[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Isolation of an Electrophoretically Homogeneous Crystalline Component of β-Lactoglobulin[2]

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Electrophoretic and ultracentrifugal studies of crystalline β-lactoglobulin by Pedersen[3] indicated that this protein was homogeneous and had a constant molecular weight of 39,000 over the *p*H range 1 to 9. The sedimentation constant, however, varied. Pedersen interpreted this as representing a change in the molecular frictional constant at about *p*H 5 and 7.5.

Li,[4] employing the more sensitive schlieren methods for boundary observation and maintaining his protein solutions at 1.5% concentration, found that crystalline β-lactoglobulin was electrophoretically inhomogeneous in acetate buffers at *p*H 4.8 and 6.5. Earlier work by Gronwall[5] on the solubility of β-lactoglobulin already had indicated the heterogeneity of this protein. These experiments were confirmed and extended by this laboratory.[6] The results of investigations with other crystalline proteins, notably serium albumin[7] and insulin[8] that demonstrate electrophoretic heterogeneity principally on the acid side of the isoelectric point, indicate the general nature and significance of this phenomenon in the field of protein chemistry.

To resolve this problem it appeared most reasonable to attempt the separation of at least one of the components in pure form, so that by a comparison of its properties with the normal β-lactoglobulin some insight to the nature of the heterogeneity of this protein might be attained. This paper describes a fractionation procedure that permits the isolation of one of the β-lactoglobulin components as a crystalline protein that is electrophoretically homogeneous over the conventional *p*H range.

Experimental

The β-lactoglobulin crystals used as starting material were prepared by salt fractionation.[9] Casein was removed from skim milk by isoelectric precipitations at *p*H 4.7. The whey proteins insoluble in 2.2 *M* ammonium sulfate at *p*H 6.0 were filtered off, and the proteins remaining in the filtrate were precipitated by raising the salt concentration to 3.3 *M*. This fraction was dissolved in water and dialyzed until salt-free. The *p*H was then adjusted to 5.2 and the solution was seeded and dialyzed until crystals formed. The crystalline β-lactoglobulin was dissolved in 0.1 *M* sodium chloride, filtered clear of insoluble particles, and recrystallized by dialyzing away the salt.

Electrophoretic experiments were performed with a Tiselius apparatus equipped with the schlieren scanning device of Longsworth.[10] The temperature of the water-bath was held at 0.8°. The crystalline protein was dissolved in a buffer at ionic strength 0.1 to a concentration of approximately 1% protein, and dialyzed against the buffer for 18 hours or longer, with stirring. Acetate buffer at *p*H 4.8[11] was used in the electrophoretic analyses designed to determine the effectiveness of the fractionation procedure. Mobilities were calculated for the descending boundaries from conductivity values determined at 0°. The *p*H was measured at 25° with a glass electrode.

Fractionation with alcohol was accomplished by slow addition of 50% alcohol at 3° with stirring, to a concentration of 10%. The solution was then cooled to −5°, and alcohol was added to the desired concentration up to 25%. Higher alcohol concentrations were then attained by the addition of 95% alcohol. Adjustments of *p*H were made with 1 *N* acetic acid or 0.5 *M* acetate buffer of *p*H 4.0. Alcoholic solutions were diluted to a final concentration of 10% before *p*H measurements were made at 25°.

Fractionation of β-Lactoglobulin.—Electrophoresis of crystalline β-lactoglobulin in acetate buffer at *p*H 4.8 showed marked asymmetry of the rising and falling boundaries (Fig. 1, S). For convenience, the normal pattern, consisting of 40% slow-moving component and 60% fast-moving component as measured on the descending boundaries, was designated β-lactoglobulin. We refer to the slow component as β_1-lactoglobulin, and the fast component as β_2-lactoglobulin. The so-called normal β-lactoglobulin was obtained readily by the salt fractionation used, with remarkably constant proportions of the two components. Attempts to separate the two components by further salt fractionation alone were not promising.

By a combination of the procedures of differential solubility at *p*H values acid and alkaline to the isoelectric point and subsequent fractionation with alcohol at low ionic strength, it was found possible to isolate pure β_1-lactoglobulin.

Preparation of Crystalline β_1-Lactoglobulin, Step 1.—Fifty grams of crystalline β-lactoglobulin containing 40% β_1-component and 60% β_2-component were dissolved in 2500 ml. of 0.1 *N* acetate buffer *p*H 4.8. The solution was then dialyzed at 3° to a relatively salt-free state. After crystallization occurred, the protein solution was warmed to room temperature. Another crop of protein crystals then formed that was removed by centrifugation (Fig. 1, SP_1).

Step 2.—The supernatant solution (Fig. 1, SF_1) containing about 1% protein was adjusted to *p*H 5.3 by the slow addition of 0.1 *N* sodium hydroxide with sufficient stirring to prevent a local excess of the base and denaturation. After standing at room temperature for a few hours a crystalline precipitate formed containing 75% β_1-component (Fig. 1, P_3).

Step 3.—The supernatant of the β-lactoglobulin crystals formed at *p*H 5.3 had a protein concentration of approximately 0.3%. This solution was equilibrated with a solution of 15% alcohol, ionic strength 0.005, *p*H 5.3 at 3°. Approximately 7 g. of crystalline protein containing 90% β_1-lactoglobulin could be removed.

(1) One of the Laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) Presented at the 116th Meeting of the American Chemical Society, Atlantic City, N. J., September 1949.

(3) Pedersen, *Biochem. J.*, **30**, 948, 961 (1936).

(4) Li, THIS JOURNAL, **68**, 2746 (1946).

(5) Gronwall, *Compt. rend. trav. lab. Carlsberg*, **24**, No. 8-11, 185 (1942).

(6) McMeekin, Polis, DellaMonica and Custer, THIS JOURNAL, **70**, 881 (1948).

(7) Luetscher, *ibid.*, **61**, 2888 (1939).

(8) Sutherland, Cori, Haynes and Olsen, *J. Biol. Chem.*, **180**, 825 (1949).

(9) Palmer, *ibid.*, **104**, 359 (1934).

(10) Longsworth, THIS JOURNAL, **61**, 529 (1939).

(11) Boyd, *ibid.*, **67**, 1035 (1945).

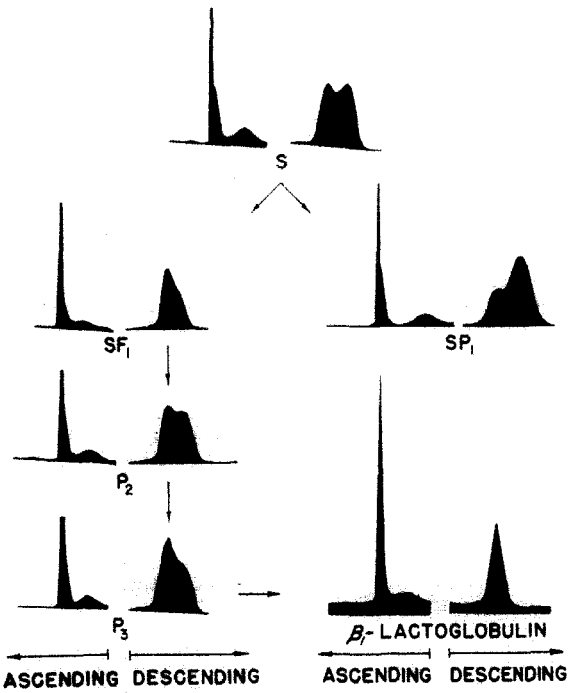

Fig. 1.—Electrophoretic patterns of β-lactoglobulin fractions in acetate buffer at *p*H 4.8, ionic strength 0.1, after electrophoresis for 180 min. at a field strength of 4.8 volts/cm.: S, stock crystals used for starting materials; SF_1, protein supernatant after dialysis at *p*H 4.8 to a salt-free state; SP_1, crystalline protein containing 70% fast component obtained by repeated crystallization at *p*H 4.8 (crystalline proteins obtained by alcohol fractionation procedures showed similar electrophoretic compositions); P_2, crystalline fraction with increased slow component precipitated from SF_1 by 17% alcohol at *p*H 5.8; P_3, crystalline fraction from P_2 supernatant precipitated by 25% alcohol at *p*H 5.3; β_1-lactoglobulin, crystalline protein finally obtained by alcohol fractionation and differential solubility at the isoelectric point.

This protein was redissolved in acetate buffer *p*H 4.8 to a final concentration of 1% protein. The three steps were repeated again once to yield 1 g. of pure crystalline β_1-lactoglobulin. A stock of P_3 β-lactoglobulin crystals could also be obtained by alcohol fractionation of SF_1. At ionic strength 0.08, *p*H 5.8 and 17% alcohol a crystalline fraction P_2 (Fig. 1) was formed. The supernatant liquor of P_2 after adjustment to 25% alcohol, *p*H 5.3, yielded a crystalline fraction (Fig. 1, P_3) that could be further purified to β_1-lactoglobulin.

Concentration of β_2-Lactoglobulin.—By recrystallization from 0.1 *M* acetate buffer at *p*H 4.8 after dialysis to remove the salt, it was found possible to increase the amount of β_2-lactoglobulin in the crystalline protein from 60 to approximately 75%. Experiments designed to obtain an electrophoretically homogeneous β_2-lactoglobulin were unsuccessful. Fractionation of the 75% β_2-lactoglobulin with ethanol gave no change or at *p*H values acid to the isoelectric point formed an insoluble and apparently denatured precipitate. The remaining protein was crystallizable but reverted back to the same electrophoretic pattern as the normal β-lactoglobulin.

Physical Chemical Properties.—Table I summarizes a comparison of the properties for the normal and β_1-lactoglobulins. The most striking variations observed are in the solubility and electrophoretic data. Differences in the solubility of the protein with change in the concentration of the β_1-component are illustrated in Fig. 2.

TABLE I

PHYSICO-CHEMICAL PROPERTIES OF CRYSTALLINE NORMAL AND β_1-LACTOGLOBULIN

	β-Lactoglobulin	β_1-Lactoglobulin
Per cent. nitrogen[a]	15.46	15.39
Molecular weight[b]	35,000	36,100
Optical rotation $[\alpha]^{25}D$[c]	−30.4	−30.7
Ratio optical density (280/250 mμ)	2.552	2.687
$E^{1\%}_{1cm.}$ at 280 mμ	9.3	9.7
Biuret color density/mg. protein nitrogen[d]	22.09	19.54
Solubility at 25°, mg. nitrogen/cc.[e]		
Water	0.12	0.27
0.02 *M* sodium chloride	1.50	2.58
Isoelectric point	5.1	5.3
Mobility, cm.²/volt/sec. × 10^5		
Veronal buffer, μ = 0.1, *p*H 8.4	−5.1	−5.6
Acetate buffer, μ = 0.1, *p*H 4.8	(+1.9, +3.0)	+1.8

[a] Nitrogen determinations were made by micro-Kjeldahl methods[12] and calculated on a moisture- and ash-free basis. These values are not intended to supplant the previously reported value of 15.65% nitrogen but indicate within the limits of precision of the method the nitrogen contents of the two crystalline proteins. [b] Molecular weights were determined by M. Halwer[13] by a light-scattering procedure. [c] Specific optical rotations were measured in acetate buffer at *p*H 4.8, ionic strength 0.1. [d] Biuret color density was measured by the procedure of Kingsley[14] on a photoelectric colorimeter with 525 filter. [e] These values were obtained in the presence of a large excess of protein crystals. At lower protein levels, neither the β_1 nor the normal β-lactoglobulin attained constant solubility; the solubility increased with increasing amounts of the crystals.

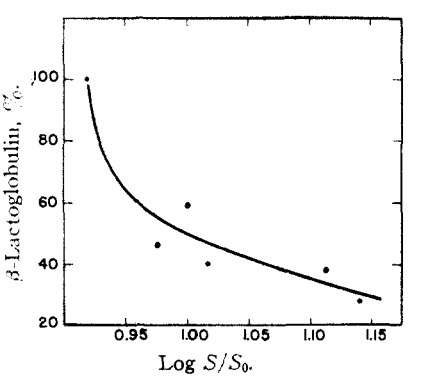

Fig. 2.—Variation of the log S/S_0 of β-lactoglobulin with electrophoretic composition at *p*H 4.8. *S* is the solubility in 0.02 *M* salt; S_0 is the solubility in water at the isoelectric point. Absolute values of *S* and S_0 for β- and β_1-lactoglobulin are given in Table I.

A unique characterization of the β-lactoglobulin components is given by the electrophoretic data. All the preparations showed a single homogeneous boundary at a

(12) Miller and Houghton, *J. Biol. Chem.*, **159**, 373 (1945).
(13) Halwer and Brice, *J. Colloid Sci.*, **4**, 439 (1949).
(14) Kingsley, *J. Lab. Clin. Med.*, **27**, 840 (1942).

*p*H alkaline to the isoelectric point in acetate or veronal buffers at ionic strength 0.1. At a *p*H acid to the isoelectric point, the β-lactoglobulin electrophoretic pattern is distinctly heterogeneous and lacks enantiography (Fig. 1, S). This heterogeneity is marked in acetate and lactate buffers to a *p*H near 3.0. In glycine buffer below *p*H 3.0, the globulin again appears homogeneous. In Fig. 3, the mobilities of normal and β_1-lactoglobulins are plotted against *p*H. The pure β_1-component moves more rapidly than the normal complex at a *p*H above 6.6 and below 2.9.

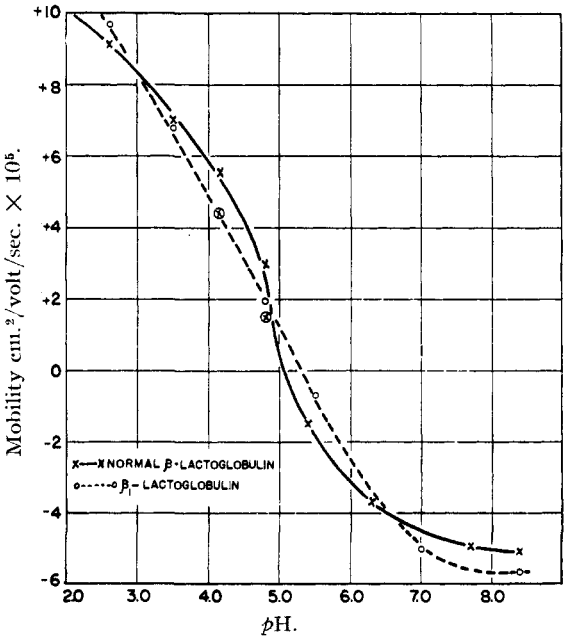

Fig. 3.—Variation of mobility with *p*H of normal β-lactoglobulin and β_1-lactoglobulin in veronal, acetate and glycine buffers at ionic strength 0.1.

The interpolated isoelectric points become 5.3 for the β_1-lactoglobulin and 5.1 for the normal β-lactoglobulin. These values agree well with the *p*H for minimal solubility

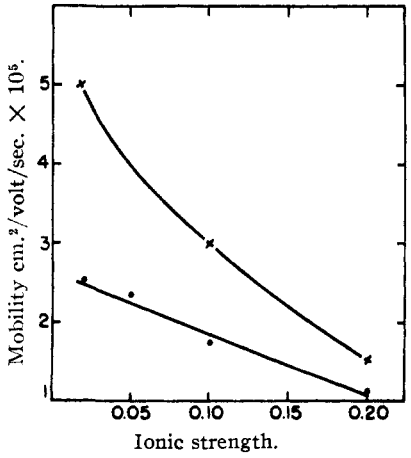

Fig. 4.—Variation of mobility with ionic strength of β_1-lactoglobulin (●—●) and β_2-lactoglobulin (×—×) in acetate buffer at *p*H 4.8. Each point represents an average of three determinations. The β_1-lactoglobulin represents the mobility change for that component in the normal complex and isolated β_1-crystalline protein.

for the β_1-component and normal β-lactoglobulin. Titration data for the two forms of β-lactoglobulin parallel the *p*H mobility curves; the isoionic points are 5.18 for normal β-lactoglobulin and 5.3 for the β_1-lactoglobulin.

The variation of the isoelectric point for the β_1- and normal β-lactoglobulins suggested a possible difference in the binding of anions by the proteins.[15] Figure 4 shows the variation of the electrophoretic mobilities with ionic strength for the normal β-lactoglobulin components and the isolated β_1-component. The curve for the pure β_1-component coincides with the curve for the β_1-component in the normal β-lactoglobulin. The variation in the slopes of the two curves may then be considered as an indication of a decreased binding by the β_1-lactoglobulin for acetate ion, as compared with the β_2-lactoglobulin.[15,16] If this concept is accepted, the nature of the electrophoretic pattern at constant ionic strength and *p*H and varied concentration of anion species becomes important. Figure 5 shows a definite decrease in the concentration of the faster moving component with the substitution of increasing amounts of chloride ion for acetate ion. A change in the acetate ion concentration from 0.1 to 0.01 *M* is reflected in an apparent change in concentration of the slow moving component from 60 to 33%.[17] It is recognized that the marked interaction of the proteins involved makes the area analysis of these electrophoretic patterns approximations. The extent of the differences observed, however, makes these variations significant.

Fig. 5.—Falling boundaries of normal β-lactoglobulin in acetate and acetate–sodium chloride buffer at *p*H 4.8, ionic strength 0.1, after electrophoresis for 180 min.: A, 0.1 μ sodium acetate–acetic acid; B, 0.05 μ sodium chloride and 0.05 μ sodium acetate–acetic acid; C, 0.09 μ sodium chloride and 0.01 μ sodium acetate–acetic acid; D, 0.098 μ sodium chloride and 0.002 μ sodium acetate–acetic acid.

Discussion

In contrast to the data reported by Li,[4] the electrophoretic patterns of β-lactoglobulin in

(15) Longsworth and Jacobsen, *J. Phy. Colloid Chem.*, **53**, 126 (1949).

(16) Velick, *ibid.*, **53**, 135 (1949).

(17) Electrophoretic composition of β-lactoglobulin (Fig. 5) as determined by area analysis and mobilities (cm.²/volt/sec. × 10⁵). (The original β-lactoglobulin preparation for *D* had an increased fast component amounting to 70% when electrolyzed in acetate buffer without sodium chloride.)

Electrophoretic pattern (Fig. 5)	β_1 Comp. %	β_1 *u*	β_2 Comp. %	β_2 *u*	β_3 Comp. %	β_3 *u*
A	40	1.8	60	3.0		
B	30	1.4	60	3.1	10	6.7
C	56	0.9	44	2.9		
D	67	1.2	33	3.3		

buffers alkaline to the isoelectric point consistently presented a single boundary. At *p*H 6.5, no heterogeneity was observed in acetate or phosphate buffer at ionic strength 0.1 after electrophoresis for three or five hours. The curves relating mobility with *p*H for β_1- and normal β-lactoglobulin intersect at about *p*H 6.6 (Fig. 3), so that if heterogeneity were to be observed at an alkaline *p*H it would seem least likely to appear at *p*H 6.5 and most likely to appear at *p*H 5.5 or 8.4.

The nature of the heterogeneity presented by β-lactoglobulin has been the subject of much conjecture.[18] It apparently is not the result of the isolation procedure. Crystals obtained by alcohol fractionation, as well as by salt fractionation of whey; crystals from the milk of a single animal, as well as mixed commercial milk; and even crystals from normal pasteurized milk, showed the same consistent electrophoretic heterogeneity at *p*H 4.8 in acetate buffer at ionic strength 0.1. The only exceptions to this highly reproducible pattern were found in the crystalline lactoglobulin isolated from the first-day colostrum of one cow and from the milk of a cow infected with brucellosis. In both cases, the β_1-lactoglobulin predominated, amounting to 80% in the brucellosis β-lactoglobulin and 100% in the colostrum. The possibility of aggregation or dissociation of the molecule is discredited by finding similar molecular weights for the normal and β_1-lactoglobulins. The isolation of one of the components in pure crystalline form that migrates with the velocity of the β-lactoglobulin in alkaline buffer, although it has a mobility similar to that of the immune globulins of whey at *p*H 4.8, refutes the postulate by Lundgren and Ward[18] that the heterogeneity of β-lactoglobulin is a result of the contamination with one or more of the immune globulins characterized by Smith.[19]

(18) Lundgren and Ward, *Ann. Rev. Biochem.*, **18**, 121 (1949).
(19) Smith, *J. Biol. Chem.*, **165**, 665 (1946).

In view of the high molecular weight (about 180,000) and the presence of carbohydrate and phosphorus in these immune globulins, it is impossible for an immune globulin to account for any component that constitutes the electrophoretic heterogeneity reported here.

The heterogeneity of β-lactoglobulin manifest at *p*H values acid to the isolectric point can be ascribed primarily to a basic difference in the net charge.

This may be attributed to a fundamental variation in the structure of the protein molecule or to the conjugation of the same molecule with another component, with resultant change in the dissociation of ionizable groups and hence difference in the net charge.

Acknowledgment.—The authors are greatly indebted to C. A. Zittle for the many helpful discussions and criticisms of this paper, and to E. S. DellaMonica for assistance in preparing the normal crystalline β-lactoglobulin.

Summary

Crystalline β-lactoglobulin, presenting a single electrophoretic boundary in buffers of ionic strength 0.1, alkaline to the isoelectric point, has been found heterogeneous in buffers acid to the isoelectric point. By means of alcohol fractionation and differential solubility at *p*H 4.8 and 5.3, one of the two components resolved by electrophoresis in acetate buffer at *p*H 4.8 has been isolated as a crystalline protein showing a single electrophoretic boundary over the conventional *p*H range in buffers of ionic strength 0.1. The per cent. nitrogen, molecular weight, optical rotation, optical density (280/250 mμ), biuret color density, solubility in water and salt, electrophoretic mobility and isoelectric point of the single-boundaried component and of the normal complex are compared.

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RECEIVED FEBRUARY 3, 1950

[CONTRIBUTION FROM THE RICHARDSON CHEMICAL LABORATORIES OF THE TULANE UNIVERSITY OF LOUISIANA]

Inorganic Complex Compounds Containing Polydentate Groups. IV. Formation Constants of Diethylenetriamine–Nickel(II) and –Copper(II) Complexes

BY HANS B. JONASSEN, R. BRUCE LEBLANC AND RUTH M. ROGAN

If a metal ion can coördinate two or more groups in the formation of complex ions, it will do so stepwise, and intermediate equilibria will be present. If M represents the metal ion and A the coördinating group the equilibria are

$$M + A \rightleftarrows MA;\ k_1 = [MA]/[M][A]$$
$$MA + A \rightleftarrows MA_2;\ k_2 = [MA_2]/[MA][A]$$
$$MA_{n-1} + A \rightleftarrows MA_n;\ k_n = [MA_n]/[MA_{n-1}][A]$$

Each of these equilibria can be represented by a constant, and the product of all the constants for the individual steps is the over-all complexity constant for the equilibrium

$$M + NA \rightleftarrows MA_N;\ k_N = [MA_N]/[M][A]^N$$

where N is the maximum number of groups coördinated.

Bjerrum[1] devised a method for measuring these step equilibria constants (or formation constants) for basic coördinating groups by measuring the *p*H of solutions which contain known amounts

(1) J. Bjerrum, "Metal Ammine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, 1941.