THE EFFECT OF ACID AND ALKALI ON THE OSMOTIC PRESSURE OF SERUM PROTEINS¹

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The effect of alterations in the state of aggregation of colloids is of great importance in connection with the exchange of water in living cells. Many conditions affect the osmotic pressure of colloidal solutions, and such changes may be partially responsible for plasmolysis and turgor in plant cells and for the laking of red blood corpuscles.²

The relation of proteins to the exchange of water between blood vessels and lymph spaces was pointed out by Starling,³ who was the first to measure the osmotic pressure of the proteins of blood serum. Moore and Parker,⁴ whilst confirming the experimental results obtained by Starling in connection with the measurement of osmotic pressure of blood serum, criticised the suggestion that the endosmotic action of proteins could account for absorption of salt solutions from the connective tissue spaces into the blood vessels.

More recently Moore and Roaf⁵ have discussed the question, whether the observed pressure readings are due to the electrolytes present in the solution. They point out that, although Reid,⁶ by repeated precipitation and re-solution, obtains protein solutions which do not give evidence of osmotic pressure, his results are probably caused by a change in the 'solution aggregate'⁷ of the protein.

Lillie,⁸ using gelatin and egg-albumin, has brought forward

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2. cf. Lillie, *Amer. Journ. Physiol.*, Vol. XX, p. 167, 1907.

3. *Journ. Physiol.*, Vol. XIX, p. 312, 1896.

4. *Amer. Journ. Physiol.*, Vol. VII, p. 261, 1902.

5. *Bio-Chem. Journ.*, Vol. II, p. 34, 1906.

6. *Journ. Physiol.*, Vol. XXXI, p. 438, 1904.

7. Moore and Parker, *loc. cit.*, p. 262.

8. *Amer. Journ. Physiol.*, Vol. XX, p. 127, 1907.

evidence which confirms and amplifies the results of Moore and Roaf. He finds that proteins give an osmotic pressure when contained inside collodium sacs, whilst the crystalloids pass through easily not affecting the final pressure recorded. Further, non-electrolytes have practically no effect on the pressure, but electrolytes markedly influence the state of aggregation thus changing the osmotic pressure.

Although his experiments and reasoning are along similar lines to those of Moore and Roaf he is unfortunately misled as to their idea of the nature of the action of electrolytes on the osmotic pressure of the colloids. He states that they consider that the electrolytes 'in some unexplained manner confer on the colloid the power of exerting osmotic pressure.' But it is evident from their paper that they consider the effect as being due to a change in solution aggregate of the protein, although they do not attempt to explain the mechanism by which the electrolyte produces this change in the protein.

The two papers are so fully in accordance on certain fundamental deductions that it is desirable to state briefly the general conclusions given in both.

(i) The existence of a homogeneous solution and diffusion is evidence of osmotic pressure influences even if the pressure is too low for measurement by any method known at the present time.

(ii) The osmotic pressure is due to colloid, as all crystalloids could pass through the membranes used and would not give a permanent pressure.

(iii) There is no sharp line of demarcation between crystalloids and colloids but that they gradually merge into each other at the margins of the groups.

(iv) Electrolytes have a marked influence on the osmotic pressure of proteins.

The experimental work to follow was commenced in order to investigate further the effect of acid and alkali on the osmotic pressure of serum proteins.

METHODS

The form of osmometer and the technique for filling it was the same as that previously described.¹

The blood serum was obtained by centrifuging stirred pig's blood. A known quantity of standardised acid or alkali was taken and made up to the required volume by means of the serum. The *amount* of acid or alkali could then be expressed in terms of normal solutions.

These solutions were then immediately placed in the osmometer, and readings taken until a permanent osmotic pressure was recorded.

At the end of the experiment a known weight of solution was evaporated to dryness at 100 C. in a tared vessel, cooled in a desiccator, and weighed; the residue was incinerated, and weighed again after cooling. In this way the weights of the solution, the total solids and ash were determined. By subtracting the weight of ash from the total solids the amount of organic matter was obtained and calculated as percentage of the weight of solution taken. The organic matter on the saline side of the membrane showed the amount of diffusible organic matter, and the percentage of this should be uniform throughout the solution; therefore, the difference between the percentages of organic matter on the serum side and on the saline side gives the percentage of organic matter unable to pass through the parchment paper, and this amount is considered to be protein in nature.

The fluid used on the outer side of the osmometer was 0.9 per cent. sodium chloride to which an amount of acid or alkali had been added to make an equal strength to that in the serum placed on the other side of the parchment paper.

There was no heating of the serum with the alkali or acid added. Hence there was no hydrolysis directly into alkali or acid albumin such as would break up the protein molecule.

The osmotic pressure was finally expressed in millimetres of mercury for each 1 per cent. of protein present.

1. Moore and Roaf, *loc. cit.*, pp. 46-51.

Experiment I.—Pig's serum against 0.9 per cent. sodium chloride.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
16	...	3	
40	...	7	
64	...	9	
88	...	14	
112	...	21	
136	...	25	
160	...	25	
184	...	25	
208	...	25	
		On serum side	On saline side
Percentage of organic matter	...	6.762	0.136
Percentage of protein by difference	...		6.626
Osmotic pressure for each 1 per cent. of protein = 3.7 mm. Hg.			

SERIES I.—EXPERIMENTS IN WHICH ALKALI WAS ADDED TO SERUM

Experiment II.—0.25 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.025 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
17	...	21	
41	...	27	
65	...	27	
89	...	27	
114	...	27	
138	...	27	
162	...	27	
185	...	27	
		On serum side	On saline side
Percentage of organic matter	...	4.619	0.157
Percentage of protein by difference	...		4.462
Osmotic pressure for each 1 per cent. of protein = 6.05 mm. Hg.			

Experiment III.—0.375 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.0375 N. ; against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
14	...	24	
39	...	30	
62	...	33	
86	...	35	
110	...	36	
134	...	37	
158	...	37	
183	...	37	
208	...	37	
		On serum side	On saline side
Percentage of organic matter	...	5.772	0.359
Percentage of protein by difference	...		5.413
Osmotic pressure for each 1 per cent. of protein = 6.83 mm. Hg.			

Experiment IV.—0.5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.05 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
15	...	30	
38	...	58	
62	...	66	
86	...	70	
110	...	70	
134	...	70	
		On serum side	On saline side
Percentage of organic matter	...	9.294	0.390
Percentage of protein by difference	...		8.904
Osmotic pressure for each 1 per cent. of protein = 7.86 mm. Hg.			

Experiment V.—0.75 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.075 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
16	...	34	
40	...	60	
65	...	64	
88	...	64	
112	...	65	
136	...	65	
160	...	65	
184	...	65	
		On serum side	On saline side
Percentage of organic matter	...	7.009	0.261
Percentage of protein by difference	...		6.748
Osmotic pressure for each 1 per cent. of protein = 9.63 mm. Hg.			

Experiment VI.—1.25 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.125 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
13	...	34	
40	...	63	
61	...	68	
85	...	73	
109	...	78	
133	...	80	
157	...	83	
181	...	86	
207	...	91	
229	...	95	
253	...	95	
277	...	95	
		On serum side	On saline side
Percentage of organic matter	...	8.308	0.325
Percentage of protein by difference	...		7.983
Osmotic pressure for each 1 per cent. of protein = 11.89 mm. Hg.			

Experiment VII.—2.5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.250 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.	
0	...	0	
12	...	31	
15	...	41	
37	...	79	
60	...	97	
84	...	107	
108	...	115	
132	...	121	
160	...	129	
184	...	130	
208	...	130	
		On serum side	On saline side
Percentage of organic matter	...	7.218	0.213
Percentage of protein by difference	...		7.005
Osmotic pressure for each 1 per cent. of protein = 18.54 mm. Hg.			

Experiment VIII.—3.75 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.375 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.	
0	...	0	
15	...	57	
39	...	78	
63	...	104	
88	...	123	
111	...	128	
135	...	132	
159	...	134	
183	...	135	
207	...	136	
231	...	136	
		On serum side	On saline side
Percentage of organic matter	...	6.262	0.511
Percentage of protein by difference	...		5.751
Osmotic pressure for each 1 per cent. of protein = 23.65 mm. Hg.			

Experiment IX.—5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.500 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
13	...	28	
37	...	54	
61	...	86	
85	...	97	
109	...	97	
133	...	91	
157	...	87	
182	...	85	
206	...	81	
229	...	75	
Percentage of organic matter		On serum side	On saline side
...		4.851	1.502
Percentage of protein by difference		...	3.349
Osmotic pressure for each 1 per cent. of protein = 22.39 mm. Hg.			

Experiment X.—7.5 c.c. of 40 per cent. sodium hydrate made up to 100 c.c. with serum, making a strength of alkali equivalent to 0.750 N, against 0.9 per cent. sodium chloride containing the same strength of alkali.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
15	...	27	
39	...	45	
63	...	67	
87	...	78	
111	...	74	
135	...	72	
159	...	71	
Percentage of organic matter		On serum side	On saline side
...		6.319	1.825
Percentage of protein by difference		...	4.494
Osmotic pressure for each 1 per cent. of protein = 15.76 mm. Hg.			

In this series of experiments it is seen that the osmotic pressure for each 1 per cent. of protein is higher the greater the amount of alkali added until a maximum is reached. Above this maximum the osmotic pressure diminishes because the alkali acts on the protein splitting it up into products that can pass through the parchment paper. Thus in Experiments IX and X no permanent pressure is produced, and after the pressure reaches a maximum it slowly falls. That this fall is due to dialysis of protein decomposition products is shown by the increased percentage of organic matter in the fluid on the saline side of the osmometer.

SERIES II.—EXPERIMENTS IN WHICH ACID WAS ADDED TO SERUM

Experiment XI.—0.5 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.02 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

This experiment was continued for a period of 264 hours during which time no evidence of osmotic pressure was noticed. At the end of the experiment no leak could be found in the apparatus.

Experiment XII.—1.0 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.04 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
17	...	0	
41	...	0	
65	...	0	
89	...	0	
113	...	1	
137	...	2	
161	...	3	
185	...	4	
209	...	5	
233	...	5	
257	...	5	
		On serum side	On saline side
Percentage of organic matter	...	6.743	0.162
Percentage of protein by difference	...		6.581
Osmotic pressure for each 1 per cent. of protein = 0.76 mm. Hg.			

Experiment XIII.—1.5 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.06 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment		Pressure in mm. of mercury	
hours		mm.	
0	...	0	
16	...	0	
40	...	9	
64	...	12	
88	...	18	
112	...	19	
136	...	24	
160	...	24	
184	...	24	
209	...	25	
232	...	25	
256	...	25	
		On serum side	On saline side
Percentage of organic matter	...	6.716	0.324
Percentage of protein by difference	...		6.392
Osmotic pressure for each 1 per cent. of protein = 3.91 mm. Hg.			

Experiment XIV.—2.0 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.08 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.	
0	...	0	
14	...	0	
38	...	1	
62	...	2	
86	...	3	
110	...	10	
134	...	15	
158	...	17	
182	...	18	
207	...	19	
230	...	23	
254	...	24	
278	...	29	
302	...	32	
326	...	32	
		On serum side	On saline side
Percentage of organic matter		5.924	0.485
Percentage of protein by difference			5.439
Osmotic pressure for each 1 per cent. of protein = 5.87 mm. Hg.			

Experiment XV.—3.0 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.12 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.	
0	...	0	
15	...	32	
43	...	42	
63	...	43	
87	...	43	
111	...	42	
135	...	43	
159	...	43	
		On serum side	On saline side
Percentage of organic matter		6.052	0.400
Percentage of protein by difference			5.652
Osmotic pressure for each 1 per cent. of protein = 7.61 mm. Hg.			

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Experiment XVI.—3.5 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.14 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	0
39	...	4
63	...	9
87	...	9
111	...	10
135	...	14
159	...	16
183	...	17
207	...	17
		On serum side On saline side
Percentage of organic matter		6.755 0.565
Percentage of protein by difference		6.190
Osmotic pressure for each 1 per cent. of protein = 2.75 mm. Hg.		

Experiment XVII—4.0 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.16 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.
0	...	0
15	...	0
39	...	0
63	...	0
87	...	1
111	...	7
135	...	13
159	...	18
183	...	18
		On serum side On saline side
Percentage of organic matter		7.612 0.276
Percentage of protein by difference		7.336
Osmotic pressure for each 1 per cent. of protein = 2.45 mm. Hg.		

Experiment XVIII.—4.5 c.c. of 14.6 per cent. hydrochloric acid made up to 100 c.c. with serum, making a strength of acid equivalent to 0.18 N, against 0.9 per cent. sodium chloride containing the same strength of acid.

Time from commencement of experiment hours		Pressure in mm. of mercury mm.	
0	...	0	
16	...	0	
40	...	0	
64	...	0	
88	...	0	
112	...	1	
136	...	2	
160	...	4	
184	...	8	
208	...	12	
232	...	16	
256	...	18	
280	...	20	
304	...	22	
328	...	23	
352	...	24	
376	...	25	
400	...	25	
424	...	25	
		On serum side	On saline side
Percentage of organic matter		6.830	0.581
Percentage of protein by difference			6.249
Osmotic pressure for each 1 per cent. of protein = 4.00 mm. Hg.			

In these experiments it is seen that the addition of a small amount of acid causes the osmotic pressure of serum proteins to fall so that only zero readings can be obtained. This amount of acid (0.02 N) is very nearly the same as that found by Moore and Wilson for the ash of serum (0.03 N)¹ using 'di-methyl' as an indicator. Further addition of acid causes a rise of pressure, but beyond a certain strength of acid the pressure drops and becomes irregular. This drop is not due to the acid splitting up the protein into hydrolytic products capable of passing through the membrane because there is no corresponding rise in the total solids of the outer fluids, such as was seen in the experiments with the stronger strengths of alkali. The solutions which gave the varying pressures showed a yellow flocculent precipitate, and the fall of pressure was therefore probably due to partial precipitation of the protein from the solution.

1. *Bio-Chem. Journ.*, Vol. I, p. 297, 1906.

The results obtained in the experiments are summarised in the following Table.

ALKALI ADDED										
No. of Experiment	I	II	III	IV	V	VI	VII	VIII	IX	X
Alkali added to serum expressed in normal concentrations ...	0	0.025	0.0375	0.05	0.075	0.125	0.25	0.375	0.50	0.75
Corresponding osmotic pressure in mm. of mercury per 1 per cent. of protein present ...	3.7	6.05	6.83	7.86	9.63	11.89	18.54	23.65	22.39	15.76
Value of solution aggregate corresponding to the osmotic pressure observed	45,887	28,063	24,859	21,601	17,631	14,279	9,157	7,179	7,583	10,773

ACID ADDED									
No. of Experiment	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	
Acid added to serum expressed in normal concentrations ...	0.02	0.04	0.06	0.08	0.12	0.14	0.16	0.18	
Corresponding osmotic pressure in mm. of mercury per 1 per cent. of protein present ...	0	0.76	3.91	5.87	7.61	2.75	2.45	4.00	
Value of solution aggregate corresponding to the osmotic pressure observed	0	223,400	43,423	28,924	22,310	61,739	69,300	42,446	

These results are plotted together in the accompanying curve (Fig. 1) where the ordinates represent pressures in millimetres of mercury for each one per cent. of protein, and the abscissae show the amount of normal acid or alkali added to the serum. The zero point marks the pressure obtained with normal serum, whilst α shows the point of neutrality of the ash of serum as obtained by Moore and Wilson. If the point α be considered as the true neutral point then the alkalinities should be corrected by adding this amount (0.03 N) to the actual quantity of alkali added, whilst the acidities should have the same amount subtracted. The value of this correction is strikingly shown in the figure, where the continuation of the acid curve practically cuts the zero pressure at the point α . The two following curves (Fig. 2) show the effect of plotting the logarithms of the amounts of alkali against the logarithms of the pressures. The upper curve is plotted using the amount of alkali actually added to the serum, whilst the lower figures are corrected for the amount of alkali present in the ash of serum. The lower curve shows a logarithmic relation between the two sets of figures, so that the neutral point of serum ash determined by Moore and Wilson must have some

0.0 shows the normal serum. The amount of acid added reads to the left, and the amount of alkali added reads to the right; x shows the neutral point of serum ash as obtained by Moore and Wilson (0.03 N). The numbers on the curve show the number of the experiment from which the point was determined.

relation to the influence of reaction on the osmotic pressure of serum proteins.

Apparently the point of exact neutrality corresponds to the condition in which the osmotic pressure is least. This would explain many points in connection with determinations of osmotic pressure of proteins. Reid¹ by repeated precipitation and washing removed the

Logarithms of the amounts of normal alkali present. Upper curve shows results obtained experimentally; lower curve shows results when the amount of alkali found by Moore and Wilson for serum ash is added to that actually used. The numbers on the curves show the number of the experiment from which point was determined.

alkali of the serum, and the partial return of osmotic pressure that he obtained on adding some of the washings to the pressure-free protein could be due to a little of the alkali in the washings acting in the same way as the alkali in the present series of experiments. Lillie² found

1. *Loc. cit.*
2. *Loc. cit.*, p. 138.

that addition of a small quantity of acid caused a marked drop of osmotic pressure, and that a little more acid caused the pressure to rise again. In the case of egg-albumin this rise was never up to the original pressure for the normal egg-albumin, but with gelatine the pressure with larger quantities of acid rose much above its original value.

The osmotic pressure of blood serum obtained by Starling was slightly greater than that obtained by Moore and Parker or Moore and Roaf. This would be due to Starling using the protein-free filtrate of serum as the solution against which the osmotic pressure was determined whilst the other authors used salt solution, and the amount of alkali would be greater in the former case than in the latter. It is interesting to note that in precipitating proteins by neutral salts it is usual to add a trace of acid in order to obtain more complete precipitation. This would neutralise the slight degree of alkalinity usually associated with natural proteins and bring about the condition where there is least osmotic pressure, and hence the colloid being in a less stable condition it is more easily precipitated.

The cause of the change in 'solution aggregate' brought about by the change in reaction has not been determined. In the case of the alkaline solutions it is not due to an ordinary hydrolysis of the protein. Hydrolytic action would be progressive, and should cause a gradual increase of diffusible organic matter as the strength of alkali is raised; but there is no increase until the alkali reaches 0.37 normal, when hydrolytic products appear in the saline solution on the outer side of the osmometer. Lillie¹ found that the similar rise of osmotic pressure produced by treating gelatin with acid, disappears slowly on removal of the acid by dialysis. If the change were due to slight hydrolysis, with weaker strengths of alkali one must assume that the amphoteric electrolytes produced neutralise the alkali so that the change does not proceed beyond an early stage and that removal of the alkali allows the protein to become re-associated into its original state. The increase in colloidal particles is unlikely to be due to the alkali lowering the surface tension between the solvent and solute, because addition of

1. *Loc. cit.*, p. 142.

inorganic salts usually raises the surface tension at an air-water surface.

The action of acid is probably of the same nature as that of alkali but owing to the onset of partial precipitation the series of experimental determinations is not so extensive. This partial precipitation points to some change which overcomes the tendency of the osmotic pressure to increase and which allows some constituent of the serum to form solution aggregates of increased size until the aggregates become of such size that they are visible to the naked eye.

The relationship of these changes in solution aggregate (osmotic pressure) to the action of neutral salts in effecting plasmolysis and laking, and to the effect of acid and alkali on cell growth and cell division, is being further studied.¹

CONCLUSIONS

1. Alkali causes an increase in the osmotic pressure of the colloidal constituents (proteins) of blood serum. This increase is not directly proportional to the amount of alkali added.

2. Acid causes the osmotic pressure of the colloidal constituents (proteins) of blood serum to fall to zero when small amounts of acid are used, but with larger quantities the pressure rises again.

3. The point at which zero pressure exists practically corresponds to the alkaline 'reactivity' of serum ash, obtained by Moore and Wilson, using di-methyl-amido-azo-benzole as an indicator.

4. When the amount of alkali added is corrected by the above-mentioned alkaline 'reactivity' the relationship of the osmotic pressure to the amount of alkali is a logarithmic one. Similarly corrected, the relationship of the increase of osmotic pressure on addition of acid is probably logarithmic to the amount of acid added, but the series of experimental observations is not long enough to give an accurate curve. These facts confirm the accuracy of the observations of Moore and Wilson in determining the reaction of blood serum ash.

1. For a comparison with the action of various agents on the swelling of gelatine (which probably depends on colloidal osmotic pressure) see W. Pauli, *Ergebnisse der Physiologie*, Vol. VI, p. 105, 1907.

5. The maximum of pressure obtained by adding alkali is limited by the breaking up of the protein into particles small enough to pass through the parchment paper membrane, whilst the maximum pressure on the addition of acid is reached when the protein is partially precipitated from solution.

In conclusion we wish to thank Professor Benjamin Moore for much help during the course of this research.