CCXXXIX. SOME OBSERVATIONS ON MIXTURES OF SERUM ALBUMIN AND GLOBULIN

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McFARLANE [1935], using normal sera and mixtures of serum proteins, and Pedersen [1936], using other protein mixtures, observed a curious phenomenon affecting the velocities of ultracentrifugal sedimentation of protein mixtures; this may be described as an apparent increase in the amount of the less rapidly sedimenting protein at the expense of that sedimenting more rapidly. It is to be presumed that the effect is caused by a decrease in the sedimentation rate of some of the latter, probably due to change of molecular size. The most surprising feature is the observation that in mixtures of proteins of different sedimentation constants, the new sedimentation rate is nearly always close to or identical with that of the protein having the smaller molecular size; it appears as if one protein exercises a specific effect on the molecular size of the other. With serum proteins the effect shows the salient features of a reversible equilibrium. No entirely convincing explanation of this effect has been put forward.

The following experiments were made on mixtures of normal serum albumin and globulin, in order to obtain further evidence as to its nature.

EXPERIMENTAL

Proteins. Albumin and pseudoglobulin from normal horse serum were used in most of the experiments. Albumin was several times recrystallized by the method of Hewitt [1936]; the product showed good crystalline form, was almost free from pigment and had a carbohydrate content of less than 0.1 %. Pseudoglobulin was separated from the same serum by several precipitations with 40 % saturated ammonium sulphate, dialysis against water and rejection of protein not soluble in distilled water. Human serum albumin was obtained by several precipitations by 2/3 saturated ammonium sulphate: human pseudoglobulin from the same serum was prepared in the same way as horse pseudoglobulin. Protein solutions were estimated by micro-Kjeldahl nitrogen determination, using a conversion factor of 6.45.

Osmotic measurements were designed to supplement those made by McFarlane on whole sera at neutrality to provide a check on the ultracentrifugal observations. Decrease of the molecular size of one component of a mixture should give osmotic pressures greater than those to be expected from osmotic pressures of solutions of the separate components.

In these experiments the proteins were dissolved in acetate buffer *pH* 4.8 of ionic strength 0.2; in these circumstances, the *pH* being close to the isoelectric points of the proteins and the ionic concentration fairly high, the effect on osmotic pressure of unequal distribution of diffusible electrolytes across the membrane can be neglected.

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The osmometer was of the design of Oakley [1936]: collodion membranes were prepared under fairly standard conditions: the temperature was $25 \pm 0.001^\circ$. Protein solutions were first dialysed against a large volume of buffer, and this same buffer solution was used in the osmometer and for making dilutions. Osmotic equilibrium was usually attained in about 4 hr.

The osmotic pressure-concentration curves for horse albumin and globulin are shown in Fig. 1—there is no sign of departure from linearity. The slopes of the curves show the albumin to be somewhat polydisperse ($M=65,000$), the

Fig.1. Concentration g./100 ml. solvent. $\circ$ Albumin. $\square$ Pseudoglobulin.

globulin considerably so ($M=97,000$). In Table I are given the results of measurements on mixtures: the concentrations and differences between observed osmotic pressures and those calculated from the curves in the figure are given. Osmotic pressures are in mm. of liquid sp. gr. 1.0: concentrations in g. per 100 ml. of solvent.

Table I

Conc. g./100 ml. of solvent		Osmotic pressure mm. H_2O at 4°		
Albumin	Globulin	Observed	Calculated	Difference
1.070	0.953	67.40	66.5	+0.90
		67.62		+1.12
1.434	1.276	94.94	89.5	+5.44
		96.21		+6.71
0.716	1.910	78.69	77.4	+1.29
1.078	1.917	96.12	92.0	+4.12
		94.51		+2.51

The ultraviolet absorption curves of solutions of the horse proteins and of some mixtures of them were measured: the object was to find whether any anomalous mixture effect could be discovered in this property. The mixtures were made up and left for at least 12 hr. to establish equilibrium; diluted to the concentration required for spectrography, and their absorption spectra photographed within

30 min. after dilution. McFarlane's results gave reason to suppose that the establishment of a new equilibrium following dilution might be very slow.

Absorption spectra were measured with a Hilger E 316 spectrograph with Spekker photometer, illumination being by a condensed spark between tungsten steel electrodes. Plates were matched with a split beam photoelectric densitometer.

No anomalous mixture effect is apparent. This is shown by analysis of the absorption curves of mixtures, in terms of those of their components, at 18 selected wave-lengths by the Gauss method of least squares. In Table II the concentrations estimated in this way are compared with their known values: the agreement is close.

Table II

Albumin conc. g. per 100 ml.			Globulin conc. g. per 100 ml.		
Known	Estimated	% diff.	Known	Estimated	% diff.
3.42	3.38	- 1.1	1.978	1.930	- 2.4
8.37	8.41	+ 0.5	0.805	0.795	- 1.3
4.23	4.21	- 0.5	3.95	3.87	- 2.0

Precipitation experiments: the object of these experiments was to discover whether the supposed change of molecular state of the globulin in mixtures with albumin produces any change in precipitability by ammonium sulphate.

The first series was directed to detecting any complex formation between albumin and globulin: a solution about 4% in both albumin and globulin was made up and left for several days before use. To 1 ml. portions of this solution in small centrifuge tubes, saturated ammonium sulphate solution was added to bring the mixtures to 40 or 50% in ammonium sulphate; after varying periods the precipitate was centrifuged down, and the composition of the clear supernatant was determined spectrographically. The results (Table III) show no significant changes in the precipitation with time of flocculation such as could be due to the presence of complexes in solution; all the globulin is precipitated by 50% saturation within 5 min.

In the second series the object was to find whether dilution of the stock solution and precipitation with ammonium sulphate at different times after dilution gave different results: these might have been ascribed to the shift of the equilibrium of the mixture effect with dilution noted by McFarlane. In this series a standard time was allowed for flocculation. Table IV shows that the results of this series were negative.

Table III. *Composition of supernatants after different flocculation times: concentrations in g./100 ml.*

Time of flocculation	Albumin			Globulin	
	Possible	Found	% diff.	Possible	Found
40% saturated ammonium sulphate					
5 min.	4.23	4.17	- 1.4	3.95	0.442
15 min.	4.23	4.09	- 3.3	3.95	0.503
16 hr.	4.23	4.20	- 0.7	3.95	0.439
50% saturated ammonium sulphate					
5 min.	4.23	4.38	+ 3.5	3.95	- 0.022
15 min.	4.23	3.96	- 6.4	3.95	- 0.088
80 min.	4.23	3.80	- 10.2	3.95	- 0.088
18 hr.	4.23	4.00	- 5.4	3.95	- 0.106

Table IV. *Composition of supernatants: standard flocculation time 20 min.: ammonium sulphate added at different times after dilution: concentrations in g./100 ml. referred back to original solution*

Dilution ×	Precipitant	Time	Albumin			Globulin	
			Possible	Found	% diff.	Possible	Found
5	40 % SAS	5 min.	4.23	3.85	-9.1	3.95	0.221
5	"	60 hr.	4.23	3.94	-6.8	3.95	0.251
2	50 % SAS	5 min.	4.23	4.34	+2.6	3.95	-0.082
2	"	3 hr.	4.23	4.30	+1.7	3.95	-0.066
5	"	5 min.	4.23	4.14	-2.1	3.95	+0.096
5	"	4 hr.	4.23	4.44	+5.0	3.95	-0.102

The small negative values found for globulin in some cases are probably due to the effect of small amounts of pigment present in the solutions. The precipitation of albumin by 40 % saturated ammonium sulphate apparent in two experiments is unexplained.

The potentiometric titration curves of solutions of human albumin (2.98 %) and of human pseudoglobulin (1.94 %) and of a mixed solution (albumin 1.19 %, globulin 1.16 %) were determined by the method of Ogston [1936]. The object was again to test how closely the mixture law is obeyed with respect to this property. The results given in Table V show that it is obeyed closely, especially between pH 4 and 8.

Table V. *Titre from pH 7: milliequivalents per g. protein*

pH	Albumin	Globulin	Mixture obs.	Mixture calc.	Difference
3.0	1.214	0.640	0.950	0.931	+0.019
3.5	0.959	0.510	0.740	0.737	+0.003
4.0	0.667	0.353	0.515	0.512	+0.003
4.5	0.431	0.232	0.330	0.333	-0.003
5.0	0.272	0.145	0.209	0.210	-0.001
5.5	0.175	0.094	0.139	0.135	+0.004
6.0	0.105	0.060	0.087	0.083	+0.004
6.5	0.049	0.029	0.040	0.039	+0.001
7.0	0	0	0	0	—
7.5	0.050	0.021	0.038	0.035	+0.003
8.0	0.090	0.043	0.071	0.067	+0.004
8.5	0.130	0.060	0.100	0.096	+0.004
9.0	0.172	0.087	0.138	0.130	+0.008
9.5	0.231	0.132	0.196	0.182	+0.014
10.0	0.327	0.210	0.285	0.269	+0.016
10.5	0.472	0.318	0.408	0.396	+0.012
11.0	0.679	0.460	0.570	0.570	0

DISCUSSION

In the discussion following it is proposed to introduce the conclusions from the above experiments as they are required for the general argument.

From the sedimentation results the phenomenon considered appears to be of the nature of a reversible reaction: the velocity with which equilibrium is established has not been studied; but the reverse reaction seems to be a very slow one, since McFarlane observed no diminution of the amount of "albumin" in the course of a run; further, continuous formation of unchanged globulin should lead to a slope in the base-line of the sedimentation diagram between the albumin and globulin boundaries. These observations were made in buffer solutions of moderate concentration covering a range of pH values.

The possibility that the effect is due to some hitherto unconsidered physical cause operating in measurements of sedimentation velocity is probably excluded by the osmotic measurements here made, taken in conjunction with those of McFarlane. Positive deviations were obtained with all mixtures; that these are due to purely physical causes is unlikely in view of the linearity of the osmotic pressure curves of simple proteins up to concentrations as great as the total protein concentrations in the mixtures. Quantitative comparison with McFarlane's sedimentation results is not possible, owing to the considerable heterogeneity of the proteins used here and to unexplained peculiarities of the sedimentation results, discussed by McFarlane. However, all evidence favours the view that in a mixture of these proteins there is some conversion of larger into smaller molecules.

It might be expected that such a change would involve some alteration of properties other than molecular dimensions. The precipitation experiments were designed to show whether there is any change in precipitability with ammonium sulphate. The results show that no such change can be detected even when the time of flocculation is as short as five minutes: with 50 % ammonium sulphate precipitation, fractionation is complete, though some albumin is removed from solution on subsequent standing; nor does precipitation at different times after dilution of a concentrated mixture reveal any significant differences. Either there is no change of solubility properties, or the high concentration of ammonium sulphate changes the very slow back reaction to a very fast one.

Similarly, the ultraviolet absorption curves show no significant difference; again this may be due to a comparatively rapid back reaction associated with dilution, though McFarlane's findings make this unlikely. No anomalous mixture effect appears in the titration curves, where there is no appreciable dilution: such an effect might have been expected at least over a certain range of *pH*. It seems that no estimable number of new titratable groups is liberated, nor are dissociation properties of titratable groups appreciably affected.

Apart from these facts, there are other considerations which make acceptance of the "reaction" hypothesis difficult: in terms of this, the sedimentation results, admittedly few, obtained to date may be summarised:

McFarlane [1935]	Globulin ($M \sim 140,000$) $\rightarrow$ Albumin ($M \sim 70,000$)
Pedersen [1936]	Albumin ($M \sim 70,000$) $\rightarrow$ Lactoglobulin ($M \sim 35,000$)
	Albumin ($M \sim 70,000$) $\rightarrow$ Clupein ($M \sim 18,000$)
	Haemoglobin $\rightarrow \frac{1}{2}$ molecules whether with clupein or other proteins (unspecified)
	Amino-acids and polypeptides have a small (unspecified) effect on other proteins (unspecified)

These could be explained by formation of complex molecules from fragments of both components of a mixture; it is difficult to understand why such molecules should take up those particular sedimentation properties, and measurable alteration of other properties would be confidently expected. The explanation favoured by Pedersen, that the influence is one of "medium", is unconvincing in view of the specificity of the effect, though the action of amino-acids and polypeptides supports it; forces producing such an effect, though specific and of sufficient strength, must be insufficient to bind molecules together in solution in any chemical or physical sense. It is difficult to guess at the nature of such forces.

It seems that the evidence at present available does not warrant the support of any known hypothesis of the nature of this effect. The line of attack on the problem most likely to be profitable lies in the further investigation of the kinetics and equilibrium properties of the phenomenon; it is suggested that combined measurements should be made by sedimentation velocity and equilibrium methods and of osmotic pressures on mixtures of such proteins as can be prepared in a state of high purity and homogeneity.

REFERENCES

- Hewitt (1936). *Biochem. J.* **30**, 2229.
McFarlane (1935). *Biochem. J.* **29**, 407, 660.
Oakley (1936). *Biochem. J.* **30**, 868.
Ogston (1936). *Biochem. J.* **30**, 1845.
Pedersen (1936). *Nature, Lond.*, **138**, 363.