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## Electrophoresis of $\beta$ -Lactoglobulin in the Presence of Calcium Chloride

### INTRODUCTION

The electrophoretic properties of  $\beta$ -lactoglobulin in the presence of calcium chloride are of particular interest in the chemistry of milk since the  $\beta$ -lactoglobulin occurs in milk together with calcium ions. The results are also of general interest since  $\beta$ -lactoglobulin is a relatively typical protein whereas the other proteins that have been studied electrophoretically in the presence of calcium ions, such as trypsin (1), myosin (2), and the blood serum proteins of the chicken (3), appear to be of a special nature. In the case of trypsin the presence of calcium prevents complicating self-digestion, but it makes a comparison with a calcium-free system inconclusive. The present electrophoretic studies indicate that calcium ion is bound to  $\beta$ -lactoglobulin above the isoelectric point, which is evident from a decrease in the mobility. This binding of calcium ions leads to an increase in the pH of the isoelectric point.

### MATERIALS AND METHODS

#### *$\beta$ -Lactoglobulin*

This protein was prepared from raw milk by the method of Palmer (4), and recrystallized several times. It was dried in the frozen state in the crystalline, isoelectric condition. The dry preparation was stored in a desiccator at a relative humidity such that the moisture content was 10%. It was homogeneous in the pH range studied in the course of 3 hr. of electrophoresis. Solutions for electrophoresis were prepared by dissolving sufficient dry material in the desired buffer-calcium chloride solution to obtain a 1% concentration, and dialyzing for 18 hr. or longer against 2 l. of the same buffer-calcium chloride solution.

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### *Preparation of Solutions*

The Veronal buffer, pH 8.5, was prepared from sodium Veronal by the addition of hydrochloric acid. The tris(hydroxymethyl)aminomethane buffer, pH 7.45, was prepared from hydrochloric acid and the required amount of the base. The cacodylate buffer, pH 6.5, was prepared from sodium hydroxide and the required amount of cacodylic acid. The acetate buffers, pH's 5.0 and 5.6, were prepared from sodium acetate and acetic acid. All buffers were used at an ionic strength of 0.05, except where noted, and additional ionic strength of 0.05 was obtained with sodium chloride or calcium chloride to make the total ionic strength 0.1 in every case. The pH values were measured at 25°.

### *Electrophoresis*

Electrophoretic experiments were performed with a Tiselius apparatus equipped with the schlieren scanning device of Longworth (5). The temperature of the water bath was held at 0.8° to give a temperature of about 4° within the cell. The mobilities were determined from photographs taken after electrophoresis had proceeded for about 3 hr.

### RESULTS

The electrophoretic mobility of  $\beta$ -lactoglobulin at constant ionic strength of 0.1 at pH 5.0–8.5 with variable calcium chloride concentrations is shown in Fig. 1. Although four different buffers, all univalent, were used in the present studies, there appear to be no specific buffer effects. Since the buffering ions would be negatively charged, binding to the negatively charged  $\beta$ -lactoglobulin would probably be slight. Binding of anions, however, is observed near the isoelectric point (pH 4.6–5.6) (7). The regular change in mobility with pH shown in Fig. 1 indicates that specific buffer effects are minimal, and replacement of sodium chloride with Veronal leads to a similar conclusion.

A precipitate appeared in the solutions at pH 8.5 with the higher calcium concentrations when the temperature was raised to 25° following 24–48 hr. at 7°. This was found to be due to denaturation, which will occur to some extent at pH 8.5 (6), but the denatured form is precipitated readily by calcium chloride only at elevated temperatures. To minimize complications caused by this irreversible precipitation, the electrophoretic cell was filled in a room at 7°.

The reduction in the mobility of  $\beta$ -lactoglobulin in the presence of calcium ions has been used to calculate the number of calcium ions bound, following the method of Longworth and Jacobsen (7). The effect of the calcium is relative to the replaced cation, sodium, which Carr (8) has shown is bound to  $\beta$ -lactoglobulin to some extent also. The proportion-

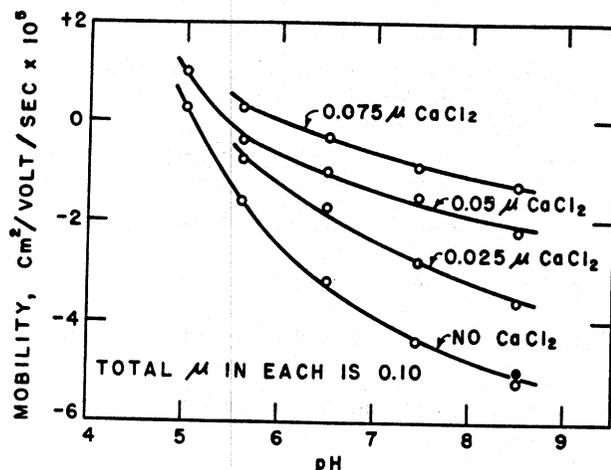
ELECTROPHORESIS OF  $\beta$ -LACTOGLOBULIN

FIG. 1. The electrophoretic mobility of  $\beta$ -lactoglobulin at ionic strength of 0.1 at several pH values in the presence of calcium chloride. The calcium chloride (0.025–0.075  $\mu$ ) replaces sodium chloride (0.05  $\mu$ ) in the buffer-salt mixture, and at the highest calcium chloride concentration replaces half the buffer as well. The concentration of the calcium chloride alone is indicated on the graph, but in each case the total ionic strength was 0.1. One point (●) is shown for 0.1  $\mu$  Veronal, no sodium chloride, which indicates that the nature of the anion does not influence the mobility. The buffer for pH's 5.0 and 5.6 was acetate, for pH 6.5 cacodylate, for pH 7.45 tris(hydroxymethyl)aminomethane, for pH 8.5 Veronal.

ality between mobility and charge is determined from a plot of the mobilities in 0.1  $\mu$  buffer-sodium chloride solutions against the equivalents,  $h$ , of acid bound per mole of  $\beta$ -lactoglobulin. The values of  $h$  used are from the titration data of Cannan *et al.* (9), reduced by 12.5% to correspond to a molecular weight of 35,000 instead of 40,000. A plot of mobility ( $u$ ) versus  $h$  is linear between pH's 5.6 and 8.5 and the value of  $\Delta u/\Delta h$  is  $2.5 \times 10^{-6}$ . Between pH 5.0 and 5.6 the value of  $\Delta u/\Delta h$  is  $3.4 \times 10^{-6}$ , close to the value of  $3.2 \times 10^{-6}$  obtained by Longworth and Jacobsen (7), in this pH region. Assuming the  $\Delta h$  is equivalent to  $\Delta z$ , the change in charge, this factor can be used to calculate the bound calcium. This has been done for the mobilities at pH 7.5, and 0.0083 and 0.0167  $M$  calcium chloride concentrations. The mobility data and the calculated bound calcium values are summarized in Table I. The decrease in charge in the presence of calcium chloride, calculated from the decrease in mobility, is given in col. 3. This decrease in charge, presumably due to the binding of calcium, divided by two since calcium is bivalent, gives the

TABLE I  
*Calcium Bound to  $\beta$ -Lactoglobulin as Calculated from the Reduction in  
 Electrophoretic Mobility (pH 7.5, Total Ionic Strength 0.1), and as  
 Measured Directly*

Ionic environment	Mobility	Decrease in charge in CaCl <sub>2</sub>	Equivalent calcium bound	Calcium bound, meas- ured directly
	sq. cm./v./sec. $\times 10^5$	equiv./mole protein <sup>a</sup>	moles/mole protein <sup>a</sup>	moles/mole protein <sup>a</sup>
NaCl-buffer	4.3	—	—	—
0.0167 <i>M</i> (0.05 $\mu$ ) CaCl <sub>2</sub> -buffer	1.5	11.2	5.6	6.3 <sup>b</sup> 6.5 <sup>c</sup>
0.0083 <i>M</i> (0.025 $\mu$ ) CaCl <sub>2</sub> -buffer	2.8	6.0	3.0	5.4 <sup>b</sup> 6.5 <sup>c</sup>

<sup>a</sup> As the molecular weight of  $\beta$ -lactoglobulin, 35,000 is used.

<sup>b</sup> Membrane electrode (10).

<sup>c</sup> Equilibrium dialysis.<sup>2</sup>

bound calcium values of col. 4. Bound calcium values obtained by direct measurements are given in col. 5 for comparison.

#### DISCUSSION

The binding of calcium by  $\beta$ -lactoglobulin calculated from electrophoretic mobilities is less than that obtained by direct measurement, and the maximal reduction in mobility, resulting from the binding, may not have been attained even at 0.0167 *M* calcium chloride concentration. Measurement of binding by a direct membrane electrode procedure (10) showed that at pH 7.4 the greatest increase in binding ended at a concentration of 0.005 *M* calcium chloride with 15 moles bound per 10<sup>5</sup> g. of  $\beta$ -lactoglobulin, equivalent to 5.3 moles/mole (35,000) of  $\beta$ -lactoglobulin. From 0.007 to 0.020 *M* calcium chloride there was a gradual further increase in the binding of 25%. Equilibrium dialysis studies<sup>2</sup> showed that calcium binding to the sodium salt of  $\beta$ -lactoglobulin at pH 7.5 appeared to be maximal at concentrations of calcium chloride as low as 0.004 *M*. The difference in the concentration of calcium chloride required to exert a maximum effect on electrophoretic mobility and on binding might mean that the binding of the positive calcium ions changes the dissociation of groups on the  $\beta$ -lactoglobulin molecule. If, for example, the binding of positive calcium ions leads to a dissociation of positive hydrogen ions, the total reduction in the negative charge would not be as great as expected from the measured calcium binding. This might also explain the

<sup>2</sup> C. A. Zittle and E. S. DellaMonica, unpublished.

gradual drop in mobility with addition of calcium compared to the abrupt leveling of the binding when measured directly (10).<sup>2</sup> Longsworth and Jacobsen (7) observed that the moles of substance bound calculated from mobility differences were considerably less than that measured directly. Differences in temperature between these electrophoretic studies, which were done at 4°, and the direct measurements of binding, which were made at 25°, are probably not significant, since previous studies (with serum albumin and various anions) have indicated that temperature has a negligible effect on binding (11).

The reduction in the electrophoretic mobility of proteins toward the positive electrode in the presence of calcium ions observed in the present study and in other studies (2, 3) presumably results from the binding of the positive calcium ions. The binding of the calcium ions will raise the isoelectric point also as observed here with  $\beta$ -lactoglobulin where the isoelectric point at 0.1 ionic strength increased from 5.1 to about 6.0. In the case of myosin the isoelectric point is reported (2) to shift from pH 5.4 in the presence of potassium chloride, to 9.5 in the presence of calcium chloride. These findings indicate, as would be expected, that the isoelectric point of any protein determined in the presence of calcium chloride is above the true value if calcium is bound to the protein.

The influence of calcium on the isoelectric point of trypsin is less clear-cut. Calcium ion is bound to trypsin quite strongly (10); at pH 7.4 and a calcium chloride concentration of 0.017 *M*, 35 moles of calcium are bound per 10<sup>5</sup> g. trypsin. The shape of the binding vs. calcium concentration curve indicates that still more is bound at higher concentrations of calcium. This binding of calcium suggests that the isoelectric point of 10.8 for trypsin in the presence of 0.03 *M* calcium chloride (1) is above the value to be obtained in a calcium-free system. Electrophoretic mobilities of trypsin in calcium-free systems are difficult to obtain for comparison because of interference from products of self-digestion of the trypsin (1). Electrophoretic studies (12) with diisopropyl fluorophosphate-inactivated trypsin in calcium-free solutions have given an isoelectric point of 10.4. If this difference in isoelectric points can be attributed to calcium it is much less than expected, and suggests that hydrogen ions are released (1*b*), or that anions are bound as well as calcium. The binding of anions might occur since trypsin is positively charged up to pH 10.5. Binding of calcium to positively charged trypsin is relatively specific [magnesium is not bound (1*b*)], compared to binding to  $\beta$ -lactoglobulin which parallels the net negative charge.<sup>2</sup> Evidence has been

presented (13) that the isoelectric point of 7 for trypsin obtained by cataphoresis is erroneous because of preferential adsorption of a contaminant onto the cataphoretic particle.

#### SUMMARY

Electrophoretic studies of  $\beta$ -lactoglobulin at constant ionic strength of 0.1, pH 5.0–8.5, have shown that the mobility is decreased by the presence of calcium ions. A concomitant increase in the isoelectric point occurs. The changes in mobility are attributed to the binding of calcium ions to the  $\beta$ -lactoglobulin molecule. The use of the electrophoretic data for calculation of the amount of calcium bound, by means of a factor for change in mobility with change in charge, leads to a value that is considerably less than the calcium known to be bound from direct measurements.

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