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Electrophoresis of Proteins in the Presence of Interacting Ions *

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The interaction of human, bovine and equine serum proteins with various protein precipitating ions was investigated by means of moving boundary electrophoresis. As these reactions are reversible, the total buffer used for the electrophoresis contained the interacting ion in the equilibrium concentration.

A detailed study of the interaction of cadmium ions with bovine serum albumin showed that at low concentrations the mass action law was obeyed with a binding constant in agreement with that found by earlier authors to apply for attachment to the histidine residues. At higher concentrations further binding, presumably to carboxyl groups, could be demonstrated.

Certain correlations were found between degree of interaction and precipitating power of the various ions.

Human, bovine, and equine serum albumin could be resolved in two separate peaks in the positive branch, when electrophoresis was performed in acetate buffer pH 6, $\Gamma/2 = 0.1$.

A change in the pattern of whole serum was observed by electrophoresis in the presence of various interacting ions.

Addition of interacting ions resulted in electrophoretic separations, which are not obtained in ordinary buffers.

It has previously been reported, that a number of anions and cations, such as tungstate, sulfosalicylate, cadmium, and lead can be used for the fractional precipitation of the serum proteins from various species [1, 2]. The interaction is reversible, and it was possible to remove the ions by dialysis, the regenerated proteins showing no signs of denaturation in subsequent electrophoresis. Marked differences were encountered between the behaviour of the various ions used, as well as between the proteins of different species.

Whether a protein is precipitated by addition of these ions under certain conditions will depend partly on the degree of interaction and partly on the solubility of the compounds formed. In order to investigate these factors, the

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interaction was studied at pH-values, where a precipitation did not occur. As the combination with the ions is accompanied by a change in the net charge of the protein molecule, electrophoresis was considered an adequate approach to the problem. One important feature of this method is the possibility to investigate the interaction with the various components of a complex protein mixture. As the examined reactions are fully reversible, all electrophoreses were performed with the total buffer containing the interacting ion in the equilibrium concentration. The observed boundary velocities would then directly yield the constituent mobilities of the complexes.

MATERIALS AND METHODS

Human, bovine and equine serum were obtained from spontaneously coagulated blood. The samples were either used immediately or stored at −20°C.

Crude albumin fractions were prepared by precipitation with 55 % saturated ammonium sulfate and dialysis of the supernatant against distilled water followed by lyophilization. A bovine albumin of high purity prepared by lead and cresol precipitation [2] was used in some of the experiments.

Crude γ-globulin fractions were prepared by repeated precipitation with 38 % saturated ammonium sulfate. The precipitate was separated by centrifugation and used immediately or stored at −20°C.

The composition of the various buffers is recorded in Table 1. The samples were dissolved in the buffer and dialyzed against an excess until equilibrium had been established. Electrophoresis was then performed using the same buffer. A moving boundary Tiselius apparatus with Philpott-Svensson optical system was employed. The temperature of the bath was 1.5°C, and the duration of the experiments 2−3 h with a potential gradient of 5−8 V/cm.

RESULTS AND DISCUSSION

Effect of ion concentration. As a typical example Fig. 1 shows the influence of increasing cadmium concentration on the mobility of bovine serum albumin at pH 6.0. There is evidence of a strong interaction. The addition of cadmium

Table 1. Composition of the buffers.

No.		pH	$\Gamma/2$
1	0.1 M NaAc + HAc to	4.1	0.1
2	0.1 M Ag lactate + lactic acid to	4.2	0.1
3	0.0333 M $PbAc_2$ + HAc to	4.2	0.1
4	0.0333 M $PbAc_2$ + HAc to	4.5	0.1
5	0.0333 M $CdAc_2$ + HAc to	4.5	0.1
6	0.1 M NaAc + HAc to	5.3	0.1
7	0.1 M $ZnAc_2$ + HAc to	5.3	0.3
8	0.05 M Sulfosalicylic acid + 0.05 M NaAc + NaOH to	5.3	0.2
9	0.048 M KH_2PO_4 + 0.009 M Na_2HPO_4 + 0.025 M NaCl	6.0	0.1
10	0.05 M NaAc + 0.05 M NaCl + HAc to	6.0	0.1
11	0.1 M NaAc + HAc to	6.0	0.1
12	0.0917 M NaAc + 0.00278 M $CdAc_2$ + HAc to	6.0	0.1
13	0.075 M NaAc + 0.00833 M $CdAc_2$ + HAc to	6.0	0.1
14	0.05 M NaAc + 0.0167 M $CdAc_2$ + HAc to	6.0	0.1
15	0.0333 M $CdAc_2$ + HAc to	6.0	0.1
16	0.0667 M $CdAc_2$ + HAc to	6.0	0.2
17	0.014 M KH_2PO_4 + 0.029 M Na_2HPO_4	7.1	0.1
18	0.02 M Sulfosalicylic acid + 0.0174 M Na_2HPO_4 + NaOH to	7.2	0.1
19	0.04 M Na_2WO_4 + 0.02 N HCl	7.1	0.1

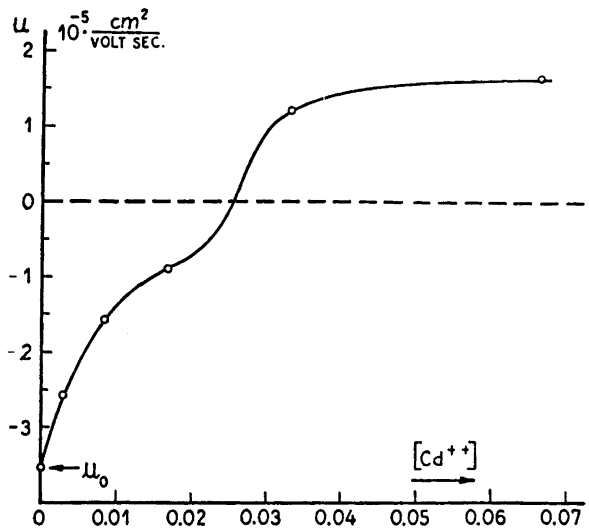

Fig. 1. Electrophoretic mobility of bovine serum albumin in acetate buffer pH 6.0 containing increasing amounts of cadmium ions. The composition of the buffers is recorded in Table 1. Nos. 11—16.

in low concentrations reduces the mobility markedly and higher concentrations even cause a reversal of the direction of migration. This shows that cadmium is still being bound after the protein compound has acquired a positive net charge.

It is possible to arrive at a quantitative interpretation of these results and to compare them with earlier investigations on the combination of cadmium ions with bovine serum albumin. From polarographic studies Tanford [3] concluded that cadmium as well as zink are bound chiefly to the histidine residues of the albumin. In the case of zink Gurd and Goodman [4] arrived at the same result by means of equilibrium dialysis.

If we thus assume that the cadmium-ions are bound by n equal sites, where they are competing with hydrogen ions, we get the following mass law equations

$$\frac{\nu}{(n-\nu-\nu_H)} = K \cdot [Cd^{++}]$$

$$\frac{\nu_H}{(n-\nu-\nu_H)} = K_H \cdot [H^+]$$

where ν denotes the number of sites occupied by cadmium, ν_H those occupied by hydrogen, and K and K_H the equilibrium constants including a possible electrostatic correction. The observed mobility change (Δu) in the electrophoresis experiment would be determined by

$$\Delta u = \frac{2\nu + \nu_H - \nu_{Ho}}{f}$$

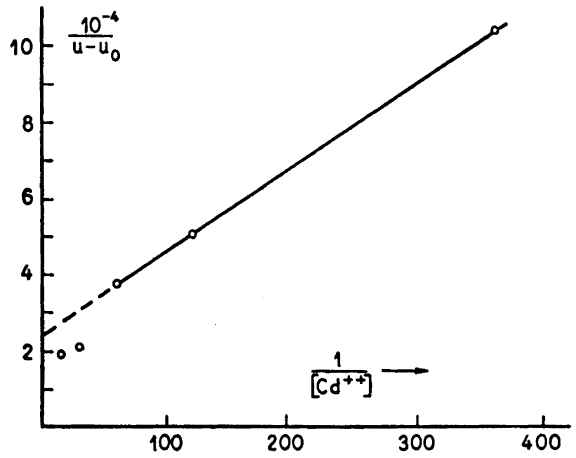

Fig. 2. Reciprocal mobility change by addition of cadmium ions to bovine serum albumin in acetate buffer plotted against reciprocal cadmium concentration.

where f denotes the number of charges corresponding to a mobility change of 1 unit, and ν_{Ho} the number of the n sites occupied by hydrogen at zero cadmium concentration. Combining these equations we get

$$\frac{1}{f \cdot \Delta u} = a \cdot \frac{1}{[Cd^{++}]} + b$$

with

$$a = \frac{(1+K_H \cdot [H^+])^2}{nK(2+K_H \cdot [H^+])}; \quad b = \frac{1+K_H \cdot [H^+]}{n(2+K_H \cdot [H^+])}$$

Plotting $1/\Delta u$ against $1/Cd^{++}$ we should expect a straight line, provided the electrostatic corrections contained in K and K_H do not change appreciably. Considering the great variation in the net charge of the protein over the experimental range this assumption appears rather questionable. However, as shown in Fig. 2, at low cadmium concentrations a straight line is obtained. Adopting the value $5.0 \cdot 10^5$ for f according to Longsworth and Jacobsen [5] and making n equal to 17 (the probable number of histidine residues in bovine albumin), the constants obtained for the working temperature of 1.5°C are log $K = 2.8$ and log $K_H = 6.7$, which is in satisfactory agreement with the intrinsic constants determined by Tanford [3].

Taking the electrostatic effect of the protein charge into account we should expect the experimental points at higher cadmium concentrations to lie above the straight line. The fact that they are situated below the line indicates, that at these concentrations further sites with lower affinity, presumably carboxyl groups, participate, resulting in binding of more cadmium ions. It should be pointed out that these concentrations greatly exceed those used by Tanford [3].

Table 2. Relative amounts of the two components obtained with various albumin samples in the positive (ascending) branch after electrophoresis in acetate buffer, pH 6.

Buffer (*cf.* Table 1)	Sample	Fast comp. %	Slow comp. %
11	Bovine serum albumin, crude	40	60
11	Bovine serum albumin, purified	43	57
10	Bovine serum albumin, crude	39	61
11	Human serum albumin, crude	50	50
10	Human serum albumin, crude	31	69
11	Equine serum albumin, crude	39	61

Heterogeneity of the serum albumin. Interestingly, all albumin preparations, which appeared quite homogeneous in phosphate buffer, showed a sharp separation into two peaks in the positive branch during electrophoresis in acetate buffer of pH 6.0 (Fig. 3). When the pH was decreased to 5.3, no separation was observed.

The total area of the two peaks was equal to that found in the negative branch and to that found in phosphate buffer. The relative amounts of the two components as determined by planimetry of the electrophoresis curves are recorded in Table 2.

When half of the acetate was substituted by chloride in the buffer the separation became less sharp. In the case of the human albumin a different

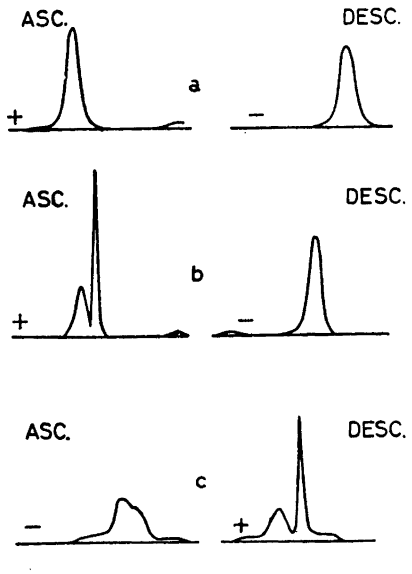

Fig. 3. Electrophoretic pattern of bovine serum albumin in (a) phosphate, (b) sodium acetate and (c) cadmium acetate buffer at pH 6.0, $\Gamma/2$ 0.1. Buffers Nos. 9, 11 and 15 (Table 1).

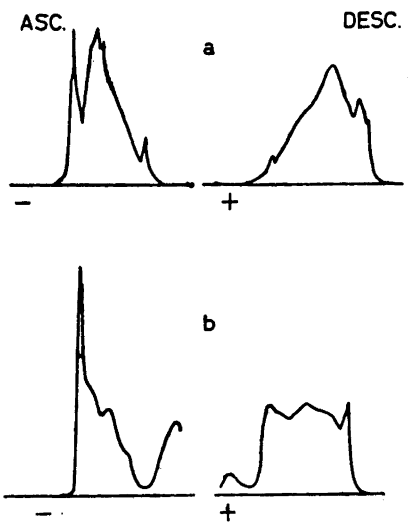

Fig. 4. Electrophoretic pattern of equine serum in (a) lead and (b) cadmium acetate at pH. 4.5, $\Gamma/2$ 0.1.

Table 3. Mobility of bovine serum albumin in various buffers. In the column denoted Δu the change in mobility is recorded relative to that found in buffers consisting of the sodium salt (cation interaction) or of phosphate or acetate (anion interaction). The values used for subtraction at pH 4.2, pH 4.5 and pH 7.2 have been interpolated.

Buffer (*cf.* Table 1)	Interacting ion	pH	u cm^2V^{-1} sec^{-1}	Δu
1	Ac	4.1	2.37	
2	Ag	4.2	5.60	3.63
3	Pb	4.2	6.76	4.79
4	Pb	4.5	5.41	4.63
5	Cd	4.5	2.33	1.55
6	Ac	5.3	−2.42	
7	Zn	5.3	0.51	2.93
8	Sulf.	5.3	−4.47	−2.05
17	Phosph.	7.1	−5.50	
18	Sulf.	7.2	−6.50	−0.85

value for the percentage of the two components was found, but on account of the poor separation of the peaks this change may not be real. On the other hand it might be explained by accepting a specific reversible ion interaction with a fraction of the albumin resulting in different mobility.

With bovine albumin exactly the same result was obtained, whether the albumin had been prepared solely by ammonium sulfate fractionation or by purification through precipitation with lead ions and cresol.

The shape of the electrophoresis curves indicates that the separation is due to a pronounced boundary sharpening effect with one albumin component. It was not obtained with whole serum and could be abolished by adding the globulin fraction. Addition of cadmium ions had no effect on the relative mobilities of the two components. Fig. 3 shows the electrophoretic diagrams of bovine serum albumin in sodium and in cadmium acetate buffer. In cadmium acetate the direction of migration is reversed, but the shape of the two peaks in the positive, now descending branch remains on the whole unaltered. They can, however, be distinguished in the negative branch and, in addition, minor components seem to separate.

It is well known that albumin is split up electrophoretically at pH 4, well below its isoelectric point. At other pH-values separations have been attained using long lasting experiments or buffers of extremely low ionic strength. A specific effect of acetate ions on the electrophoretic pattern at pH 4.7 was reported by Phelps and Cann [6]. In the present experiments other conditions are described, which lead to similar separation effects. The heterogeneity of bovine serum albumin is thus emphasized. Experiments by Christiansen *et al.*[7] indicate that molecules of different size may exist in solutions of bovine serum albumin. Investigations are in progress to show whether this could explain the present observations.

Correlation between ion interaction and precipitation behaviour. One purpose of the present work was to see if electrophoresis experiments of this kind may yield information, which can be used for fractionation by means of specific precipitations.

The interaction of bovine serum albumin with cadmium ions was described above. When compared with earlier fractionation experiments [2] it seems probable that precipitation produced by cadmium addition is mainly due to the ions bound to carboxyl groups, as at this pH an extensive binding as well as precipitation occurs only at high cadmium concentrations.

This assumption is confirmed by experiments with lead. At pH 4.5 where the metal ions will be attached chiefly to carboxyl groups, the change in mobility with 0.033 M lead acetate is thrice that noted with cadmium at the same concentration (Table 3). Correspondingly, lead ions added to bovine serum precipitate the albumin selectively at a pH about 5 in contrast to cadmium [2] which, in the same concentration range, precipitates the albumin only at pH-values above 7. Gurd and Murray [8] using purified human mercaptalbumin found intensive binding of lead ions to carboxyl groups and insolubility of the compounds formed, in close analogy to the present findings.

Various anions which previously [1] had proved suitable for serum protein fractionation were included in the investigation.

Sulfosalicylate showed a marked interaction with bovine serum albumin at pH 5.3, well above the isoelectric point (Table 3). At pH 7.0 the mobility still changed considerably when sulfosalicylate was partly substituted for phosphate in the buffer.

With bovine γ-globulin a notable interaction was observed with various ions. The isoelectric point of the preparation was found to be about pH 5.9, indicating that it contained mainly γ_1-globulin. The mobilities measured in phosphate and sulfosalicylate buffer at pH 7.2 were —1.69 and —2.66 $cm^2 V^{-1} sec^{-1}$, respectively. The change in mobility is thus of the same magnitude as that of albumin at the same pH (Table 3).

In tungstate buffer at pH 7.1 the mobility increased even to —3.37 $cm^2 V^{-1} sec^{-1}$ which is in accordance with the fact that γ-globulin is precipitated from bovine serum by tungstate [1] at relatively high pH.

The electrophoresis experiments reported have thus proved valuable for estimating the interaction with the various anions used for precipitation, and distinct correlations have been found. It is on the other hand evident that the mobility change due to addition of a certain ion is no strict criterion of the efficiency of this ion as a precipitating agent, as the solubility of the compound will also greatly depend on the binding sites and on other factors.

In this connection electrophoresis experiments performed in phosphate buffer pH 7.7 in the presence of phenol may be mentioned. Addition of phenols is known to influence profoundly the precipitation properties of the serum proteins [9, 10]. The experiments demonstrated that the mobilities of the proteins were unchanged by phenol addition. The change in their solubility is thus in the present case not associated with variation in the net charge.

Experiments with whole serum. In a number of experiments electrophoresis of whole sera was performed in the presence of interacting ions at different pH-values. The purpose was to see if better separations could be obtained, and if any qualitative differences from the usual electrophoretic diagrams could be observed. Addition of lead or cadmium at pH 4.5 caused striking changes in the electrophoretic pattern, corresponding to the different interaction with

the various components (Fig. 4). However, the exact interpretation of the electrophoresis diagrams awaits further experimental study.

Mehl and Golden [11] recommended electrophoresis of pathological sera at pH 4.5 as a valuable supplement to those generally performed at pH 8—9. The present results render it probable, that further informations may be gained by addition of suitable ions such as lead or cadmium.

Electrophoresis of bovine serum in the presence of tungstate at pH 7 gave a pattern rather similar to that achieved with phosphate buffer. Since tungstate interaction is strongest with the slower moving globulins, a more compressed picture was obtained, which made it desirable to prolong the analysis. On the other hand a considerable boundary sharpening was observed, which made the various fractions better discernible than in phosphate buffer.

Specific separation effects. It might have been expected, that mixtures of protein components of very similar mobilities would show greater variations when combined with added ions, resulting in better electrophoretic separation. The result with bovine albumin in cadmium buffer (Fig. 3) indicates, that this is indeed the case, and that the procedure can be used analytically. With human albumin in cadmium acetate buffer at the same pH a similar effect was obtained. Electrophoresis of bovine γ-globulin in the presence of sulfosalicylate made it possible to distinguish several subfractions, but the separation was poor. In this case the effect of the added sulfosalicylate may possibly be due to a suppression of the protein-protein interactions between the various γ-globulin components. A number of further experiments indicate that the best conditions for such separations will probably be found with low ionic strengths of strongly interacting ions.

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