

THE COLLOIDAL BEHAVIOR OF SERUM GLOBULIN.

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I.

INTRODUCTION.

The theory of the colloidal behavior of proteins developed by Loeb¹ has been shown to apply to the proteins, gelatin, casein, egg albumin, and edestin.² It may be recalled that this theory is based on the idea that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form ionizable salts, and on Donnan's theory of membrane equilibrium.³ The object of the present work was to find out whether the theory would explain the behavior of a serum globulin as well.

The globulin was prepared from serum which was obtained by whipping and centrifuging fresh ox blood. The serum was diluted and the globulin was precipitated near its isoelectric point by CO₂ and purified as described by Robertson.⁴ The product was therefore the insoluble serum globulin or euglobulin. The isoelectric point of this globulin was found by Rona and Michaelis⁵ to be at a hydrogen ion concentration of 0.36×10^{-5} (pH 5.44). 1 per cent suspensions of the present preparation in distilled water were found to have a pH of 5.41 or 5.42 at 33°C. The removal of salts was assured by a measurement of the conductivity of a 1.628 per cent suspension

¹ Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922; *J. Gen. Physiol.*, 1918-22, i-iv.

² Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 597.

³ Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

⁴ Robertson, T. B., *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, 1918, 40.

⁵ Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914, 56.

of the globulin in distilled water; the specific conductivity at 34° was found to be 1.2×10^{-5} reciprocal ohms. The globulin was kept in suspension in distilled water saturated with thymol, and was preserved in an ice box. To obtain the concentrations of globulin required for the experiments, the bottle was shaken and samples were withdrawn by a pipette. The accuracy of this method of measuring the globulin was checked by dry weight determinations on two 25 cc. samples; each was found to contain 0.407 gm. of dry globulin.

Inasmuch as this globulin preparation did not give clear solutions with either acid or alkali, it is probable that it had become partly denatured or changed in some way during the process of purification. A second lot was prepared which gave nearly clear solutions in acid or alkali. Nevertheless, the first preparation could still be used to show whether or not a protein prepared from ox serum obeyed the same laws as other proteins.

II.

Titration of Globulin with Acids and Bases.

Titration curves were obtained by measuring with the hydrogen electrode, at 33°, the pH of 1 per cent solutions of the globulin in HCl and H₃PO₄ of various concentrations, and of a 0.5 per cent solution of the globulin in H₃PO₄. The pH values were referred to 0.1 M HCl, its pH being taken as 1.036. The results are given in Table I.

In order to determine how much of the HCl was combined with the globulin, the amounts of HCl required to give the same pH to 100 cc. of water, without protein, were subtracted from the total amounts of HCl in Table I. The figures for the acid-water curve have been given in a previous paper.⁶ In the case of H₃PO₄ it was pointed out in connection with the titration of edestin² that this method does not give the true amounts of combined acid, on account of the repression of the ionization of the weak acid H₃PO₄ by the H₂PO₄⁻ ion from the protein phosphate. Accordingly the amounts

⁶ Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 733.

TABLE I.
Titration of Globulin with Acids.

0.1M HCl in 100 cc. of 1 per cent globulin, cc.....	0	0.60	0.60	1.20	1.20	2.39	2.51	4.18	4.54	6.57	6.57	8.95	8.97	11.35	11.36	16.14	16.95	22.12	29.85	37.52	61.3	
	pH.....	5.41	5.42	3.99	4.07	3.71	3.80	3.43	3.39	3.16	3.07	2.85	2.86	2.60	2.62	2.35	2.36	2.04	2.08	1.85	1.67	1.52
0.1M H ₃ PO ₄ in 100 cc. of 1 per cent globulin, cc.....	1.39	2.78	5.56	9.72	16.7	24.3	34.0	43.6														
	pH.....	3.63	3.33	2.98	2.65	2.33	2.14	1.98	1.88													
0.1M H ₃ PO ₄ in 100 cc. of 0.5 per cent globulin, cc.....	0.69	1.39	2.78	4.16	6.94	11.11	18.05	27.8	41.7	69.4												
	pH.....	3.67	3.41	3.12	2.89	2.60	2.37	2.15	1.99	1.84	1.67											

of combined H_3PO_4 were calculated by the equation⁷ which was used in the case of edestin

$$x = \frac{kc}{h+k} - h$$

Here x = concentration of H_2PO_4^- from protein phosphate (assumed to be completely ionized).

k = primary ionization constant of $\text{H}_3\text{PO}_4 = 0.01$.

c = total concentration of H_3PO_4 .

h = concentration of H^+ = concentration of H_2PO_4^- from H_3PO_4 .

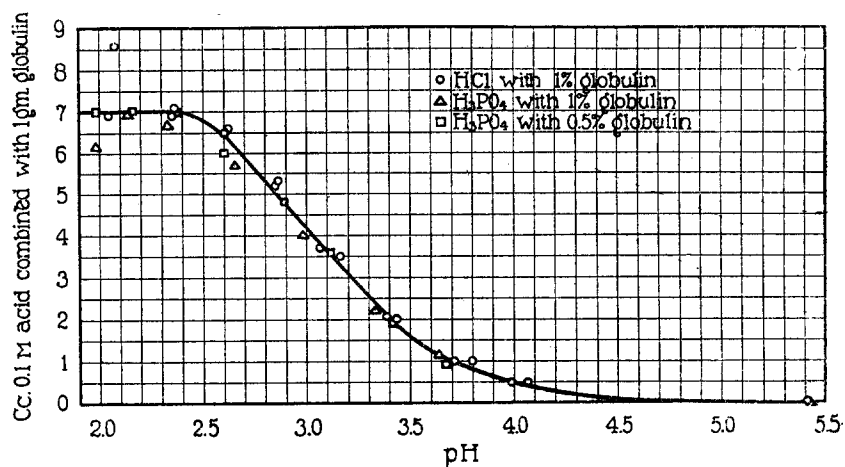


FIG. 1. Combination of globulin with acids.

The values obtained in this way, together with the values for HCl, are plotted in Fig. 1 in terms of cc. of 0.1 M acid combined with 1 gm. of globulin. Since the points fall on one curve it is to be inferred

⁷An equation identical with this was used by Pauli and Hirschfeld (Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245; Pauli, W., *Kolloidchemie der Eiweisskörper*, Dresden and Leipsic, 1920, pt. 1, 57) to calculate the amounts of acetic acid combined with horse serum albumin. However, since they plotted the amounts of combined acid against the total concentration of acid added instead of against the pH, they were not able to show that the protein was combined with chemically equivalent amounts of weak and strong acids.

that the globulin reacts stoichiometrically with equal numbers of molecules of the two acids. In other words, both HCl and H_3PO_4 react with globulin as monobasic acids. The combination curve appears to become horizontal at about 7 cc. of 0.1 M acid, indicating a combining weight of about 1,400 for the globulin. However, the height of the maximum is more or less uncertain. In attempting to carry the curve into the region of lower pH, it was found that the points for HCl varied irregularly on both sides of the value 7 cc.,

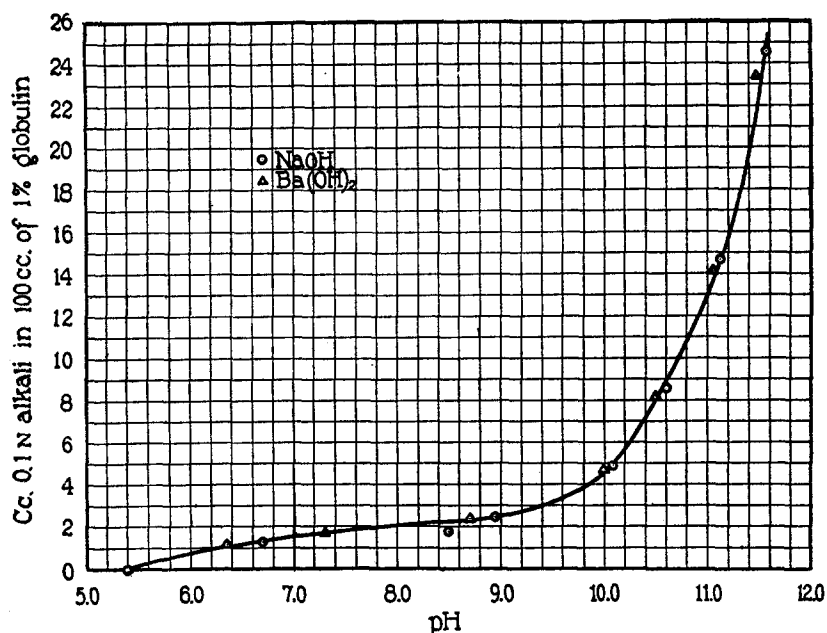


FIG. 2. Titration of globulin with alkalis.

while those for H_3PO_4 seemed to descend toward the axis of abscissæ. The former deviations are evidently due to the magnification of errors involved in taking the differences between two steep curves; the apparent decrease in the amount of H_3PO_4 combined is probably due to incomplete ionization of the globulin phosphate in the presence of much H_3PO_4 , while in the calculation it was assumed that the protein phosphate was completely ionized. At any rate the results in Fig. 1 show that between pH 2 and 4 the globulin reacts stoichiometrically with equimolecular amounts of the two acids.

It should be added that a potentiometric titration of serum globulin with HCl was made by Robertson.⁸ He calculated the amount of combined HCl simply by taking the difference between the total acid concentration present and the hydrogen ion concentration found in the presence of the protein. This involves the assumption of complete ionization of the HCl, which is probably correct for the concentrations he used, 0.01 M or below. His values fall at points higher than those in Fig. 1, and do not appear to reach a maximum in the range of acidity which they cover. The cause for this difference may lie in differences in the globulin preparations, or possibly in differences in temperature or in the standards of hydrogen ion concentration.

Fig. 2 represents the titration of 1 per cent globulin with NaOH and Ba(OH)₂. The curve is the original titration curve; no attempt was made to calculate the amount of combined alkali. These two strong alkalies appear to give the same titration curve with globulin when the concentrations are plotted in terms of normality, indicating that the globulin combines with them in equivalent, not in molecular proportions.

These titration experiments indicate that serum globulin behaves like gelatin, casein, egg albumin,¹ and edestin² in its stoichiometric reactions with acids and bases.

III.

Membrane Potentials.

In order to determine whether the Donnan equilibrium applied to the behavior of serum globulin, experiments were carried out by the method which had been used by Loeb¹ with gelatin and egg albumin and followed by the present writer² with edestin. Solutions were prepared containing 1 gm. of globulin in 100 cc. of HCl of various concentrations. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl free from protein but of about the same pH as the protein solutions. The beakers were placed in a water bath at $25^{\circ} \pm 1^{\circ}\text{C.}$, and about 20 to 24 hours were allowed for the

⁸ Robertson, T. B., *J. Phys. Chem.*, 1907, xi, 437; *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, 1918, 99.

attainment of equilibrium. Then the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes, and the P.D. between the inside and outside solutions was measured with the aid of saturated KCl-calomel electrodes and a Compton electrometer. The P.D. measurements were made at about 25°. The pH of the inside and outside solutions, at 33°, was then measured with the hydrogen electrode and potentiometer. The calculated P.D. values

TABLE II.
Effect of pH on P.D. and Osmotic Pressure of 1 Per Cent Globulin Chloride.

pH inside.....	4.57	4.38	3.86	3.55	3.35	3.06	2.67	2.19	1.72	1.28
pH outside.....	4.27	4.12	3.54	3.21	3.02	2.75	2.44	2.07	1.67	1.28
Observed P.D., millivolts .	6.5	7.0	16.5	21.0	21.0	20.0	15.0	10.5	4.0	3.0
Calculated P.D., millivolts .	17.5	15.0	19.0	20.0	20.0	18.0	13.5	7.5	3.0	0
Osmotic pressure, mm. . . .	9	12	23	38	57	73	71	48	27	45

Effect of pH on P.D. and Osmotic Pressure of 0.5 Per Cent Globulin Phosphate.

pH inside.....	3.57	3.26	2.95	2.76	2.50	2.30	2.09	1.97	1.83	1.66
pH outside.....	3.32	3.00	2.70	2.54	2.35	2.19	2.02	1.90	1.78	1.64
Observed P.D., millivolts .	12.0	13.0	13.0	11.5	9.0	7.0	5.0	4.0	3.5	2.5
Calculated P.D., millivolts .	15.0	16.0	14.5	13.0	8.5	7.0	4.5	4.0	3.0	1.5
Osmotic pressure, mm. . . .	9	16	29	33	25	22	19	18	16	14

Effect of pH on P.D. and Osmotic Pressure of 1 Per Cent Globulin Acetate in 0.01 M Sodium Acetate.

pH inside.....	4.63	4.34	4.02	3.69	3.35					
pH outside.....	4.61	4.31	4.01	3.63	3.31					
Observed P.D., millivolts .	1.0	1.5	2.0	2.0	3.0					
Calculated P.D., millivolts .	1.0	2.0	1.0	4.0	2.0					
Osmotic pressure, mm. . . .	6	6	7	9	11					

represent the differences between the E.M.F. readings obtained for the inside and outside solutions with the hydrogen electrode, corrected to 25°; in other words, the calculated P.D. values are calculated from the measurements of the hydrogen ion concentration according to the formula deduced by Donnan (which is identical with Nernst's formula).

The results of experiments on the effect of acidity on the P.D. are given in Table II.

The results show that with protein and acid alone the agreement between the observed and calculated values for the P.D. is fairly good except in the region near the isoelectric point, and the last experiment in Table II shows that agreement may be obtained in this range of pH also by the use of buffered solutions.⁹

The effect of salt concentration on the P.D. was tested by a few experiments with NaCl, CaCl₂, and LaCl₃, and the results are given in Table III. Again the agreement between the observed and calcu-

TABLE III.
Effect of Different Chlorides on the P.D. and Osmotic Pressure of 1 Per Cent Globulin Chloride.

Concentration of salt.	O	N/1,024			N/256			N/32		
		NaCl	CaCl ₂	LaCl ₃	NaCl	CaCl ₂	LaCl ₃	NaCl	CaCl ₂	LaCl ₃
pH inside.....	3.68	3.58	3.60	3.55	3.82	4.10	3.80	3.66	3.69	3.57
pH outside.....	3.34	3.36	3.38	3.33	3.76	3.93	3.73	3.63	3.63	3.54
Observed P.D., millivolts	21.0	13.5	12.5	13.0	4.0	3.0	3.5	1.0	1.0	1.0
Calculated P.D., millivolts	20.5	13.0	13.0	12.5	4.0	10.0	4.0	1.5	3.5	1.5
Osmotic pressure, mm.	52	25	26	28	20	16	19	4	4	4

TABLE IV.
Effect of pH on the P.D. and Osmotic Pressure of 1 Per Cent Sodium Globulin.

pH inside.....	9.20	9.98	10.47	11.05
pH outside.....	9.81	10.34	10.85	11.31
Observed P.D., millivolts	-32.0	-23.0	-18.0	-11.5
Calculated P.D., millivolts	-36.5	-21.0	-22.5	-16.0
Osmotic pressure, mm.	220	174	165	152

lated values for the P.D. is sufficient to show that the P.D. is due to the Donnan equilibrium. Moreover, the different chlorides, at equivalent chloride ion concentrations, have identical effects in depressing the P.D. This proves that here too, as in the case of other proteins, the P.D. is affected only by the ion of opposite charge to that of the protein ion.

Table IV gives results which indicate that the predictions of the Donnan theory are fulfilled on the alkaline side of the isoelectric

⁹ Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 617.

point. Here the pH inside was found to be less than the pH outside, and the observed p.D. was opposite in sign to that observed with the acid solutions, the inside solution now being negative with respect to the outside. While the quantitative agreement between the observed and calculated values is not so good as on the acid side, this is probably due to the effect of CO₂ from the air on the pH values. The solutions were protected by soda lime tubes while osmotic equilibrium was being reached, but were open to the air during the p.D. measurements.

IV.

Osmotic Pressure.

The osmotic pressure of these solutions was affected in the same sense by changes in pH as in the case of other proteins. On the acid side the osmotic pressure increased from small values near the isoelectric point to a maximum in the neighborhood of pH 3, and decreased with further increases in acidity. This is qualitatively in accord with the Donnan theory. The actual calculation of the osmotic pressure from the hydrogen ion measurements, however, gave values which were much higher than those observed, the maximum in the case of globulin chloride being over 3.5 times as high as that observed. The explanation for this discrepancy may lie in the existence of large aggregates in the globulin solutions. Loeb has shown¹⁰ that the presence of undissolved particles of gelatin in place of dissolved gelatin has very little effect on the p.D. of a gelatin chloride solution, but that it materially decreases the osmotic pressure, the undissolved particles appearing to have but a slight share in the osmotic pressure as measured by the manometer. Since many of the globulin solutions were very opaque it is quite probable that much of the globulin was in the form of particles which produced no measurable osmotic pressure. However, a second preparation of globulin gave nearly clear solutions in HCl which still had an osmotic pressure much less than that calculated from the pH measurements.

The small osmotic pressure which was measured, however, was affected by salts in the way predicted by the theory. Table III shows that equivalent concentrations of different chlorides had the

¹⁰ Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 769.

same effect in decreasing this osmotic pressure, the decrease being evidently dependent on the concentration of the ion of opposite sign of charge to that of the protein ion.

The osmotic pressure observed in the experiments with alkali was more nearly of the same magnitude as that calculated on the basis of the Donnan theory, indicating that the sodium globulinate probably contained fewer large aggregates than the globulin chloride. Quantitatively, however, the agreement was poor; possibly this may be due to the presence of sodium carbonate.

SUMMARY.

1. The globulin prepared from ox serum by dilution and precipitation with carbon dioxide has been found, by electrometric titration experiments, to behave like an amphoteric electrolyte, reacting stoichiometrically with acids and bases.

2. The potential difference developed between a solution of globulin chloride, phosphate, or acetate and a solution of the corresponding acid, free from protein, separated from the globulin by a collodion membrane, was found to be influenced by hydrogen ion concentration and salt concentration in the way predicted by Donnan's theory of membrane equilibrium. In experiments with sodium globulinate and sodium hydroxide it was found that the potential difference could be similarly explained.

3. The osmotic pressure of such solutions could be qualitatively accounted for by the Donnan theory, but exhibited a discrepancy which is explicable by analogy with certain experiments of Loeb on gelatin.

4. The application of Loeb's theory of colloidal behavior, which had previously been found to hold in the case of gelatin, casein, egg albumin, and edestin, has thus been extended to another protein, serum globulin.

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