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Molecular Interactions in β-Lactoglobulin. I. The Electrophoretic Heterogeneity of β-Lactoglobulin Close to its Isoelectric Point

An electrophoretic study of β-lactoglobulin was carried out close to its isoelectric point. It was shown that the heterogeneity observed at *p*H 5.3–5.6 is due primarily to the presence of the two genetic species. Calibration curves for composition analysis have been prepared and the various nomenclatures found in the literature have been reconciled in terms of the Aschaffenburg β-lactoglobulins A and B.

Introduction

The electrophoretic heterogeneity of crystalline preparations of β-lactoglobulin has been observed by a number of investigators.[2–9] Depending on the conditions, two, three or even four electrophoretic components can be observed, especially if the experiment is permitted to proceed to maximal resolution, which in some cases requires as long as 24 hours.[8] A certain amount of disagreement exists among the various authors as to whether β-lactoglobulin is heterogeneous electrophoretically at any given set of conditions, especially above its isoelectric point (*p*H 5.1–5.3). Thus, Li[2] reported that, while at the *p*H's of 5.3 and 5.6, this protein was homogeneous, at *p*H 4.8 and 6.5 it resolved into three components, the composition being different at the two *p*H's. Polis and coworkers,[5] working at *p*H 4.8 in a 0.1 ionic strength acetate buffer, concluded that β-lactoglobulin consists of two components with mobilities of 1.9 and 3.0×10^{-5} cm.^{2}/v. sec. under those conditions. They assigned the name of β_1-lactoglobulin to the slow component and β_2-lactoglobulin to the rapid component. Furthermore, they reported the isolation of β_1-lactoglobulin in pure form and its identification as a true molecular entity. These authors, however, did not observe any heterogeneity in the *p*H region alkaline to the isoelectric point, as Li had reported.[2] Smithies[7] reported that, contrary to the findings of Li and of Polis, this protein was heterogeneous electrophoretically in the *p*H region of 5.3–5.5 but concluded that the patterns were too complicated to determine from the composition of the protein. Ogston and Tilley[9] have carried out electrophoretic experiments as a function of temperature at *p*H 4.66 and correlated these with some ultracentrifuge experiments. They concluded that at least part of the heterogeneity of β-lactoglobulin observed at that *p*H is the result of reversible association favored by low temperature, low *p*H and high protein concentration. They concluded further that the situation is complicated by the presence of probably two species of β-lactoglobulin, only one of which is capable of associating. Working as a function of *p*H, the present authors[10] have found that the association observed by Ogston and Tilley[9] is limited to the *p*H region between 3.5 and 5.2, thus eliminating the possibility of intermolecular interactions as the explanation for electrophoretic heterogeneity in the *p*H region below 3.5 or above 5.2.

In 1955, Aschaffenburg and Drewry,[11] working with milk obtained from individual cows, showed by paper electrophoresis (*p*H 8.6, 0.05 ionic strength veronal buffer) that β-lactoglobulin consists of two genetically different proteins and that furthermore different cows may produce either one of the two species of this protein or a mixture of the two. These authors initially assigned the names of β_1-lactoglobulin to the rapidly migrating protein and β_2-lactoglobulin to the slowly migrating one but later changed the nomenclature to β-lactoglobulin A (β-A) and β-lactoglobulin B (β-B), respectively.[12] Each one of these components, however, was still

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) C. H. Li, This Journal, **68**, 2746 (1946).

(3) T. L. McMeekin, B. D. Polis, E. S. Dellamonica and J. H. Custer, *ibid.*, **70**, 881 (1948).

(4) L. G. Longsworth and C. F. Jacobsen, *J. Phys. and Colloid Chem.*, **53**, 126 (1949).

(5) D. Polis, H. W. Schmukler, J. H. Custer and T. L. McMeekin, This Journal, **72**, 4965 (1950).

(6) R. A. Alberty, E. A. Anderson and J. W. Williams, *J. Phys. and Colloid Chem.*, **52**, 217 (1948).

(7) O. Smithies, *Biochem. J.*, **58**, 31 (1954).

(8) S. N. Timasheff, unpublished experiments.

(9) A. G. Ogston and J. M. A. Tilley, *Biochem. J.*, **59**, 644 (1955).

(10) R. Townend and S. N. Timasheff, *Arch. Biochem. Biophys.*, **63** 482 (1956).

(11) R. Aschaffenburg and J. Drewry, *Nature*, **176**, 218 (1955).

(12) R. Aschaffenburg and J. Drewry, *ibid.*, **180**, 376 (1957).

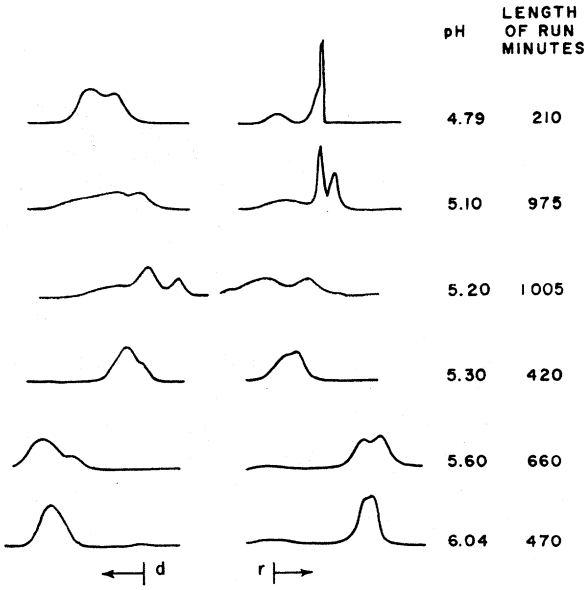

Fig. 1.—Tracings of electrophoretic patterns obtained with β-lactoglobulin, Preparation I: protein concn. *ca.* 10 g./l.; 6.9 v./cm. The starting positions are indicated by the arrows. Polarity was reversed between *p*H 5.10 and 5.20.

found to be heterogeneous in electrophoresis experiments at *p*H 4.66 by Ogston and Tombs[13] as confirmed by Klostergaard and Pasternak,[14] as well as the present authors.[15] Klostergaard and Pasternak, however, reversed the nomenclature of Aschaffenburg and Drewry.[11]

A detailed study of the heterogeneity of β-lactoglobulin and its association properties in the *p*H region below its zone of denaturation (*p*H 7.0 and above) was undertaken in this Laboratory and the results of these studies are reported in the present series of papers. Since, in order to understand fully the behavior of this system, it was necessary to be able to analyze individual β-lactoglobulin preparations into true electrophoretic components, a thorough electrophoretic investigation of this protein was carried out in the region close to its isoelectric point. It is the purpose of the present paper to describe this work, as well as to attempt to disentangle the presently existing confusion on the nomenclature used with respect to various β-lactoglobulin components.

Experimental

Materials.—β-Lactoglobulin samples used in these studies were a commercial preparation obtained from Pentex Laboratories (referred to as Preparation I) and material given to us by Dr. W. G. Gordon, who prepared it from pooled milk by ammonium sulfate precipitation followed by repeated recrystallizations from distilled water[16] (referred to as Preparation II).

The partially fractionated material used was the "β_2-enriched" material of Polis and co-workers[5] (containing 75% of their β_2-lactoglobulin).

The pure β-A and β-B were samples prepared by Dr. R. Aschaffenburg according to his procedure[17] and kindly given by him to us.

(13) A. G. Ogston and M. P. Tombs, *Biochem. J.*, **66**, 399 (1957).

(14) H. Klostergaard and R. A. Pasternak, THIS JOURNAL, **79**, 5671 (1957).

(15) S. N. Timasheff and R. E. Townend, *ibid.*, **80**, 4433 (1958).

(16) A. H. Palmer, *J. Biol. Chem.*, **104**, 359 (1934).

(17) R. Aschaffenburg and J. Drewry, *Biochem. J.*, **65**, 273 (1957).

Electrophoresis.—The electrophoresis experiments on Preparation I were carried out in a Klett[18] Tiselius electrophoresis apparatus at 2°. Those on all other protein samples were carried out in a Perkin–Elmer[18] apparatus, Model 38A, at 0°. The mobilities and area distributions were measured from enlarged projected tracings. All mobilities were calculated from the descending boundaries.

Results

The electrophoretic patterns of β-lactoglobulin (Preparation I) shown in Fig. 1, were obtained at a series of *p*H's close to the isoelectric point in acetate buffers[19] of 0.1 ionic strength. It can be seen that an increase in *p*H causes a change from very "abnormal" patterns to ones which could be given by a two-component system. At *p*H 4.79, two broad peaks of approximately equal area are found on the descending side, while on the rising side there is a hypersharp leading boundary with a small trailing shoulder. Such a pattern has been attributed by Ogston and Tilley[9] to the association of β-lactoglobulin at this *p*H. At the *p*H's of 5.10 and 5.20, the patterns are again highly non-enantiographic with as many as three "components" being detectable. In this *p*H region a small amount of association is probably still present at low temperatures.[10] At *p*H's 5.30, 5.60 and 6.04, two-component diagrams of more normal appearance are obtained. Since intermolecular interactions occur below *p*H 5.2,[10,20] and above *p*H 6.04 heterogeneity is evidenced only by a strong skewness of the boundaries, the *p*H's selected for analytical purposes were 5.3 and 5.6, 0.1 ionic strength acetate.

A series of electrophoretic experiments were carried out on Preparation II as a function of concentration at *p*H 5.60. The patterns obtained were analyzed as two-component systems. The mobilities, extrapolated to zero protein concentration, were -1.87×10^{-5} and -1.55×10^{-5} cm.^{2}/sec. v. The mobilities at a protein concentration of 10 g./l. were -1.77×10^{-5} and -1.38×10^{-5} cm.^{2}/sec. v. The area analyses, however, led to different compositions in the rising and descending channels even on extrapolation to zero protein concentration. Analysis of the Aschaffenburg β-A and β-B at identical conditions resulted in mobilities of -1.67×10^{-5} and -1.46×10^{-5} cm.^{2}/sec. v., respectively, at a protein concentration of 10 g./l.

Examination of the electrophoretic patterns obtained with the β-lactoglobulins at identical concentrations, shown in Fig. 2c, revealed that while βA produces sharp, almost symmetrical peaks in both channels at *p*H 5.60, βB shows definite skewing toward the rapid side in the descending limb. Superposition and addition of the two descending patterns results in an apparent composition highly enriched with respect to fast component (Fig. 2c), and quite similar to that given by a 1:1 mixture of the two proteins (Fig. 2b). However, the sum of the patterns on the rising side does not quite equal that of the mixture. This might indicate some sort of interaction between the proteins, causing a change in

(18) Mention of this company does not constitute an endorsement of its product to the possible detriment of other companies not mentioned.

(19) The run at *p*H 6.04 was made in a cacodylate buffer.

(20) S. N. Timasheff and R. Townend, Abstracts, 132nd Meeting, Am. Chem. Soc., New York, 1957, p. 19-I.

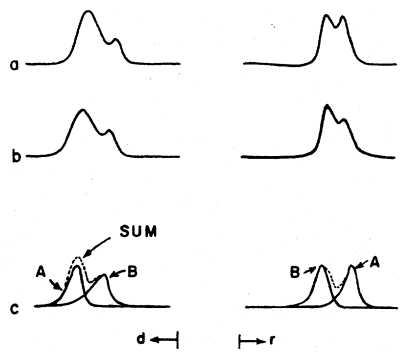

Fig. 2.—Tracings of electrophoretic patterns obtained with β-lactoglobulins in *p*H 5.6 acetate buffer of 0.1 ionic strength; 300 min.; 10 v./cm.; protein concn.: 10 g./l. The starting positions are indicated by the arrows: (a) pooled β-lactoglobulin, Prepn. II; (b) 1:1 mixture of β-A and β-B; (c) β-A and β-B, superposition of patterns.

the rising mobility of one when the other is present.

Similar experiments were carried out in *p*H 5.30 acetate buffer of 0.1 ionic strength. The results are shown in Fig. 3. The patterns obtained with β-A display a small amount of skewness in the forward direction on the descending side. Those for β-B resolve into two "components" on each side. On the descending side the bulk of the β-B migrates with a mobility of -0.12×10^{-5} cm.^{2}/sec. v., while *ca.* 30% of the material under the boundary has a mobility of -0.44×10^{-5}. The situation is reversed on the rising side. Examination of ten samples of β-B obtained from the milk of individual β-B producing cows of various breeds showed that all behaved in an identical manner.[21] Direct sampling experiments definitely showed that resolution into two peaks does not represent true molecular heterogeneity[22] but that it is rather due to some other factors, such as an isomerization similar to that reported by Aoki and Foster[23] for serum albumin or an interaction with buffer components as found by Cann.[24] A detailed study of this behavior has been undertaken and will be reported on later.

The presence of these minor "components" complicates the analysis of the protein from pooled milk, since neither the rising nor the descending limb shows patterns corresponding to actual composition (Figs. 2 and 3).

In order to circumvent this difficulty in analysis, it is possible to construct a calibration graph[25,26] of area distribution against known composition. A series of electrophoretic runs on synthetic mixtures of β-lactoglobulins A and B was carried out at *p*H's 5.30 and 5.60 in a 0.1 ionic strength acetate buffer. The total protein concentration was 10 g./l. in the experiments done at *p*H 5.60, and 6–7 g./l. in the case of the *p*H 5.30 runs. The results of the area

(21) This is part of a study on the genetic characterization of dairy cows now being carried out jointly with Drs. N. D. Bayley, R. D. Plowman and C. A. Kiddy of the Dairy Cattle Research Branch, U. S. D. A.

(22) See, for example, R. A. Brown and S. N. Timasheff, in M. Bier 'Electrophoresis," Academic Press, Inc., New York, N. Y., 1959, p. 317.

(23) K. Aoki and J. F. Foster, THIS JOURNAL, **79**, 3385 (1957).

(24) J. R. Cann, *ibid.*, **80**, 4263 (1958).

(25) L. Pauling, H. A. Itano, S. J. Singer and I. C. Wells, *Science*, **110**, 543 (1949).

(26) R. M. Bock and R. A. Alberty, *J. Biol. Chem.*, **193**, 435 (1951).

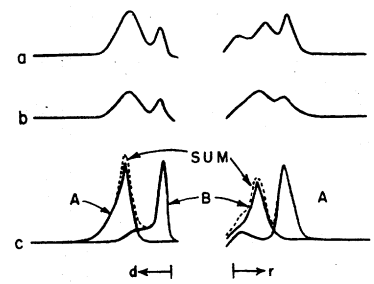

Fig. 3.—Tracings of electrophoretic patterns obtained with β-lactoglobulins in *p*H 5.3 acetate buffer of 0.1 ionic strength; 420 min.; 10 v./cm.; protein concn.: 10 g./l. The starting positions are indicated by the arrows: (a) pooled β-lactoglobulin, Prepn. II; (b) 1:1 mixture of β-A and β-B; (c) β-A and β-B, superposition of patterns.

analyses of both sets of rising and decending patterns are plotted as a function of the known composition in Fig. 4. It can be seen that none of the four area distribution curves correspond to the true composition of the mixture. However, the experimental curves presented in Fig. 4 can be used as standards for the analysis of a β-lactoglobulin preparation. For this purpose, any given sample of β-lactoglobulin is analyzed electrophoretically at *p*H 5.30 and 5.60 in a 0.1 ionic strength acetate buffer at the proper protein concentration at the same field strength and for the same length of time as the standard experiments. Area analyses of the two sets of rising and descending boundaries, when compared with the standard curve, given in Fig. 4, should result in identical compositions.

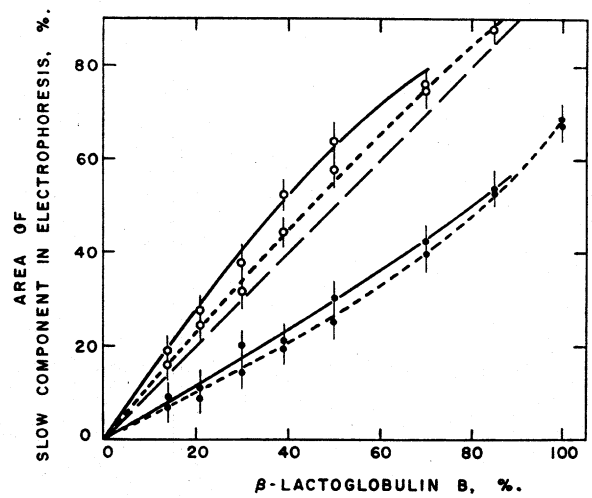

Fig. 4.—Standard calibration curves for β-lactoglobulin compositions: -----, *p*H 5.3; ———, *p*H 5.6; —— ——, ideal area distribution (slope = 1); O, rising patterns; ●, descending patterns. The analyses were carried out as described in the text and in Table I.

Such analyses have been carried out with Preparation II and the Polis "β_2-enriched" protein. The results are given in Table I. It can be seen that in both cases good agreement between the four deduced analyses has been obtained. Thus, pooled β-lactoglobulin (Preparation II) contains *ca.* 59% β-A and 41% β-B, while the Polis "β_2-enriched" material contains *ca.* 77% β-A and 23% β-B.

TABLE I

AREA-COMPOSITION DATA FOR β-LACTOGLOBULIN SAMPLES

Pooled β-lactoglobulin (prep. II)	pH 5.3 Rising	pH 5.3 Descending	pH 5.6 Rising	pH 5.6 Descending
% Area slow[a]	42.8	23.9	51.3	23 6
% β-lactoglobulin B[b]	38.3	45.5	39.2	40.3
			Av.	40.8 ± 2.8
Polis "β_2-enriched"				
% Area slow[a]	26.1	13.0	32.2	12.2
% β-lactoglobulin B[b]	23.0	25.7	23.8	21.0
			Av.	23.4 ± 1.7

[a] Measured planimetrically on electrophoretic diagrams. It was found that the most consistent results were obtained if the area analyses were carried out in the following manner. pH 5.3, descending: construction of symmetrical slow peak by projection of its trailing edge across a perpendicular dropped from the apex and construction of rapid "component" by subtraction of the slow peak; rising: similar procedure, with projection of rapid moving peak. pH 5.6, descending: procedure identical with pH 5.3 descending; rising: area divided into two portions by dropping perpendicular from minimum between the two peaks. [b] Deduced from Fig. 4.

Electrophoretic analyses were also carried out in pH 8.6 veronal buffer of 0.1 ionic strength. The results presented in the last column of Table II show that β-lactoglobulin A is the faster moving protein under those conditions in Tiselius electrophoresis as well as in paper electrophoresis.[11] Pooled milk β-lactoglobulin did not resolve into components at this pH but gave a mobility slightly below that of β-A. This difference in mobilities, however, is within experimental error.

TABLE II

ELECTROPHORETIC MOBILITIES OF VARIOUS β-LACTOGLOBULIN PREPARATIONS

Protein	Mobility × 10^5, cm.²/sec.-v. pH 5.3[a]		pH 5.6[a]	pH 8.6[b]	
Preparation II	−0.63	−0.22	−1.77	−1.38	−5.31
Polis "β_2-enriched"	− .80[c]	− .36[c]	−1.81	−1.30	....
β-Lactoglobulin A	− .63	.....	−1.67	....	−5.34
β-Lactoglobulin B	(− .44)	− .12	....	−1.46	−5.19

[a] 10 g./l. protein solutions in 0.1 ionic strength acetate buffer. [b] 10 g./l. protein solutions in 0.1 barbital buffer. [c] pH = 5.35.

Discussion

β-Lactoglobulin has been found in the present study to be heterogeneous electrophoretically in the entire pH range between 4.79 and 6.04. These results are in good agreement with various previous reports on the heterogeneity of this protein.[2–9] The previous disagreement between Li[2] and Polis and co-workers,[5] on one hand, and Smithies,[7] on the other, on the heterogeneity of β-lactoglobulin in the pH region of 5.3 to 5.6 could possibly be due to the fact that under these conditions it is necessary to carry out the runs for long durations in order to observe heterogeneity. In shorter runs, the patterns obtained do not show any resolution into "components," nor even any pronounced skewing.

Below pH 5.3, the interpretation of the patterns becomes complicated by the appearance of as many as three or four peaks, if the runs are extended sufficiently long (20–24 hr.).[8] Between pH 5.3 and 5.6, however, the observed heterogeneity manifests the presence of real molecular components and, at these conditions, true composition analysis can be carried out with the use of standard reference area-composition curves. This method of analysis leads to the true composition of a given β-lactoglobulin sample in terms of the Aschaffenburg β-lactoglobulins A and B.[11,12] It has been adopted, therefore, in the present study on the association of β-lactoglobulins of various composition, and, as will be shown in the next two papers, leads to good agreement between electrophoretic, ultracentrifugal and light scattering experiments.

The electrophoretic patterns of the individual Aschaffenburg components of β-lactoglobulin are much simpler than those of the pooled protein but still exhibit characteristics of non-homogeneity in the pH region close to the isoelectric point. Both genetic components exhibit electrophoretic heterogeneity in acetate buffer at pH 4.65–4.80. In the case of β-A, this is due to molecular association.[15,20,27] In β-B the reasons for skewness of the pattern are not clear at the present time, but it is known from ultracentrifuge measurements[15] that it is not a result of association processes. In the pH region of 5.3–5.6, β-A seems to be essentially electrophoretically homogeneous, in contrast with β-B (see Figs. 2 and 3), which exhibits two peaks. It should be noted that Tombs[28] has reported from solubility studies that β-A consists of 90% of a principal component and 10% of a minor component and that β-B also seems to exhibit some heterogeneity.[29] Since the apparent heterogeneity of the genetic components is constant from sample to sample,[15,21,27] analysis of β-lactoglobulin preparations into β-A and β-B can be effected by methods such as shown in Fig. 4.

The difference in mobilities of the two genetic components of β-lactoglobulin at pH 5.3 and 5.6 (Table II) would correspond to a difference of 1–2 charges per molecule of 36,000 molecular weight, with β-A being the more negatively charged protein. A similar charge difference could be inferred from the reported isoelectric points of the two proteins.[14] These estimates are in reasonable agreement with the findings of Tanford[30] who has established from titration data that β-A has two more carboxyl groups than β-B per molecule of 36,000 molecular weight.

It becomes possible now to establish the mutual identity of the various β-lactoglobulins mentioned in the literature, using the mobility criteria given in Table III. One complication arises in the identification of the material which Polis and co-workers had isolated and named β_1-lactoglobulin.[5] This material was found to be electrophoretically homogeneous at pH 4.8. At that pH β-A exhibits a strongly "heterogeneous" pattern due to association, while β-B gives only a skewed peak.[15] At pH 8.4, however, Polis and co-workers reported that their β_1-lactoglobulin had a more negative mobility than whole β-lactoglobulin. Since, in that pH region, β-A has a more negative mobility than β-B (see Table II), the Polis β_1 protein can be related only to β-lactoglobulin A by this criterion. However, the electrophoretic homogeneity of the Polis β_1 protein

(27) S. N. Timasheff and R. E. Townend, Abstracts, I tional Congress of Biochemistry, Vienna, Sept. 1958, p. 25.

(28) M. P. Tombs, *Biochem. J.*, **67**, 517 (1957).

(29) M. P. Tombs, *ibid.*, **69**, 491 (1958).

(30) C. Tanford and Y. Nozaki, *J. Biol. Chem.*, **234**,).

TABLE III

LITERATURE NAMES AND CHARACTERISTICS OF β-LACTOGLOBULIN COMPONENTS

Authors	Component names		Ref.
Aschaffenburg and Drewry	A	B	12
Aschaffenburg and Drewry	1	2	11
Ogston and Tombs	1	2	13
Klostergaard and Pasternak	2[a]	1[a]	14
Polis and co-workers	1,2 (?)[b]	2(?)[b]	5
Townend and Timasheff	Fast	Slow	20
*p*H	Relative mobility		
8.6, Tiselius electrophor.	Fast	Slow	This paper
5.6, Tiselius electrophor.	Fast	Slow	This paper
5.3, Tiselius electrophor.	Fast	Slow	This paper
4.65–4.8, Tiselius electrophor.	Slowest and fastest	Intermediate	15
8.6, Paper electrophor.	Fast	Slow	11

[a] Based on electrophoretic data. [b] The "β_2-enriched" protein was found to be enriched in β-lactoglobulin A; β_1 could be best identified as 10% non-associating fraction of β-A.

at *p*H 4.8 indicates that it does not associate strongly as does the bulk of β-A.[15] Thus, it becomes impossible at the present time to identify unequivocally the components reported by Polis and co-workers with the genetically different materials. The electrophoretic data indicate possibly that the Polis β_1 protein corresponds to the minor (10%) component of β-A which, according to Tombs' postulate,[28] does not associate and thus might give a "homogeneous" electrophoretic pattern at *p*H 4.8.

Because of the confusion which exists in the literature and the above mutual identification of various β-lactoglobulins, it is suggested that the nomenclature of β-lactoglobulin A and β-lactoglobulin B, as proposed by Aschaffenburg,[12] be adopted generally for the genetic species of this protein, especially since this nomenclature was chosen specifically to be consistent with generally accepted genetic usage.[12]

Acknowledgments.—We would like to thank Dr. W. G. Gordon for giving us samples of pooled milk β-lactoglobulin preparations, Dr. T. L. McMeekin for a sample of the "β_2-enriched" Polis fraction and Dr. R. Aschaffenburg for samples of β-lactoglobulins A and B.