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The Interaction of Serum Albumins with Calcium

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Pribram in 1871 postulated that some part of the serum calcium was bound to the serum proteins. Rona & Takahashi (1911) produced experimental evidence justifying Pribram's suggestion. Numerous authors (Loeb, 1926; Marrack & Thacker, 1926; Greenberg & Greenberg, 1931; McLean & Hastings, 1935; Weir & Hastings, 1936; Drinker, Green & Hastings, 1939; Ludewig, Chanutin & Masket, 1942) have published papers on qualitative and quantitative studies of the distribution of calcium in human serum, and the extent to which it may combine with human and other proteins.

McLean & Hastings (1935) gave tables of pK values for calcium proteinate based on a hypothetical mass law relationship

$$\frac{[\text{Ca}^{++}] [\text{Protein}^{-}]}{[\text{Ca protein}]} = K,$$

and in the same paper developed a nomogram based on this mass law to explain the relationships in human serum. Weir & Hastings (1936) produced further data to support this relationship, but revised the pK values. Ludewig *et al.* (1942) re-examined the relationship of calcium to protein in human serum, using the ultracentrifuge to study the equilibrium. They concluded that the system was too complex for the simple mass law to be applied.

Recent developments in the technique of low-temperature fractionation of proteins (Pillemer & Hutchinson, 1945; Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946; Kekwick & MacKay, 1949) offered fresh opportunity for the examination of the relationships of calcium to the individual serum proteins. Of these, albumin seemed the most satisfactorily characterized for careful study. In the present investigation we started with the object of discovering whether the calcium-binding capacities of albumins of different species

are the same, and whether albumins from the same species, separated and purified by different techniques, have the same calcium-binding capacity.

EXPERIMENTAL

The albumins examined had been prepared by the methods of Cohn *et al.* (1946), Pillemer & Hutchinson (1945) and Kekwick & MacKay (1949). The purity of the specimens was judged by electrophoretic analysis carried out in the 11 cm. analytical cell in phosphate buffer, ionic strength 0.2, at pH 8.0, using the moving-boundary technique of Tiselius (1939).

The Ca distribution was studied by dialysis. These dialyses were carried out at 2° in a two-chambered system separated by a semi-permeable membrane. The time taken to establish equilibrium was measured by controls run in parallel. Though equilibrium in the majority of cases was established in 72 hr. all specimens were left for 6 days before analysis of the ionic distribution between the two chambers was carried out. During this period the samples were stirred frequently to help to establish equilibrium. The ratio of the volume of fluid in the protein-free compartment to that in the protein-containing compartment was 15 : 1.

Determinations of pH were carried out on all samples at the completion of each experiment, using a glass electrode and the Cambridge potentiometer.

Ca determinations on the fluid in each compartment were made by a modification of the method of Trevan & Bainbridge (1926). Fig. 1 shows the percentage deviation of Ca estimated by our method, from Ca concentrations estimated gravimetrically on large samples.

Chloride determinations were carried out on the fluid of both compartments by the method of Van Slyke (1923).

The protein concentrations in the inner compartment were determined at the completion of each equilibrium by N determination using the standard micro-Kjeldahl technique. In each instance the fluid in the outer compartment was examined, and any experiment in which transference of N-containing materials could be demonstrated was rejected. The albumins had been dialysed against 0.15M-NaCl prior

to setting up the equilibrating systems. The final molarities ranged from 0.15 to 0.155. The only cations present were Ca and Na and the only anions were chloride and protein.

Fig. 1. Deviations of the Ca concentrations found by the method used in this work from the true Ca concentrations determined gravimetrically, expressed as percentages of the gravimetric values.

The distribution of freely diffusible ions at equilibrium across a semi-permeable membrane in the presence of one non-diffusible ion was investigated by Donnan & Harris (1911). They proved that if the distribution of a univalent anion was measured, then the distribution of a univalent cation present in the same system would be inversely proportional to that of the anion

$$\frac{[\text{Na}^+]_1}{[\text{Na}^+]_2} = \frac{[\text{Cl}^-]_2}{[\text{Cl}^-]_1}$$

Donnan & Garner (1919) extended the theory to the study of bivalent ions and showed that the distribution of bivalent

cations at equilibrium was proportional to the ratio of the square of the univalent ions

$$\frac{[\text{Ca}^+]_1}{[\text{Ca}^+]_2} = \frac{([\text{Na}^+]_1)^2}{([\text{Na}^+]_2)^2} = \frac{([\text{Cl}^-]_2)^2}{([\text{Cl}^-]_1)^2}$$

The corrections applied in this paper have been based on the distribution of chloride between the two chambers, assuming that at the concentrations employed there was complete ionization of CaCl_2 and that the salt does not form complexes when equilibrium has been established.

RESULTS

Human, bovine and equine albumins were examined. The results are arranged in Tables 1–3. Three specimens of bovine albumin and two of horse albumin were examined and compared with seven specimens of human albumin. The crude albumins prepared by methanol fractionation of horse and ox both had calcium-binding capacities of the order of 11 mg./g. protein nitrogen, which is markedly different from the calcium-binding capacities of three human albumins examined, all of which had calcium-binding capacities of the order of 4.5 mg./g. protein nitrogen. Two preparations of human albumin had no demonstrable calcium-binding capacity and the remaining two preparations, the one crystallized with the aid of decanol and the other in the presence of mercury, had calcium-binding capacities of the order of 1.0 and 9.0 respectively.

Tables 1 and 2 show the results on bovine and horse albumins and on crude and purified human albumin, respectively. Table 3 shows the results on a human albumin believed to have been denatured in the course of fractionation.

Table 1. Calcium-binding capacities of bovine and horse serum albumins

Material	pH	N of sample in sac (g./l.)	Ca (mg./l.)		Donnan coefficient calculated from chloride distribution	Calculated free Ca inside sac (mg./l.)	Bound Ca N (mg./g.)
			Outside sac	Inside sac			
Bovine albumin							
Methanol fractionation	6.70	0.80	125.2	137.0	1.01	126.9	12.6
(94-97%) (Pillemer &	6.76	0.80	124.1	134.2	1.01	125.4	11.0
Hutchinson, 1945)	6.84	2.33	134.0	162.5	1.02	137.2	10.9
	6.86	2.33	134.0	164.2	1.03	137.6	11.5
Ethanol fractionation	6.92	9.97	130.6	179.8	1.08	141.4	3.9
(95%) (Cohn <i>et al.</i> 1946)	6.95	9.97	130.6	179.2	1.09	142.0	3.7
Crystalline (100%) (Cohn,	6.10	4.57	144.7	158.6	1.04	148.7	2.2
Hughes & Weare, 1947)	6.30	9.13	138.1	167.3	1.07	147.1	2.2
Horse albumin							
Methanol fractionation	6.76	1.50	136.2	156.5	1.02	138.8	11.8
(94-97%) (Pillemer &							
Hutchinson, 1945)							
Crystallized carbohydrate-	6.32	1.07	94.9	107.4	1.02	96.3	10.4
poor (100%) Kekwick,	6.34	1.07	95.0	108.1	1.02	96.4	11.0
1938)	6.40	2.14	92.8	119.8	1.02	94.7	11.8
	6.42	3.21	90.8	130.1	1.03	92.5	11.8
	6.43	4.28	87.5	141.4	1.03	90.1	12.0

Table 2. *Calcium-binding capacities of human serum albumins*

Specimen	Material	pH	N of sample in sac (g./l.)	Ca (mg./l.)		Donnan coefficient calculated from chloride distribution	Calculated free Ca inside sac (mg./l.)	Bound Ca N (mg./g.)
				Outside sac	Inside sac			
1	Methanol fractionation (94-97%) (Pillemer & Hutchinson, 1945)	6.64	2.33	138.4	150.9	1.01	139.9	4.7
		6.69	4.70	137.3	164.4	1.04	142.1	4.7
2	Ether fractionation (85%)* (Kekwick & MacKay, 1949)	6.63	3.13	168.3	187.8	1.04	174.9	4.1
		6.71	6.27	174.8	214.8	1.08	188.7	4.2
3	25% (American Red Cross, for therapy) (95-97%)	5.86	11.0	124.4	135.9	1.09	135.6	Nil
		6.35	11.0	123.1	132.0	1.07	131.9	Nil
		6.41	11.0	123.1	132.0	1.08	132.9	Nil
		7.16	11.0	124.1	133.0	1.08	134.0	Nil
		7.24	11.0	123.1	133.0	1.08	132.9	Nil
		7.41	11.0	125.0	134.0	1.07	134.8	Nil
		7.50	11.0	125.0	134.0	1.07	134.8	Nil
		7.77	11.0	122.1	131.2	1.08	132.0	Nil
		7.84	11.0	127.2	136.1	1.08	137.4	Nil
4	Ethanol fractionation, 5 re-crystallizations (100%) (Cohn <i>et al.</i> 1946)	6.80	3.83	134.9	156.5	1.03	139.3	4.5
		6.76	5.07	130.6	159.0	1.04	135.5	4.6
		6.74	7.70	137.0	183.4	1.08	147.5	4.7
		6.76	10.07	130.6	185.5	1.06	138.5	4.7
5	Ethanol fractionation, 10 re-crystallizations (100%) (Cohn <i>et al.</i> 1946)	6.26	3.23	134.3	140.7	1.02	136.7	1.2
		6.30	6.37	130.6	140.3	1.03	134.2	1.0
		6.30	6.43	134.3	146.6	1.03	138.6	1.2
		6.33	10.03	138.1	152.1	1.04	142.0	1.0
6	Ethanol fractionation; re-crystallizations as mercury salt (100%) (Hughes, 1947)	6.79	4.67	138.9	185.6	1.03	142.7	9.2
		6.72	7.03	138.9	206.1	1.06	146.6	8.5

* 85% albumin from ether fractionation. If an arbitrary correction is made on the assumption that all the Ca is bound to the albumin only, the figure for mg. of Ca bound to g. of albumin N (last column) would be 4.8. This assumption is not strictly warranted on the data presented.

Table 3. *Calcium-binding capacity of substandard human albumin serum from ether fractionation*

Material	pH	N of sample in sac (g./l.)	Ca (mg./l.)		Donnan coefficient calculated from chloride distribution	Calculated free Ca inside sac (mg./l.)	Bound Ca N (mg./g.)
			Outside sac	Inside sac			
Ether fractionation (90%) (Kekwick & MacKay, 1949)	6.58	3.23	136.0	138.0	1.02	138.2	Nil
	6.50	4.83	136.0	140.1	1.03	139.6	Nil
	6.40	4.83	136.0	140.1	1.03	139.6	Nil

DISCUSSION

Although, by adopting the arguments and making the assumptions of McLean & Hastings (1935) and Weir & Hastings (1936), pK values could be derived from the data presented here, we do not feel that such a procedure is justified or useful in the present state of our knowledge. It would have been proper to study the calcium-binding over a wider range of pH. This would have involved the introduction of buffering ions in addition to those already present. We considered the use of phosphate buffers, but rejected them because of the known complexity of the binding of calcium with phosphates (Marrack & Thacker, 1926; Greenwald, 1942) and the imponder-

ables that such complexes might introduce into the system. Within the limited range of pH at which we explored and obtained calcium binding, though small fluctuations were observed they did not seem to bear any relation to changes in pH. This is in keeping with the findings of Ludewig, Chanutin & Masket (1942) in this range. Materials fractionated by identical methods in the same hands showed species variation. Within the limits of the conditions of our experiments there is clear evidence that there are species differences in calcium-binding capacity of albumins, and that these differences are well outside the limits of experimental error. It is interesting that ox and horse albumin showed the same order of calcium binding, differing sharply from human.

The recent studies of Scatchard, Scheinberg & Armstrong (1950) on the interaction of protein with chloride ions would, in a detailed treatment, entail a correction of Donnan coefficients, but as all our experiments were carried out over a limited and comparable range of pH and chloride concentration we have not introduced such a correction.

The variations in binding shown in the varying samples of ox albumin suggest two possibilities: that the different techniques used for separation of albumin have modified the calcium-binding capacity of the molecule to different extents, or that the fractionating methods are separating out different albumins. The other fractionated albumin shown in Table 3 was kindly given to us by Dr R. A. Kekwick. He had rejected the specimen because for independent reasons it was regarded as below standard. Electrophoretic analysis was perfectly satisfactory, and there was no indication from the behaviour of the material in solution that it had been modified; nevertheless, as the table shows, we were unable to demonstrate any calcium binding. The ether-fractionated concentrates (2), which had been handled with care, are shown in Table 2, and give calcium-binding capacities comparable with those of the methanol-fractionated (1) and the ethanol-fractionated five times recrystallized product (4). It seems certain, therefore, that molecular modification can occur during fractionation. Table 2 shows analyses of three carefully purified albumins given us by the courtesy of Prof. W. L. Hughes of Harvard. The albumin ten times recrystallized with decanol (5) had a calcium-binding capacity lower than either

the five times recrystallized albumin (4) or the ether-fractionated (2) and methanol-fractionated (1) crude materials. These albumins had comparable calcium-binding capacities. The product recrystallized with mercury (6) had a considerably higher calcium-binding capacity. All these three materials stem from an ethanol fractionation and had been prepared with every care. The possibility arises, therefore, that the process of recrystallization had resulted in the separation of two albumins present in normal serum and differing in their calcium-binding capacity. We appreciate that without accurate yield tables for these purified specimens it is not possible to be dogmatic, but we submit that the data are indicative of the presence of at least two albumins in normal human serum.

SUMMARY

1. Purified albumins from differing species have been examined for their calcium-binding capacities. A species difference is observed.

2. Albumins from individual species purified by varying techniques show variations in calcium-binding capacities.

3. These variations are discussed, and it is suggested that, while some results suggest modifications of the albumin during purification, there is evidence that certain techniques of crystallization result in the fractionation of distinct albumins.

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