.107	0.182	0.191
	Dextrose 2.0 N	1.147	1.170	0.187	0.123

† The solutions with NaCl are buffered by 0.025 molar  $\text{NaHCO}_3$  at pH 8.3. The solutions with KCl are buffered with phosphates at pH 7.9. The dextrose solutions are unbuffered.

TABLE III  
*Osmotic Pressures of Haemoglobin in Equilibrium with  $\alpha/10$   
KCl buffered with 0.0613 molar  $\text{Na}_2\text{HPO}_4$  + 0.00533 molar  $\text{KH}_2\text{PO}_4$*

Expt. No.	Grams Hb per 100 cc. solution	Litres of solution per gram mol	Pressure $p_p$ in mm. Hg at 0°C.	" $b$ "	$\alpha'$
300	8.12	823.0	27.8	143	2.1
298	24.00	279.0	140.9	158	2.4
299	34.41	194.0	332.6	211	3.2

" $b$ " = volume correction in litres, calculated by the equation  $p_p(V-b) = RT$ .

$$\alpha' \text{ (the apparent value of } \alpha) = \frac{b \times 1000}{66,800}$$

in equilibrium with the protein solution must be affected by the amount of water combined with the protein. In the ideal case of an isoelectric protein which has no effect on the activity coefficients of the crystal-

loids, the relationship can be expressed in the simple form given below.

$$\frac{D' - D''}{x} = 1 + W - \alpha D'' \quad (7)$$

$x$  = grams of dry protein per cubic centimeter of solution.

$W$  = grams of water of hydration per gram of dry protein.

$\alpha$  = volume in cubic centimeters of protein hydrate per gram of dry protein.

Table II gives a number of observations of the densities of solutions of haemoglobin. The numbers of the experiments have been recorded in order to facilitate reference to the tables given in a previous paper (Adair<sup>7</sup>), which give osmotic pressures and other data not recorded in Table II. The observations in Table II can be represented by Formula 7 with an error not exceeding 0.1, if  $\alpha$  is equal to 0.963 cc. and  $W = 0.22$  cc. of water per gram of protein. These figures may require correction when we have more data concerning the effect of proteins on the activities of crystalloids, but it is interesting to notice that  $\alpha$  calculated from density determinations with different solvents is approximately equal to the value 0.965 calculated from chloride distribution measurements recorded in a previous paper<sup>7</sup> and is much less than the apparent values ( $\alpha'$ ) calculated from osmotic pressure measurements given in Table III by the formula  $p(V - v_p) = RT$ . In addition to the 3 experiments recorded in Table III, 15 similar experiments are recorded in tables numbered 10 and I in previous papers (Adair<sup>5,7</sup>). The values of  $\alpha'$  calculated from the earlier less accurate experiments such as those numbered 200-205 are not very different from the figures recorded in Table III.

#### SUMMARY

The osmotic pressures and the membrane equilibrium of chloride ions have been determined for solutions of gelatin in equilibrium with solutions of magnesium chloride containing from 4.0 to 9.0 equivalents per litre.

The pressures increase more rapidly than the concentration, an effect represented by a high value of the term " $b$ " in van der Waals' equation  $p(V - b) = RT$ . Calculations made by a thermodynamical formula which makes allowances for deviations from the ideal solution

laws show that the high value of " $b$ " is not due to an unequal distribution of diffusible ions.

The theory that the high values of the hydration estimated from viscosity formulae account for the magnitude of " $b$ " has been examined and the conclusion has been reached that the term " $b$ " for gelatin as well as for haemoglobin is considerably larger than the volume of the protein hydrate.