CCXII. THE ANALYSIS OF THE OSMOTIC PRESSURES OF THE SERUM PROTEINS, AND THE MOLECULAR WEIGHTS OF ALBUMINS AND GLOBULINS.

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I. *Introduction.*

The determinations of the molecular weights of serum albumin and globulin made by Sørensen [1925], Adair [1926] and Svedberg and Sjögren [1928] show a considerable range of variation which Svedberg has attributed to the instability of these proteins. In the work recorded in this paper, methods for eliminating errors due to instability of proteins have been investigated, and comparisons have been made of molecular weights of serum albumin and globulin from different sources, purified by different methods. The values of the molecular weights given by Sørensen and by Adair were obtained by osmometric methods, which have also been used in this work.

If the protein be equilibrated with a salt solution at the physiological hydrogen ion concentration, a part of the observed osmotic pressure, symbolised p_i, may be attributed to the unequal distribution of the diffusible ions. In ideal solutions p_i is determined by the formulae of Donnan [1911]. It has been suggested that these formulae can be applied to the serum proteins [Hecht, 1925] but the effects of slight deviations from the ideal solution laws are exaggerated in the calculation of p_i, which represents a small difference between two large pressures.

Experiments recorded in this paper show that the protein systems are beyond the range of application of Donnan's formulae. The calculations of p_i given below have been made by a thermodynamical formula derived by Adair [1929, 2]: The partial osmotic pressure of the protein ions is equal to the observed osmotic pressure minus the pressure, p_i, due to diffusible ions.

II. *Methods.*

The methods of preparation of the proteins from horse-serum have been described in detail in a previous paper [Adair and Robinson, 1930]. Serum proteins from three different sources have been examined, and comparisons

have been made between preparations of albumin crystallised once, twice and four times by a modification of the method of Hopkins [1900]. In order to prevent decomposition, the proteins were kept at 0° throughout the preparation, except during the crystallisation of albumin, and during the process of centrifuging.

The electrolyte content of the protein solutions was fixed by dialysis in collodion membranes against standard phosphate buffers, a method that is less likely to cause changes in the protein than the alternative procedure in which the protein is first dialysed against water until free from electrolytes and then mixed with the salts required in the final osmometric experiment.

The measurements of osmotic pressure were made with collodion membranes fixed to manometers as described by Adair [1925]. The osmometers were kept in a room at 0° for 15 days or more, the pressures being observed daily. After equilibrium had been established for a number of days, the osmometers were dismantled and determinations of the capillarity correction, the refractive index, the density and the nitrogen content were made on the protein solution. The refractive index and the density of the dialysate were also measured.

The protein concentrations have been stated in terms of g. of dry protein per 100 cc. of solution. In the majority of cases, the protein concentration was calculated from Kjeldahl nitrogen determinations. The factors necessary for this calculation have been redetermined on specially purified material [Adair and Robinson, 1930] and it was found that crystalline horse-serum albumin dried at 110° contained 15·60 % N. The nitrogen content of uncrystallised albumin of horse-serum and that of the albumin of ox- and sheep-serum has not yet been determined; the factor $\frac{100}{15{\cdot}6}$ has been used provisionally for the calculations of the concentrations of these proteins. The nitrogen content of total globulin of horse-serum has been redetermined as 15·13 %. This figure has been used in provisional calculations of the concentration of ox- and sheep-serum globulin.

In a few experiments, the protein concentration was determined by the refractometric method, as described in detail in a previous paper [Adair and Robinson, 1930].

In certain cases, the measurements of osmotic pressure were supplemented by determinations of the membrane potential made with a slightly modified form of the apparatus described by Loeb [1922] in which saturated calomel electrodes are dipped into the protein solution and into the dialysate.

The distribution of ions across the membrane was studied in three experiments in which serum albumin was equilibrated with an ammonium phosphate buffer of p_H 7·3. Ammonia was estimated colorimetrically with Nessler's reagent and phosphates were estimated by the method of Bell and Doisy [1920].

III. *The stability of the serum proteins.*

According to the observations of Svedberg and Sjögren [1928], serum proteins are unstable substances, easily decomposed during the process of purification. In this work, three methods for testing the stability of the proteins have been employed: firstly, the degree of constancy of their osmotic pressures has been observed for periods from 12 days to 3 months; secondly, the dialysis fluids surrounding the membranes have been tested for protein derivatives; thirdly, comparisons have been made of the molecular weights of preparations from different sources, and purified by different methods.

The first test was applied to all the osmometric experiments. In the few cases where the condition of constancy of osmotic pressure was not fulfilled, the cause was attributable to imperfections of the membrane. A rapid fall of osmotic pressure indicated that the membrane was permeable by protein, and in such cases protein could be demonstrated in the dialysis fluid. A gradual fall of pressure was caused in a few cases by absorption of the protein by the membrane, as shown by the reduction in concentration of the protein solution. Typical osmometric readings, yielding constant pressures, are recorded in Table I.

Table I. *Stability of albumin and globulin.*

Osmometer readings in cm. of solution at 1·0°.

Exp. HA 20. Horse-serum albumin. Once crystallised		Exp. HA 31. Horse-serum albumin. 4 times crystallised		Exp. G 19. Horse-serum globulin.	
Date 1929	Osmometer reading	Date 1929	Osmometer reading	Date 1929	Osmometer reading
Aug. 9	26·30	Oct. 31	16·40	Nov. 1	10·58
10	19·80	Nov. 1	14·82	2	7·82
11	18·10	2	14·60	3	7·20
12	17·20	3	14·50	4	7·08
13	16·97	4	14·47	5	7·05
14	16·90	5	14·44	6	7·02
15	16·82	7	14·41	7	7·00
	Reset		Reset		Reset
15	20·25	7	12·92	7	4·80
17	17·20	8	14·09	8	6·49
18	17·05	9	14·40	9	6·85
19	17·00	10	14·42	10	6·91
21	16·90	12	14·45	14	7·02
22	16·80	13	14·43	28	7·02
23	16·80	14	14·43		
24	16·80	15	14·43		

In the second test, positive results for the biuret, the xanthoproteic, the ninhydrin and Millon's reactions were obtained only in the few cases in which the osmometer readings indicated that the membranes were permeable by protein. In all the remaining experiments, completely negative results were obtained with these tests, for once crystallised and for four times crystallised albumin, and also for globulin. These negative results in the tests for protein derivatives are of significance, because they indicate that our preparations of four times crystallised albumin did not give rise to the decomposition products

of low molecular weight referred to by Svedberg and Sjögren [1928]. If these derivatives were present, it is probable that they would diffuse across the membranes. If they were not diffusible, their presence would be manifested by discrepancies between the osmotic pressures of the once and the four times crystallised albumin.

The results obtained in the third test, given in detail in Section V, show that the molecular weight of four times crystallised horse-serum albumin is the same as that obtained for once crystallised material. There was furthermore a close agreement between the molecular weight of the albumin prepared from horse-serum obtained from the University Field Laboratories and from Messrs Burroughs and Wellcome. Horse-blood obtained from a knacker gave an albumin with a molecular weight of 67,000 instead of 72,000, but in this case, it was observed that the protein was contaminated with a coloured impurity, even after three recrystallisations.

The results of all three tests show that the stability of these preparations of albumin and globulin appears to be distinctly greater than that of the proteins as prepared by Svedberg and Sjögren. These authors state that in dilute solution serum albumin is decomposed reversibly into particles of low molecular weight. In a truly reversible reaction, which should be governed by the law of mass action, the amount of protein decomposed in unit time should be proportional to the protein concentration. Svedberg's experiments indicate that 29 % of the protein is decomposed in 0·25 % solution, whereas no decomposition takes place in 1·25 % solution. These figures are inconsistent with the law of mass action, and suggest that the decomposition is due to some external factor.

IV. *Methods for the estimation of the molecular weights of serum proteins in non-ideal solutions.*

In calculations of the molecular weight of a protein from measurements of osmotic pressure made with membranes of collodion and similar substances, there are at least three factors which must be considered, the "ion pressure difference," p_i, due to the excess of diffusible ions inside the membrane, the effects of the volume of the protein hydrate and the effects of attractive and repulsive forces between the molecules represented by the osmotic coefficient g_p in formula (1)

$$p - p_i = p_p = RTg_p m_p \qquad \text{.....(1),}$$

p = the observed osmotic pressure, p_i = the ion pressure difference, p_p = the partial osmotic pressure of the protein ions, m_p = g.-mols. of protein per litre of "solvent," calculated by deducting the volume of the protein hydrate from the volume of the solution.

If the molecular weight of the protein is constant, that is, if the degree of polymerisation is unaffected by changes in concentration, and if the effects of the ion pressure difference and of interionic forces are not too great, it is possible to apply the method of extrapolation described below, whereby the

effects of all the three factors are eliminated simultaneously. The conditions under which this method gives valid results have been studied in previous investigations on haemoglobin [Adair, 1924, 1928], and it has been applied to egg-albumin [Marrack and Hewitt, 1929].

It is necessary to work with systems of well-defined hydrogen ion concentration, such as the phosphate buffer mixtures, because the electric charge or valence of the protein ions may vary over a wide range in unbuffered solutions. As a general rule, the concentration of diffusible salts should be greater than 0·05 equivalents per litre of dialysate in order to reduce the ion pressure difference, but in the region of the isoelectric point it is possible to work with more dilute salt solutions.

The most important experimental determinations required are a series of measurements of the osmotic pressures of protein solutions, with a wide range of protein concentration, in equilibrium with a standard solution of electrolytes of well-defined composition. If the relationship between the pressure and the protein concentration approximates towards a straight line, at low protein concentrations, it is probable that the molecular weight of the protein can be calculated by the modification of van't Hoff's law given below:

$$M = 10RT/\pi_0 = 170{,}330/\pi_0 \qquad \text{.....(2),}$$

M = molecular weight in g. of dry protein.

$RT = 760 \times 22{\cdot}412 = 17{,}033$ at 0°.

π_0 = the limiting value of the ratio π in the infinitely dilute solution.

$\pi = p/C$ where p = the observed osmotic pressure in mm. Hg at 0°.

C = g. of dry protein per 100 cc. of solution.

The factor 10 must be included in the formula, as it is customary to express the protein concentration in g./100 cc. rather than in g./litre. The determination of π_0 by extrapolation is discussed below.

The assumptions concerning the ion pressure difference can be tested by the method for the determination of this pressure from membrane potential measurements, described in Section IX. The constancy of the molecular weight of the protein can further be verified by estimation of the ratio π_0 at different concentrations of electrolytes.

Since variations in the concentrations of electrolytes cause large changes in the activity coefficients of the protein ions, as deduced from solubility measurements, it is necessary to verify the assumption that at all concentrations of salts the osmotic coefficient g_p approaches unity as the protein concentration is reduced. In the case of haemoglobin, it has been shown [Adair, 1928] that g_p approximates to unity in solutions at different hydrogen ion concentrations and at salt concentrations ranging from 0·001 to 1·0 g.-mols./litre. This apparent discrepancy between the activity and the osmotic coefficients can be accounted for by the analysis of the activity coefficients [Adair, 1929, 1].

Determinations of π_0 for protein solutions have been made by extrapolation

from a curve showing the relationship between the osmotic pressure and the protein concentration, or by plotting the ratio π as ordinate and C as abscissa [Adair, 1924, 1928]. In the case of the measurements of the osmotic pressure of serum albumin which have been made in this work, it was found advisable

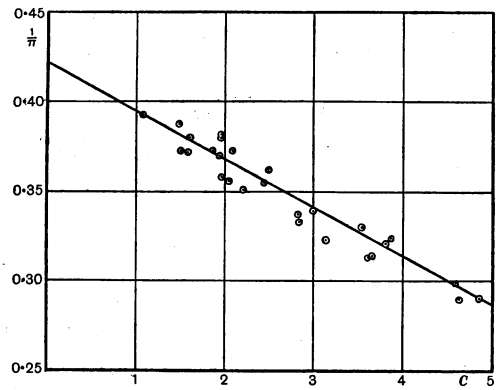

Fig. 1. Crystalline serum albumin at p_H 7·4. Ordinate = pressure-concentration ratio $1/\pi$ (formula 5) at 1°. Abscissa = C = protein concentration in g. per 100 cc. solution.

to adopt a third method, in which $1/\pi$ is plotted as ordinate and C as abscissa. Fig. 1 shows that this method gives a straight line, which can be extrapolated with greater accuracy than the curves which are obtained by application of the first or of the second method. In a recalculation of Sørensen's measurements of the osmotic pressures of concentrated solutions of albumin at the isoelectric point, we have found that the best approximation to a straight line is obtained by a fourth method, in which C_v, the protein concentration in g./100 cc. of solvent, is plotted as the abscissa and the ratio p/C_v is plotted as the ordinate.

All the methods described above yield a mean value for the molecular weight. In order to facilitate the comparison of different preparations, a slight modification of the general method has been devised, in which a value for the molecular weight in each experiment is calculated by formula (3)

$$p = \phi RT C_p = \phi RT\,[10C/M] \qquad \text{.....(3),}$$

C_p = g.-mols. of protein per litre of solution.

ϕ = a coefficient which represents the sum of the osmotic effects due to the ion pressure difference, the volume of the protein hydrate and the deviations from the ideal solution laws.

Theoretically, ϕ is equal to unity, if an infinitely dilute solution of the protein is equilibrated with a standard salt solution. At higher protein concentrations ϕ is equal to π_0/π, if M is constant.

The estimation of ϕ from the observed osmotic pressures may be simplified by the application of empirical formulae. If the pressures can be represented by formula (4) [Adair, 1928], the value of ϕ can be calculated by formula (6)

$$p = \frac{\pi_0 C}{1 - K_b C} \qquad \text{.....(4).}$$

K_b = an empirical constant. This constant has the same effect on the pressures as the volume correction "b" in van der Waals' equation. K_b represents the sum of the effects due to a number of factors, including the ion pressure difference.

Within the range of application of formula (4), there is a straight line relationship between $1/\pi$ and C, defined by formula (5)

$$\frac{1}{\pi} = \frac{1}{\pi_0} - \frac{K_b C}{\pi_0} \qquad \text{.....(5).}$$

The observations recorded in Fig. 1 show that a straight line is obtained in the case of solutions of serum albumin, if C is not greater than 5. The constant $1/\pi_0$ is equal to 0·422, the value of $1/\pi$ at $C = 0$, determined by extrapolation. The constant K_b can then be determined by applying the formula $K_b = \frac{\pi_0}{C}\left(\frac{1}{\pi_0} - \frac{1}{\pi}\right)$ to any point on the line where C is greater than 0. It will be observed that $1/\pi$ is equal to 0·287 when C is 5·0, and it follows that $K_b = 0{\cdot}064$.

Within the range of application of formula (5), the coefficient ϕ can be calculated by formula (6)

$$\phi = \frac{\pi}{\pi_0} = \frac{1}{1 - K_b C} \qquad \text{.....(6).}$$

$K_b = 0{\cdot}064$ in the case of serum albumin at p_H 7·4. In the case of serum globulin at the isoelectric point, $K_b = 0{\cdot}0282$.

The values of ϕ calculated by formula (6) and recorded in Table II must represent a mean value for all the experiments rather than the best value for any one preparation, and therefore the variation in the calculated molecular weights represents the sum of the variations in ϕ as well as the variations in M, but in spite of this source of error, the deviations from the mean value of $M = 71{,}980$ are within 4 %.

V. *The molecular weight of serum albumin.*

The first determination of the molecular weight of serum albumin in a system of well-defined composition is due to Sørensen [1925] who gave the figure 45,000. Adair [1926] obtained a higher value, 62,000. Svedberg and Sjögren [1928], investigating the problem by the ultra-centrifugal method, found that once crystallised serum albumin is a homogeneous substance of molecular weight 68,000, but stated that material three times crystallised was a mixture which contained decomposition products of low molecular weight. They therefore suggested that the proteins studied by Sørensen and by Adair were partially decomposed.

In this work, measurements of osmotic pressure have been made with serum albumin preparations crystallised once, twice and four times. The purity of the material was studied by the refractometric method described in a previous paper [Adair and Robinson, 1930]. The osmotic pressures given by each preparation are recorded in Table II. Fig. 1 shows that if $1/\pi$ be plotted as ordinate, and the concentration C as abscissa, a straight line is obtained over the range

from $C = 1{\cdot}0$ to $C = 5{\cdot}0$. By extrapolation it appears that $1/\pi = 0{\cdot}422$ when $C = 0$, and therefore $\pi_0 = 1/0{\cdot}422 = 2{\cdot}37$. By substitution in formula (2), the molecular weight at 1·0° is equal to 170,960/2·37 = 72,100.

In order to facilitate the comparison of the molecular weights in different experiments, calculations have been made by formulae (3) and (4) as described in the previous section. The results obtained with albumin prepared from fresh serum obtained from the Cambridge Field Laboratories and from Messrs Burroughs and Wellcome are recorded in Table II. It will be seen that the range of variation observed (from 69,200 to 74,900) is small, although some of the measurements have been made on material which has been crystallised four times. The estimations of the refraction increments given in a previous paper [Adair and Robinson, 1930], show that the preparations can be divided into two groups. In the first group, which includes preparations HA 1, 2, 3·2, 4·52, 4·0 and 4·5, attempts were made to remove lipins by washing the serum with ether or by recrystallisation. In these experiments, the refraction increment is about 0·00177, and the molecular weight varies from 70,100 to 74,900 (average 72,800). In the second group, which includes preparations HA 3·1, 3·3 and 4·1,

Table II. *Osmotic pressures and molecular weights of crystalline horse-serum albumin, equilibrated at 1·0° with a phosphate buffer mixture,* p_H *7·4.*

$\frac{0{\cdot}8}{15}M\ Na_2HPO_4 + \frac{0{\cdot}2}{15}M\ KH_2PO_4$.

Preparation No.	Exp. No.	C protein g./100 cc.	p pressure mm. Hg	π ratio p/C	ϕ correction formula (6)	Mol. weight	No. of crystallisations
HA 1·0*	2	2·08	5·58	2·68	1·15	73,700	1
HA 2·0*	5	4·86	16·76	3·45	1·45	72,100	1
,,	6	3·54	10·70	3·03	1·29	73,100	1
,,	7	2·49	6·87	2·76	1·19	73,700	1
,,	9	1·07	2·69	2·52	1·07	72,800	1
HA 3·1	12	2·19	6·25	2·85	1·17	69,900	1
,,	13	1·94	5·24	2·70	1·14	72,500	1
,,	14	4·64	16·00	3·45	1·42	70,600	1
,,	15*a*	3·66	11·65	3·18	1·31	70,400	1
HA 3·2*	16	2·83	8·50	3·00	1·22	73,800	1
,,	17	2·99	8·80	2·95	1·24	71,600	1
,,	18	1·50	4·02	2·68	1·11	70,600	1
,,	19	1·48	3·83	2·58	1·11	73,300	1
HA 3·3	20	3·62	11·52	3·19	1·30	69,900	1
,,	21*a*	1·96	5·46	2·79	1·14	70,200	1
,,	22*a*	1·97	5·18	2·63	1·14	74,400	1
HA 4·52	23	2·05	5·74	2·81	1·15	70,100	2
HA 4·00	24	1·86	5·00	2·68	1·14	72,400	4
,,	25	1·96	5·12	2·61	1·14	74,900	4
,,	26	3·87	11·96	3·09	1·33	73,600	4
,,	27	3·81	11·88	3·12	1·32	72,600	4
HA 4·5	28	1·58	4·25	2·69	1·11	70,700	4
,,	29	1·60	4·19	2·63	1·12	72,500	4
,,	30	2·82	8·37	2·97	1·22	70,200	4
,,	31	3·13	9·69	3·10	1·25	69,200	4
HA 4·1	34	4·61	15·40	3·34	1·42	72,700	1
,,	35	2·44	6·88	2·82	1·19	71,900	1

* Serum washed with ether.

no attempt was made to remove lipins. The refraction increments are about 3 % greater than the normal value, and the molecular weights range from 69,200 to 74,400 (average 71,200). If the lipins exert any osmotic pressure, the molecular weight of the protein calculated from the uncorrected pressure must be too small. The agreement between the figures quoted above shows that the lipins present in the impure preparations exert no appreciable osmotic pressure.

It may be observed that in three preparations, HA 3·1, 3·2 and 3·3, about half of the yield of crystals was dissolved by washing with a diluted solution of ammonium sulphate. According to Sørensen [1925], crystalline serum albumin can be separated into three fractions which have different solubilities but identical osmotic pressures. The partial fractionation effected in the preparations 3·1, 3·2 and 3·3 caused no significant deviation from the mean molecular weight.

The determinations of the molecular weight in Table II show that the results obtained by Svedberg and Sjögren are more accurate than the earlier figures given by Sørensen and by Adair, but Svedberg's suggestion that the earlier experiments were made with decomposed proteins is not supported by the experiments made in this work, which prove that the protein is not necessarily decomposed by the process of recrystallisation. It is probable that the low values of 45,000 given by Sørensen and 62,000 given by Adair are due to the approximations made in their provisional calculations. Adair [1928] published a revised estimate of 68,000 a short time before the appearance of Svedberg's paper.

In this work the methods for the determination of the molecular weight by extrapolation have been improved, as stated in Section IV, and it appears that in the case of Sørensen's measurements on albumin solutions containing 5 and 10 % of ammonium sulphate, the most probable values for the molecular weights are 76,000 and 72,000 respectively. The degree of accuracy of these figures is uncertain, because Sørensen gives no data for solutions containing less than 8 % of protein. They are about 60 % above the figure 45,000 calculated by Sørensen himself, but it should be observed that he states most emphatically that his result is a mere estimate of the order of magnitude. His calculation should give correct results in an ideal colloidal solution of the type described by Donnan, but errors of 60 % or more are by no means improbable in colloidal solutions of finite concentration.

In addition to the measurements on crystalline horse-serum albumin recorded in Table II, a number of preliminary determinations were made with other forms of albumin. The percentage of nitrogen in these proteins is not known; provisional estimates of the protein concentration have been made by assuming that, like crystalline horse-serum albumin, they contain 15·60 % of nitrogen.

The conalbumin of horse-serum, prepared from the mother liquor after the crystals have been removed, is probably a mixture of proteins. The average

molecular weight of this mixture (80,000 ± 8000) is greater than that of the crystalline fraction.

Rough estimates of the molecular weight of the sheep-serum albumin and of ox-serum albumin have been made by formula (3) on the assumption that ϕ is the same for all of the albumins at the same protein concentration, and it seems that, within the limits of experimental error, the size of the particles is the same in each species.

Molecular weights of about 70,000 were obtained in the case of both the total albumin of the sheep-serum and the fraction obtained in the form of an amorphous precipitate when acetic acid was added to a solution of sheep-serum albumin half-saturated with ammonium sulphate.

It has been found that the p_H can be reduced to 6·1 and the salt content increased to 1·0 equiv. without altering the molecular weight. Svedberg and Sjögren [1930] have stated that serum albumin is stable over the range p_H 4–9.

VI. *The molecular weight of serum globulin.*

A number of determinations of the molecular weight of globulin have been made by previous workers; Du Nouy [1924] obtained the figure 35,000 by applying his theory of the surface tensions of protein solutions; Sørensen [1925], Adair [1926] and Svedberg and Sjögren [1928], have obtained much larger values. Even if no corrections are made for deviations from the ideal solution laws in measurements of osmotic pressure, Sørensen's estimates range from 80,000 to 140,000. The corrections increase the estimates by about 60 % in the case of serum albumin, and it is probable that the estimates for globulin should be increased. The measurements required for making these corrections are not available.

In the systems investigated by Adair [1926], the molecular weight of euglobulin was about 174,000, and pseudoglobulin varied from 130,000 to 150,000, when the proteins were equilibrated at 0°, with $M/15$ phosphate buffer mixtures at p_H 7·4 and p_H 7·8.

Svedberg and Sjögren [1928] obtained the value 103,800 ± 3000, in the case of solutions of globulin in $\frac{1}{5}$ molar phosphate buffer mixture at p_H 5·5, centrifuged at temperatures of 18–26°. They state that although "total globulin" prepared by their method is a homogeneous substance of molecular weight 103,800, eu- and pseudo-globulins are mixtures of decomposition products, and they suggest that the results of previous workers were affected by the decomposition of the protein. In a recent paper, Svedberg and Sjögren [1930] state that the molecular weight of pseudoglobulin varies from 53,200 to 99,300. A further examination of the problem by osmometric methods has been undertaken in this work.

In order to minimise the possibility of errors due to changes in the protein, a number of preparations of total globulin were made by a rapid method, described by Svedberg and Sjögren [1928] and by Adair and Robinson [1930]. Although the protein is not perfectly stable under all conditions, the investiga-

tions referred to in Section III indicate that the degree of stability appears to be sufficient for the purpose of osmometric determinations at 0°.

Fig. 2 and Table III record observations of the osmotic pressure of unfractionated horse-serum globulin, equilibrated for periods of 2–12 weeks with the phosphate buffer mixture at the isoelectric point used by Svedberg. It will be seen that the variations from the mean pressure are comparatively small.

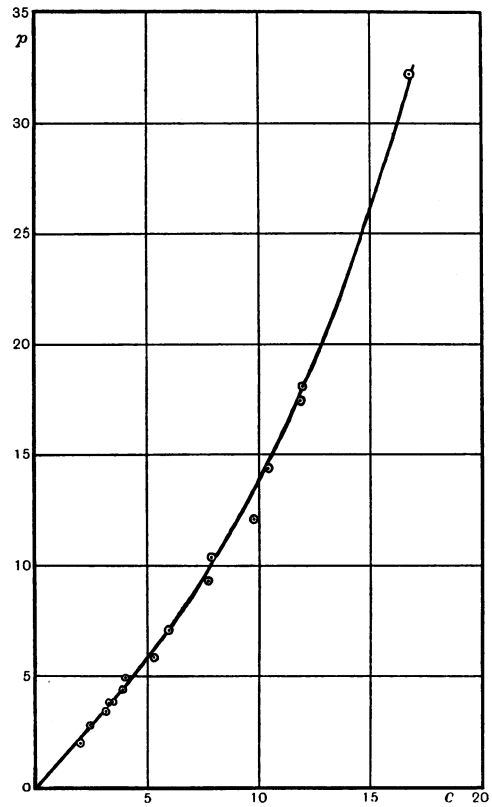

Fig. 2. Osmotic pressure of unfractionated serum globulin (horse) at p_H 5·35. Ordinate = pressure in mm. mercury at 1·0°. Abscissa = C = protein concentration in g. per 100 cc. solution. Curve calculated from formula 4.

Two methods for the preparation of euglobulin have been investigated. In the first method, which gave irregular values for the molecular weight, the euglobulin was precipitated by dilution and acidification of serum. Hardy [1905] has shown that the degree of dispersion of euglobulin prepared by this method is variable, and in this work it was found that the solutions remained opalescent even after the addition of salts at the physiological hydrogen ion concentration, and that the osmotic pressures were irregular. In the case of 2 % solutions, the pressures ranged from 1 to 2 mm. at p_H 7·4. In later experiments, a rapid method was adopted, in which the euglobulin was precipitated from fresh serum (diluted with an equal volume of water) by $\frac{1}{3}$

saturation with ammonium sulphate. The results obtained by this method are shown in Fig. 3.

Table III. *Osmotic pressures of isoelectric solutions of horse-serum globulin (unfractionated).*

Solvent 0·19 *M* KH_2PO_4 + 0·009 *M* Na_2HPO_4. p_H 5·35 at 1·0°.

Preparation No.	Exp. No.	*C*	*p*	π	ϕ	Mol. weight
2*	G 1	3·30	3·78	1·15	1·10	163,000
2*	G 2	3·39	3·84	1·14	1·10	165,000
1	G 3	4·02	4·96	1·24	1·12	154,000
3	G 6	5·33	5·86	1·10	1·17	181,000
3	G 7	16·98	32·28	1·90	1·92	172,000
3	G 9	11·92	17·51	1·47	1·50	174,000
3	G 10	9·84	12·24	1·24	1·38	189,000
3	G 11	7·74	9·32	1·20	1·27	180,000
3*a*	G 12*a*	2·04	1·92	0·94	1·06	192,000
3*a*	G 13*a*	3·15	3·44	1·09	1·09	171,000
4	G 16	12·00	18·11	1·51	1·51	170,000
4	G 17	7·93	10·42	1·31	1·28	167,000
4	G 18	5·98	7·09	1·19	1·20	171,000
4	G 19	3·92	4·39	1·12	1·12	171,000
4	G 20	4·01	4·53	1·13	1·13	169,000
4	G 21	10·45	14·40	1·38	1·42	174,000

* Serum treated with ether.
Preparations 1 and 2 = Serum from University Field Laboratory.
Preparations 3 and 4 = Serum from Burroughs and Wellcome.
Preparation 3*a* = Protein stored for 8 months.
$\phi = 1/(1 - 0{\cdot}0282C)$ formula (6).

Pseudoglobulin was obtained by half-saturation with ammonium sulphate. The results were rather variable, as shown by the data recorded in Fig. 3.

Before the molecular weight of a protein can be determined by the methods of extrapolation referred to in Section IV, it is necessary to prove that the degree of aggregation is independent of the protein concentration. Marrack and Hewitt [1929] suggest that the degree of aggregation of globulin is variable. A number of investigations have been made in this work which show that under certain conditions the observed osmotic pressures of globulin follow the laws which are characteristic of a protein of constant molecular weight, namely haemoglobin [Adair, 1928].

(*a*) In dilute solutions, containing less than 4 % of protein, the pressure is practically proportional to the protein concentration if the reaction is not too far from the isoelectric point, as shown by the data in Figs. 2 and 3.

(*b*) The pressure-concentration ratio π is increased by an increase in the protein concentration at the isoelectric point. According to the aggregation theory π should be diminished.

(*c*) The relationship between the pressures and concentrations in Fig. 2 is determined by a simplified form of van der Waals' equation $p\,(v - b) = RT$, which is applicable to solutions of haemoglobin at the isoelectric point, over a similar range of protein concentrations. Since v is equal to $1/C_p$, van der Waals' formula is equivalent to the expression $p = RTC_p/(1 - bC_p)$. If the protein concentration is given in g./100 cc., this equation may be restated in the form given in equation (4), $p = \pi_0 C/(1 - K_b C)$.

The determination of π_0 and K_b is described in Section IV. In the case of globulin, the results obtained are 1·0 and 0·0282 respectively. It will be seen that the line in Fig. 2, calculated by formula (4), is in agreement with the observed points. The constant K_b is equal to $100\,M\,b$, where b is van der Waals' constant, expressed in cc./g.-mol. It follows that if $K_b = 0{\cdot}0282$, there is a volume correction of 2·82 cc. per g. of globulin; in the case of haemoglobin,

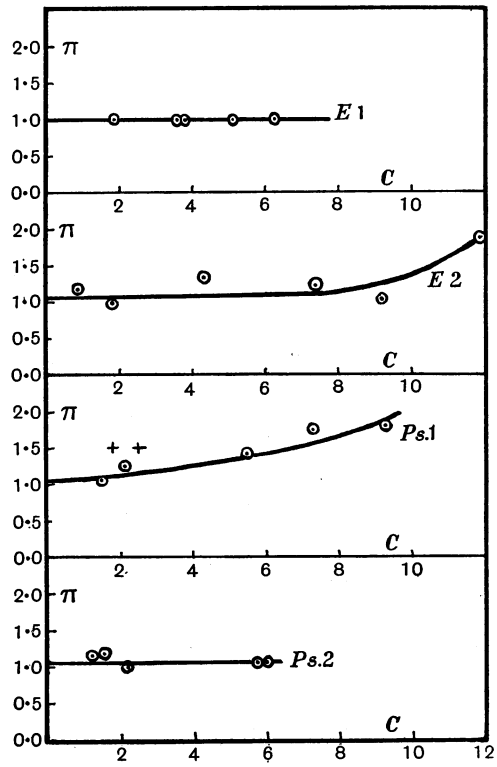

Fig. 3. Pressure-concentration ratios for fractionated globulin. Curves *E* 1 and *E* 2, euglobulin (sheep). Curve *Ps* 1, pseudoglobulin (sheep). Curve *Ps* 2, pseudoglobulin (ox). Solvent for *E* 1, *E* 2 and *Ps* 1, *M*/10 KCl + 0·95/15 *M* Na_2HPO_4 + 0·05/15 *M* KH_2PO_4, p_H 8·0. Solvent for *Ps* 2, 0·8/15 *M* Na_2HPO_4 + 0·2/15 *M* KH_2PO_4, p_H 7·4. Ordinate = π (formula 2). Abscissa = *C*, the protein concentration in g. per 100 cc. of solution.

K_b is about 0·026, and the volume correction is 2·6 cc. According to van der Waals, the value of b is four times the volume of the molecules. It is probable that the volumes of the hydrated globulin and haemoglobin are of the order of 1·3 and 0·96 cc./g. respectively [Weber and Nachmannsohn, 1929; Adair, 1928]. The volume corrections given above are less than four times the volume of the molecules, but there are many factors which might diminish the observed values of b.

The calculations in Table III show that the molecular weight of globulin in concentrated solutions is constant within the limits of error, if it is assumed

that the correction coefficient ϕ in formula (3) is determined by the formula $\phi = 1/(1 - 0{\cdot}0282C)$.

(*d*) Supplementary measurements indicate that the molecular weight of globulin remains constant over an appreciable range of concentrations of hydrogen ions and salts. Measurements of osmotic pressure of the same preparation of horse-serum globulin (No. 2) at p_H 5·35 and p_H 7·4 are recorded in Table III and Fig. 6. In the case of solutions containing 9 g./100 cc. of pseudoglobulin, at p_H 7·8, the pressures were 16·1, 16·5 and 18·2 mm. Hg respectively, when the concentrations of diffusible salts were 0·06, 0·23 and 1·12 g.-equiv. per litre of solvent. The changes in pressure caused by salts are relatively small, and it is almost certain that they are due to changes in the osmotic coefficient of the protein [Adair, 1928].

It is difficult to test the effects of temperature on the degree of aggregation of globulin by direct osmometric methods, because the purified protein is rather unstable at temperatures exceeding 0°, but according to Marrack and Hewitt [1927] it appears that variations from 0° to 37° do not alter the state of aggregation of the mixture of albumin and globulin present in serum. Svedberg finds no change in the molecular weight over the range 18–26°.

On the whole, the evidence in favour of the hypothesis that the molecular weight is constant appears to be sufficient to justify the estimation of the ratio π_0 and the calculation of the molecular weight of globulin from the experiments recorded in Figs. 2 and 3, although it is not inconceivable that a combination of the effects of changes in aggregation and repulsive forces between the particles might produce the effects predicted for a system in which the molecular weight is a constant.

A summary of the provisional calculations is given in Table IV. It will be seen that the molecular weight of euglobulin prepared by the second method described above is practically the same as the molecular weight of total globulin. The preparation of pseudoglobulin is a longer process and the lower values obtained may be due to decomposition, as suggested by Svedberg. The absolute values of the molecular weight are 60–70 % greater than Svedberg's value 103,800; at present it is not possible to explain this difference.

Table IV. *Provisional estimations of the molecular weight of globulin dissolved in phosphate mixtures at 0°.*

No. of determinations	Fraction	p_H	Mol. weight	Notes
13	Total globulin	5·35	175,000 ± 17,000	Horse-serum*
3	,,	5·35	163,000 ± 9,000	Horse-serum†
2	,,	7·4	163,000 ± 4,000	,,
1	,,	7·4	174,000	Ox-serum
1	,,	7·4	160,000	Sheep-serum
4	Euglobulin	7·8	170,000 ± 10,000	,,
6	,,	7·8	162,000 ± 20,000	,,
10	Pseudoglobulin	7·8	155,000 ± 47,000	,,
5	,,	7·4	150,000 ± 20,000	Ox-serum

* Burroughs and Wellcome. † Field Laboratory.

VII. *The relationships between fractionated albumins and globulins and the proteins of natural serum.*

Since the work of Hardy [1905] on the globulins, it has been recognised that the separation of serum into fractions may be correlated with changes in the state of aggregation of the proteins. According to Moore and Roaf [1907] the process of separation increases the aggregation of the proteins, but the opposite view that the albumins and globulins are fractions of a single protein has received more support [Starling, 1926]. Sørensen [1925] has suggested that complexes of euglobulin and pseudoglobulin may be formed.

In this work, the state of aggregation of the proteins in unfractionated serum has been studied by comparing the observed osmotic pressure of the unfractioned protein with the theoretical pressure p, calculated by applying Dalton's Law of partial pressures, as stated below

$$p = p_i + p_a + p_g \qquad \text{.....(7).}$$

p_i = the ion pressure difference, p_a and p_g the partial pressures of albumin and of globulin. The calculation of p_i is described in Section IX. The estimation of p_a and p_g is exceedingly difficult in the case of normal serum, which contains about 8 % of protein, on account of the interionic forces, and until the effects of these forces have been subjected to more detailed investigation, it is necessary to work with more dilute solutions. In the limiting case of an infinitely dilute solution of the protein, the term p_i is zero and the pressure is determined by the formula:

$$p = RT\,[10\,C_a]/M_a + RT\,[10\,C_g]/M_g \qquad \text{.....(8).}$$

C_a and C_g = g. of albumin and globulin per 100 cc. of diluted serum. M_a and M_g denote the molecular weights of albumin and globulin in serum. In applying this formula it has been assumed that $M_a = 72{,}000$ and $M_g = 170{,}000$. The theoretical value of the ratio π_0 calculated by formula (8) is equal to $2{\cdot}36\,x_a + 1{\cdot}00\,(1 - x_a)$, where x_a is equal to g. of albumin per g. of serum protein, and hence $1 - x_a$ is equal to the g. of globulin per g. of serum protein. The values of x_a for horse-serum and for ox-serum, given in Table V, are the averages of the results obtained by Bächer and Kosian [1924]; the value for human serum is that given by Marrack and Hewitt [1927].

The observed values of π_0 for horse-serum and ox-serum have been obtained by applying the method illustrated in Fig. 1 to the experiments recorded in Tables VI and VII. We have attempted to estimate the value of π_0 for human serum protein from the data published by previous workers, including Verney [1926], Mayrs [1926] and Marrack and Hewitt [1927]. The results obtained from their measurements are $1{\cdot}8 \pm 0{\cdot}2$, $1{\cdot}89 \pm 0{\cdot}2$ and $1{\cdot}9 \pm 0{\cdot}2$ respectively. In all cases the pressures have been recalculated in mm. Hg and reduced to 0°.

The results summarised in Table V show that the observed and the theoretical values of π_0 agree within the limits of experimental error. The theo-

retical values are 2–5 % higher than those observed, but differences of this order might be accounted for if the proportion of globulin in the actual specimens used were slightly higher than usual. Moreover, it appears that a part of the albumin designated "conalbumin" may have a molecular weight greater than 72,000 (Section V).

Since the observed and the theoretical values of π_0 agree in the case of sera of three different species, it seems justifiable to infer firstly that the methods used in this work cause little or no change in the state of aggregation of albumin and globulin and secondly that the proteins in diluted serum are a mixture rather than a compound of albumins and globulins. The osmotic pressures of concentrated solutions of serum protein are represented in Fig. 7, and the form of the curves obtained is consistent with the hypothesis that no change in aggregation takes place. The osmotic pressures of undiluted sera with different proportions of albumin have been studied by Govaerts [1925, 1927], who states that his formula is empirical, and without any physical significance. It may be noted that in his formula the ion pressure difference p_i has been neglected. The results recorded below show that p_i is relatively large in undiluted sera.

Table V. *Application of Dalton's law of partial pressures to dilute solutions of unfractionated serum proteins.*

Species	x_a albumin g./g. of total protein	π_0 theoretical formula (8)	π_0 observed
Horse	0·525	1·72	1·64 ± 0·1
Ox	0·507	1·69	1·63 ± 0·1
Man	0·688	1·94	1·90 ± 0·2

Table VI. *Osmotic pressure of unfractionated ox-serum proteins.*

Outer fluid $\frac{0·8}{15}$ *M* $Na_2HPO_4 + \frac{0·2}{15}$ *M* KH_2PO_4 at 0°.

Exp. No.	C	C_v	p	π	E_m	E_m calculated by formula $E_m = 0·141 C_v$
32	0·96	0·97	1·2	1·25	−0·15	−0·14
33	1·16	1·17	2·2	1·90	—	−0·165
28	2·10	2·15	3·7	1·76	—	−0·303
29	2·17	2·21	3·9	1·80	−0·3	−0·312
27	3·71	3·85	7·6	2·05	−0·75	−0·543
26	4·31	4·50	8·4	1·95	−0·50	−0·635
23	8·30	9·06	20·9	2·52	−1·30	−1·28
22	8·62	9·45	22·4	2·59	−1·05	−1·33
31	15·37	18·15	59·1	3·85	−2·20	−2·56
30	16·75	20·10	72·9	4·35	−2·90	−2·83

Table VII. *Osmotic pressure of unfractionated horse-serum proteins.*

Outer fluid $\frac{0·2}{15}$ *M* KH_2PO_4. + $\frac{0·8}{15}$ *M* Na_2HPO_4. at 0°.

Exp. No.	C	p	π
1	9·82	24·2	2·46
3	4·9	9·9	2·02
4	4·93	9·55	1·93
5	2·14	4·05	1·89
6	2·11	3·52	1·67

VIII. *The conditions for the calculation of the ion pressure difference by Donnan's formulae.*

In ideal solutions, the ion pressure difference symbolised p_i must be determined by the formula of Donnan [1911]. In a later paper Donnan and Allmand [1914] have given formulae for the activities of the ions applicable to non-ideal solutions, but Donnan's treatment of osmotic pressures refers to infinitely dilute solutions. It has been suggested [Hecht, 1925; Marrack and Hewitt, 1927], that Donnan's formulae can be applied to the osmotic pressures of the serum proteins, but the experimental evidence is not conclusive, because the observed pressures are affected by an unknown factor, the osmotic pressure of the protein ions themselves. It is possible to investigate the problem without making any assumptions concerning the partial pressures of the protein ions, if determinations of the distribution of the diffusible anions and cations are correlated with electrometric measurements of the membrane potentials.

The results obtained by the equilibration of ammonium albuminate and an ammonium phosphate buffer mixture at p_H 7·3 are recorded in Tables VIII and IX. The experiments show that the ammonium albuminate is ionised, and that there is an excess of diffusible ions inside the membrane. If the system is within the range of application of Donnan's equations, the pressure p_i should be determined by formula (9)

$$p_i = RT\{[NH_4]_c' + [H_2PO_4]_c' + [HPO_4]_c' - [NH_4]'' - [H_2PO_4]'' - [HPO_4]''\} \quad \ldots\ldots(9),$$

$[NH_4]''$, $[H_2PO_4]''$ and $[HPO_4]''$ denote the concentrations of the ions in g.-mols./litre of dialysate.

$[NH_4]_c'$, $[H_2PO_4]_c'$ and $[HPO_4]_c'$ denote the corrected concentrations of the ions in the protein solution. A correction is necessary, as stated by Sørensen [1917], on account of the volume occupied by the protein referred to below. According to Donnan's theory, a measurement of the distribution ratio for one of the ions makes it possible to calculate the ratios for all the ions. If the ratio $[NH_4]_c'/[NH_4]''$ is equal to λ, the distribution ratios for H_2PO_4' and HPO_4'' ions are λ^{-1} and λ^{-2} respectively. These ratios are correlated with the membrane potential by formula (11). It follows that if the concentrations of all the ions in the dialysate are known, their concentrations in the protein solution can be calculated by a number of different methods.

In the first place the corrected[1] concentration of each ion can be calculated by formula (10)

$$m_i' = c_i' \times \frac{100}{100 - V_1C} \quad \ldots\ldots(10),$$

m_i' = corrected concentration of the ion in the protein solution, in g.-mols./litre of "solvent." c_i' = concentration in g. mols./litre of solution, determined by chemical analysis. V_1C = % of the total volume occupied by protein.

[1] This method of correction has been used instead of the calculation of the molalities of the ions, in g.-mols./kg. of water, because it facilitates the estimation of the partial pressures of the protein ions.

C = g. protein per 100 cc. solution. V = effective volume occupied by 1 g. of protein plus the water combined with it. According to Weber and Nachmannsohn [1929] $V_1 = 1{\cdot}0$ cc. in the case of serum albumin.

The data given in Table VIII show that the sum of the corrected concentrations in experiment SA 42 is 0·1989. The sum of the concentrations in the dialysate is 0·185. The value of p_i calculated from the difference is $17{,}033 \times 0{\cdot}0139 = 236$ mm. Hg. In experiment SA 43, p_i is approximately 311 mm.

In the second method the corrected concentrations of the ions are calculated from measurements of the membrane potential by formula (11), a slight modification of Donnan's equation for the membrane potential

$$\log m_i' = \log c_i'' - n_i FE/2{\cdot}303\, RT \qquad \ldots\ldots(11).$$

E = the membrane potential in volts. n_i = the valence of the diffusible ion (negative for anions). $2{\cdot}303\, RT/F$ = a constant = 0·05416 at 0°. c_i'' = g.-mols. of the diffusible ion per litre of dialysate.

The measurements in Table VIII show that the "ideal values" of m_i' calculated by this formula are smaller than the corresponding values calculated by formula (10). The differences show that the protein must diminish the "activity coefficient" of the diffusible cations by about 4 %. In the case of the anions the diminution is about 12 %. Similar changes have been observed by Hastings, Salvesen, Sendroy and Van Slyke [1927] and by Adair [1928].

If the ion pressure difference is calculated by applying formula (9) to the

Table VIII. *Sheep-serum albumin, equilibrated with* $\frac{0{\cdot}75}{15} M\ (NH_4)_2HPO_4 + \frac{0{\cdot}25}{15} M\ NH_4H_2PO_4$, p_H *7·3 at 0°.*

Exp. No.	SA 42	SA 43	SA 44
p osmotic pressure mm. Hg	56·5	54·6	53·3
C g. protein per 100 cc. solution	10·09	10·00	10·18
C_v g. protein per 100 cc. solvent	11·22	11·11	11·35
Density of inner fluid at 0°	1·0337	1·0326	1·0334
Density of outer fluid at 0°	1·0057	1·0055	1·0062
E_m = membrane potential millivolts	−2·25	−2·55	−2·35
p_H of outer fluid minus p_H of inner fluid	0·0416	0·0470	0·0435
$[NH_4]''$ outer, g.-mols./litre	0·1183	0·1145	0·1172
$[NH_4]'$ inner, g.-mols./litre	0·1214	0·1213	0·1311
$[PO_4]''$ outer, g.-mols./litre	0·0667	0·0669	0·0663
$[PO_4]'$ inner, g.-mols./litre	0·0575	0·0582	0·0567
*Ratio $\frac{HPO_4''}{\text{total } PO_4}$ outer	0·775	0·712	0·768
*Ratio $\frac{HPO_4'}{\text{total } PO_4}$ inner	0·753	0·689	0·736
$[NH_4]_c'$ observed [Formula (10)]	0·135	0·135	0·145
$[NH_4]_c'$ ideal [Formula (11)]	0·130	0·128	0·130
Ratio $\frac{[NH_4]_c \text{ ideal}}{[NH_4]_c \text{ observed}}$	0·96	0·95	—
$[PO_4]_c'$ observed	0·639	0·647	0·632
$[PO_4]_c'$ ideal	0·563	0·557	0·556
Ratio $\frac{[PO_4]_c' \text{ ideal}}{[PO_4]_c' \text{ observed}}$	0·882	0·861	0·896

* Ratio $HPO_4/HPO_4 + H_2PO_4$ = fraction of total phosphate in bivalent form, calculated by correlating the direct measurements of the ratio for the outer fluid and the membrane potentials by formula (11).

"ideal values" of m_i' recorded in Table VIII the result obtained is 29 ± 3 mm. In addition to the results obtained by methods 1 and 2, described above, calculations have been made by other methods and are summarised in Table IX. It will be seen that the results vary from 1·4 to 273 mm. The range of variation is large in comparison with the differences between the ideal and the observed concentrations, because small errors in the theoretical assumptions are exaggerated in the calculation of p_i which represents a small difference between two relatively large pressures. A number of modifications of formula (9) have been suggested [Loeb, 1922; Hecht, 1925; Marrack and Hewitt, 1927], but the degree of accuracy of the assumptions involved is rather uncertain.

Table IX. *Calculations of the ion pressure difference by Donnan's formulae.*

Sheep-serum albumin equilibrated at 0° with $M/15$ ammonium phosphate buffer, p_H 7·3.

Method	Calculated p_i
Observed excess of ions inside membrane	273 ± 37
Ammonia distribution ratio	60
Membrane potential	29 ± 3
Equivalents ammonium proteinate	15 ± 4
Total phosphate distribution ratio	1·4

IX. *The calculation of the ion pressure difference in non-ideal solutions.*

In systems where the deviations from the ideal solution laws are not great, it is possible to estimate the value of p_i by a simple modification of Donnan's theory [Adair, 1928]. The deviations shown in Table X are so large that it is necessary to adopt an entirely different method of treatment in order to obtain a formula for the ion pressure difference. The derivation of the more accurate formula (12) has been given in a previous paper [Adair, 1929, 2]

$$p_i = RT \int_0^u m_p n_p du \qquad \text{.....(12).}$$

m_p = g.-mols. of protein per litre of solvent [*cf.* formula (13)]. n_p = the mean valence of the protein ions. The symbol u is defined as the value of $E \times F/RT$, where E, the membrane potential, is a function of the protein concentration, determined under conditions where the protein concentration is varied from 0 to m_p, and the activities of water and of the diffusible salts are kept constant by the equilibration of the protein solutions, enclosed in membranes, with a solution of electrolytes of constant temperature, pressure and composition.

Materials required for the application of the formula to horse-serum albumin have been summarised in Table X. It will be observed that there are slight variations in the refractive index and in the p_H of the outer fluids, due to the evaporation of water and to traces of alkali from the glass and other factors, but the variations in the osmotic pressures and membrane potentials due to these changes are within the limits of experimental error.

Table X. *Osmotic pressures and membrane potentials of crystalline horse-serum albumin.*

Outer fluid $\frac{0{\cdot}8}{15}$ *M* $Na_2HPO_4 + \frac{0{\cdot}2}{15}$ *M* KH_2PO_4 at 0°.

Preparation No.	Exp. No.	C	C_v	p	π	E_m membrane potential in millivolts	p_H outer fluid final value	Difference between refractive indices of outer fluid and of water
HA 4·1	32	9·14	10·05	45·8	5·01	−2·0	7·46	0·00168
,,	33	6·60	7·07	28·2	4·27	−1·55	7·45	0·00168
,,	34	4·60	4·82	15·38	3·34	−1·10	7·55	0·00167
,,	35	2·44	2·50	6·88	2·82	−0·73	7·49	0·00170
,,	36	7·41	8·00	31·15	4·20	−1·85	7·49	0·00170
HA 5	37	9·97	11·09	60·2	6·04	−2·65	7·41	0·00173
,,	38	6·65	7·12	30·3	4·56	−1·72	7·43	0·00170
,,	39	5·64	5·98	25·6	4·54	−1·50	7·42	0·00171
,,	40	3·84	3·99	13·9	3·61	−1·10	7·40	0·00170
,,	41	2·39	2·45	7·5	3·13	−0·73	7·40	0·00174

The values of C_v given in this table are defined by formula (13) (*cf.* formula (10)).

$$C_v = C \times \frac{100}{100 - V_1 C} \qquad \text{.....(13),}$$

C_v = g. dry protein per 100 cc. solvent.

If the molecular weight M is known, the corrected concentration m_p can be calculated by the formula $m_p = 10 \times C_v/M$.

The determination of the valence n_p and the equivalent concentration of the protein ions $n_p m_p$ requires further consideration. Theoretically, $n_p m_p$ must be equal to the difference between the equivalent concentrations of the diffusible anions and of the diffusible cations. The determination of the concentrations of the ions by chemical methods is open to criticism, on the ground that a certain proportion of the ions may be bound by the protein. Electrometric measurements are not affected by the bound ions but the result may be difficult to interpret on account of the effects of interionic forces. The effects of these forces can be represented by the activity coefficient f_i', defined by the formula $a_i' = m_i' f_i'$, where a_i' represents the activity of the diffusible ions in the protein solution. The activities of hydrogen and of chloride ions can be determined by direct electrometric methods, but if direct methods are not available calculations can be made by the thermodynamical formula of Donnan and Allmand [1914], restated below

$$\log m_i' f_i' = \log c_i'' f_i'' - n_i EF/2{\cdot}303\, RT \qquad \text{.....(14).}$$

f_i'' = the activity coefficient of the diffusible ion in the dialysate.

If f_i' is equal to f_i'', and if E is less than 0·002, formula (14) can be replaced by the series formula $m_i' = c_i'' [1 - n_i u + \frac{1}{2} n_i^2 u^2]$, and under these conditions, $m_p n_p$ is determined by formula (15). Similar formulae have been referred to by Bjerrum [1924], by Rinde [1926] and by Adair [1928]

$$m_p n_p = uJ \qquad \text{.....(15).}$$

J = the ional concentration, or the sum of the concentrations of the ions in the dialysate multiplied by the squares of their valences.

Formula (15) may be subject to considerable errors if the solutions are not ideal, but it has been observed that the difference between f_i' and f_i'' diminishes as the protein concentration is diminished, if the concentration of salts in the dialysate is kept constant [Adair, 1928]. According to formula (14) the effects due to the interionic forces cancel out, if $f_i' = f_i''$. Provisionally therefore it seems reasonable to assume that formula (15) can be applied to the limiting values of the ratio $(E_m/C_v)_0$ defined by formula (16)

$$n_p = \frac{uJ}{m_v} = 0{\cdot}00425\, M\, J\, (E_m/C_v)_0 \qquad \text{.....(16).}$$

0·00425 is a constant equal to $(F/10{,}000\, RT)$ at 0°. M = the molecular weight of the protein (72,000). E_m = the membrane potential in millivolts = $E \times 1000$, C_v = g. dry protein per 100 cc. of solvent. $(E_m/C_v)_0$ = the limiting value of the ratio (E_m/C_v) at $C_v = 0$, determined by extrapolation. The curve in Fig. 4 indicated that the limiting value of this ratio is about 0·324 ± 0·1. This ratio indicates that $n_p = -34{\cdot}3$, in the case of a very dilute solution of serum albumin equilibrated with the phosphate buffer at p_H 7·4.

The calculation of the valence in more concentrated protein solutions requires further consideration, because an alteration of the protein concentration from $C_v = 0$ to $C_v = 12$ is correlated with a change in the membrane potential and a diminution of the p_H of the protein solution from 7·4 to 7·35. It has been shown that the amount of base bound by serum albumin is proportional to the observed p_H minus 4·8 [Cohn, 1925] and it follows that n_p may be diminished by about 2 %. This change is within the limits of experimental error.

After the values of u, m_p and n_p have been determined, formula (12) can be integrated by graphical methods, but in many cases, the calculation can be shortened by the application of an empirical formula, of the type stated below

$$m_p n_p = Ju + Jku^2 \qquad \text{.....(17),}$$

k = an empirical constant. Within the limits of experimental error the measurements in Fig. 4 are represented by the curve calculated by the empirical formula $(C_v/E_m) = 3{\cdot}09 + 0{\cdot}7\, E_m$; it follows that k is about 5·3.

Within the range of application of formula (17) the ion pressure difference is determined by formula (18)

$$p_i = RT \int_0^u J\,(u + ku^2)\, du = RTJ\, \tfrac{1}{2}u^2 + RTJk\, \tfrac{1}{3}u^2 \qquad \text{.....(18).}$$

If the pressures are expressed in mm. Hg at 0°, formula (18) is equivalent to formula (19)

$$p_i = 15{\cdot}4\, JE_m^2 + 0{\cdot}436\, JkE_m^3 \qquad \text{.....(19).}$$

In the system referred to in Table X, J is equal to 0·3466, and k is approximately 5·3 and it follows that $p_i = 5{\cdot}35\, E_m^2 + 2{\cdot}32\, E_m^3$. A number of calculations of p_i made by this formula are recorded in Table XI. In this table

the partial pressure of the protein ions, symbolised p_p, has been calculated from the observed osmotic pressure p by the formula $p_p = p - p_i$. The osmotic

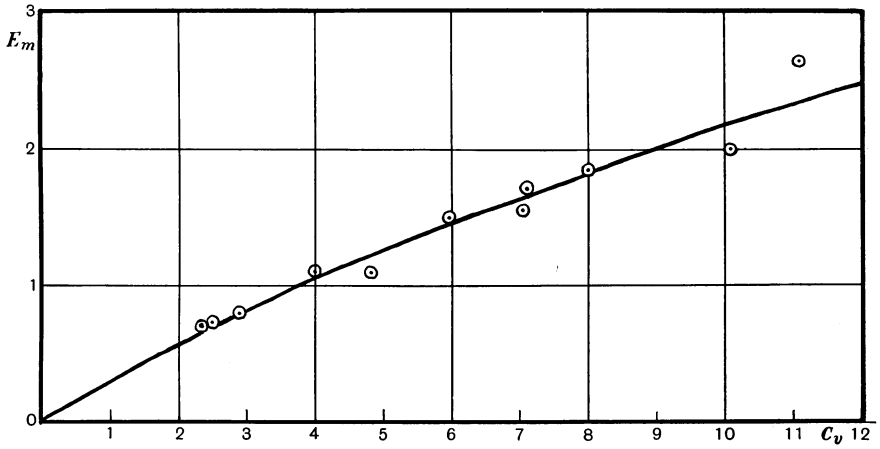

Fig. 4. Membrane potentials of crystalline serum albumin at p_H 7·4. Ordinate = membrane potential in millivolts at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. solvent. Curve calculated from formula $C_v = 3{\cdot}09\, E_m + 0{\cdot}7\, E_m^2$.

coefficient g_p, defined by the formula $p_p = RTg_p m_p$, has been given in the last column of Table XI. It will be observed that in fairly dilute solutions in which the concentration m_p is less than 0·0005, g.-mols./litre of solvent, the osmotic coefficient diminishes as the protein concentration is increased, in accordance

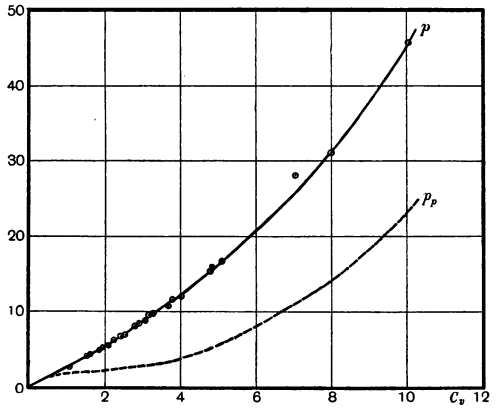

Fig. 5. Partial osmotic pressure of crystalline serum albumin at p_H 7·4. Ordinate = pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. solvent. p = observed osmotic pressure. $p_p = p - p_i$ = partial pressure due to protein ions.

with the interionic attraction theory of Debye and Hückel [1923]. In more concentrated solutions, the effects of the electrical forces described by Debye and Hückel are masked by other factors and the osmotic coefficients tend to increase. According to their theory, the interionic forces depend upon the squares of the valences of the ions; although their equations cannot be applied to

protein systems without modification, it is interesting to note that there is a marked difference between serum albumin (valence − 37 at p_H 7·4) and haemoglobin (valence − 7 at p_H 7·8), in accordance with the theory. In the case of haemoglobin, the effects of the interionic forces are masked by other factors even in dilute solutions, and the osmotic coefficients and the activity coefficients exceed unity at all protein concentrations [Adair, 1927, 1928, 1929, 1].

Table XI. *The ion pressure difference in solutions of crystalline horse-serum albumin equilibrated with* $\frac{0.8}{15}M$ $Na_2HPO_4 + \frac{0.2}{15}M$ KH_2PO_4. p_H *7·4 at 0°.*

C_v g. protein per 100 cc. solvent	m_p g.-mols protein per litre solvent	E_m membrane potential millivolts	p observed osmotic pressure mm. Hg	p_i ion pressure difference	p_p partial pressure of protein ions	RTm_p ideal value of p_p	g_p osmotic coefficient p_p/RTm_p
1·7	0·00024	−0·5	4·4	2·2	2·2	4·1	0·54
3·8	0·00053	−1·0	11·2	7·6	3·6	9·0	0·40
6·2	0·00086	−1·5	22·4	13·2	9·2	14·7	0·63
9·0	0·00125	−2·0	38·0	19·9	18·1	21·2	0·85
10·8	0·00150	−2·3	50·5	24·6	25·9	25·5	1·01

Theoretically, the osmotic coefficients of the ions of globulin at p_H 7·4 should be intermediate between the values obtained for albumin and for haemoglobin, if the valence of the globulin has an intermediate value. The provisional estimates recorded in Fig. 6 are in agreement with this hypothesis.

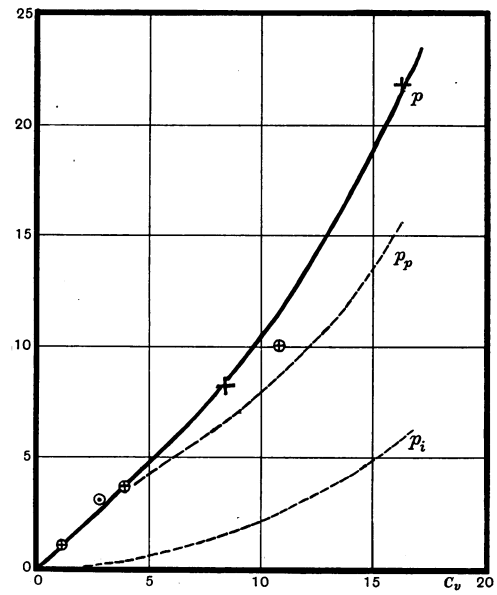

Fig. 6. Partial osmotic pressure of unfractionated globulin at p_H 7·4. Ordinate pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. of solvent (formula 10). p = observed osmotic pressure; p_p = partial pressure of protein ions; p_i = diffusible ion pressure difference. Crosses = globulin of sheep. Circles = globulin of ox, except point at C_v = 2·8, which represents 2 observations on preparation *G* 2 (horse).

Hardy [1905] did not detect any ionic globulin in serum by the method of kataphoresis. Preliminary measurements of the membrane potentials indicate that the ratio (E_m/C_v) is about 0·065 ± 0·025, in the case of globulin at p_H 7·4, and from this result it appears that the valence of the ions may be about − 16·3, a value which is not large in comparison with the size of the molecule. It is possible that this low ratio of the charge to the mass and the effects of the ion atmosphere, described by Debye, prevent any appreciable movement in the electric field.

A series of 8 measurements of the membrane potentials of the total proteins from ox-serum has been recorded in Table VI. It is possible that the ratio E_m/C_v is a function of the protein concentration as in the case of serum albumin, but as a first approximation, the observed values of the membrane potential may be represented by the empirical formula $E_m = 0{\cdot}141\ C_v$ in which the coefficient 0·141 is the mean value of the ratio E_m/C_v over the whole range of protein concentrations. Approximate values of p_i at different protein concentrations have been calculated by formula (20)

$$p_i = 15{\cdot}4\ JE_m^2 \qquad \text{.....(20).}$$

Since $J = 0{\cdot}3466$, $p_i = 5{\cdot}33\ (0{\cdot}141\ C_v)^2$. The results are recorded in Fig. 7.

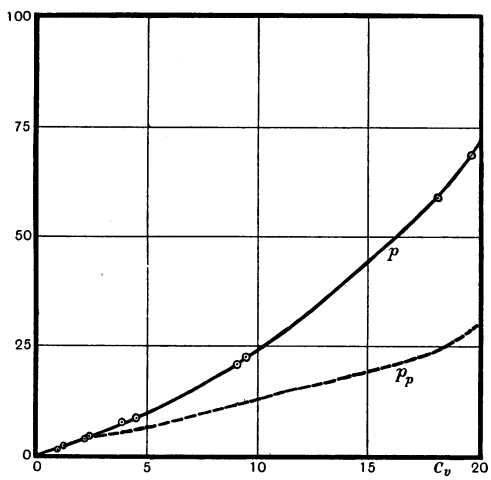

Fig. 7. Osmotic pressure of unfractionated proteins of ox-serum at p_H 7·4. Ordinate = pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in g. per 100 cc. of solvent (calculated from formula 10). p = observed osmotic pressure. $p_p = p - p_i$ = partial pressure due to protein ions.

This curve is of interest in connection with the theories advanced by previous workers concerning the high pressures of concentrated solutions of serum proteins which have been observed by Starling [1899] by Krogh and Nakazawa [1927] and other workers. Verney [1926] has suggested that the effect may be due to the hydration of the protein. Marrack and Hewitt [1927] have suggested that the effect may be accounted for by the ion pressure difference. Their hypothesis is confirmed by the results recorded in Fig. 7. The

curve for the partial pressure p_p is a much closer approximation to a straight line than is the case with pure serum albumin or haemoglobin. It is probable that the apparent approximation to a straight line in concentrated solutions is partly accidental. It may be due to errors in the estimation of the ion pressure difference, or it may be due to a balancing of the effects of attractive and repulsive forces which may take place in certain ranges of protein concentrations.

Summary.

The stability of the serum proteins has been examined by a number of methods, and it has been found that serum albumin can be recrystallised four times without change in the molecular weight. The preparations of albumin made in this work appear to be more stable than those made by Svedberg and Sjögren, who state that three crystallisations lead to decomposition of albumin.

The methods for the determination of molecular weights of proteins in non-ideal solutions [Adair, 1924, 1928] have been developed, with special reference to the estimation of the molecular weight of serum proteins in the physiological range of hydrogen ion and salt concentrations.

The mean value of the molecular weight of horse-serum albumin in 27 experiments has been determined as 72,000 $\pm$ 3000. One preparation gave a value of 67,000. Sørensen's provisional estimate of 45,000 is subject to a correction of about 60 % on account of deviations from the ideal solution laws. The corrected value (about 74,000) agrees with the results obtained in this work. Preliminary determinations indicate that the serum albumins of the ox and sheep have molecular weights of about 70,000.

The mean value of the molecular weight of the unfractionated globulin of the horse is 175,000. In a series of 17 experiments the maximum and minimum values observed were 192,000 and 154,000 respectively. The unfractionated globulins of the ox and the sheep gave similar results. Euglobulin, prepared by a rapid method, resembles total globulin. Svedberg's value for total globulin, 103,800, is lower than that obtained in this work. The factors which might account for the difference have been studied, but at present no explanation can be offered.

The osmotic pressure of the total protein of serum before fractionation has been studied from the point of view of Dalton's law of partial pressures, and it appears that serum protein is not a compound of albumins and globulins. The state of aggregation of the proteins in the untreated serum appears to be the same as their state of aggregation in the purified proteins prepared by the methods used in this work.

The protein solutions which have been examined are beyond the range of application of Donnan's formulae for osmotic pressures. Attempts to estimate the pressure due to the excess of diffusible ions inside the membrane (p_i) by these formulae yield contradictory results.

Methods for the estimation of p_i in non-ideal solutions are described, and calculations of p_i and of p_p, the partial pressure of the protein ions, have been made. The application of the theory of Debye and Hückel to these partial pressures has been discussed.

A method has been suggested for the estimation of the valence of the protein ions in non-ideal solutions.

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