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Physicochemical Comparison of Goat β-Lactoglobulin with the Bovine Varieties

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β-Lactoglobulin was prepared from mixed goat milk and multiple amino acid analyses were carried out. One difference in basic residues was found from the analysis previously reported. Ultracentrifugal, light-scattering, and optical rotatory dispersion experiments showed that the goat protein is quite similar to the bovine β-lactoglobulins in most of its physical properties, but indications were obtained that it is conformationally less stable at low pH values than its bovine analog. Titration experiments indicate that six of the free carboxylic acid (Asp and Glu) residues in monomeric bovine β-lactoglobulin B do not occur in titratable form in the caprid β-lactoglobulin. These experiments also indicate a low pH-induced conformational change to a molecule of slightly greater radius.

The recent report of the apparent identity of the β-lactoglobulin from the milk of the Indian buffalo with the B form of the genetically controlled bovine variants (1) has stimulated interest in the β-lactoglobulins from other ruminant species, since comparative biochemistry of milks is currently recognized as being able to give clues to the ancestry of various breeds within species (2). Furthermore, since the β-lactoglobulins of cow's milk are among the best understood proteins from a physicochemical point of view, a comparison of their conformational and dissociative behavior (3–6) with β-lactoglobulins from other species can lead to greater knowledge about this general class of structurally well-behaved proteins.

MATERIALS AND METHODS

Caprid β-lactoglobulin (G-β) was prepared from goats' milk as follows: 20 liters of skimmed herd milk was acidified to pH 4 (conc. HCl) and the caseins filtered off. The whey was adjusted to pH 6 and powdered $(NH_4)_2SO_4$ added slowly to approximately 75% saturation. The heavy precipitate was centrifuged off, dissolved in 10 times its weight of water, dialyzed essentially salt-free, and lyophilized. The powder was dissolved to a 5% solution in water, adjusted to pH 4 with HCl, and allowed to stand overnight (4°) at which time most of the α-lactalbumin precipitated.

Acrylamide gel electrophoresis (pH 8.5, veronal, 0.025 ionic strength) showed that a component running ahead of the main β-lactoglobulin band—probably serum albumin—was still present in the supernatant, and two subsequent crystallizations at 75% saturation of $(NH_4)_2SO_4$ failed to remove it. A portion of the crop was then fractionated by $(NH_4)_2SO_4$ precipitation (at pH 6) into three fractions: 50–57%, 57–65%, and 65–75% saturation. The 50–57% fraction, representing one-half the protein, was free of electrophoretically detectable contaminants and was used for most of the experiments reported here.

A crystalline preparation from herd goat milk, prepared by a slightly different procedure (7) by one of us (JJB) was also used for portions of the work. No difference could be detected by amino acid analysis, gel electrophoresis, or titration behavior between the two preparations.

Two-mg samples of the protein were hydrolyzed in sealed capillaries for 24, 36, 72, or 96 hours at

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110° and analyzed on a Phoenix amino acid analyzer[2]. Serine, threonine, valine, and isoleucine were determined by extrapolation in the usual manner.

Ultracentrifugal experiments were carried out in a Spinco Model E ultracentrifuge at 59,780 rpm and Tiselius electrophoresis experiments in a Spinco Model H at 1°. Optical rotations and circular dichroism were measured in Rudolph 200S and Jasco ORD 5 instruments, and other spectral measurements were made in Cary Model 14 and Zeiss PMQII spectrophotometers. Titration experiments were carried out under nitrogen using a Radiometer Model 4 pH meter and Radiometer electrodes. Light scattering was done in the Brice photometer with solutions clarified by filtration through ultrafine sintered Pyrex filters.

RESULTS AND DISCUSSION

To determine if the behavior of the goat β-lactoglobulin is the same as that of other β-lactoglobulins, a series of ultracentrifugal runs was carried out at 25° and 10 g/liter protein concentration as a function of pH in $\Gamma/2 = 0.1$ NaCl-HCl solutions. The results are shown in Fig. 1A, along with data for pooled bovine β-lactoglobulin published previously (6). It can be readily seen that, in solutions of 0.1 ionic strength, the G-β has a significantly lower sedimentation coefficient than the bovine proteins at pH values below 5. The filled circle point is material which was taken to pH 2, allowed to stand 3 hours at 25°, and readjusted to pH 5 before running. The recovery of the native value of the sedimentation coefficient shows that the low pH drop in $s_{20,w}$ is, as in the bovine case, reversible. These data are in agreement with those of Phillips and Jenness (8) who find an $s_{20,w}$ slightly below the bovine protein at several pH values[3]. It should be noted, however, that below pH 2.5, where the bovine β-lactoglobulin sedimentation coefficient at 10 g/liter levels off at a value near 2.3S, the data on G-β continue downward with no inflection appearing at the lowest pH points measured[4].

A single light-scattering experiment was carried out in a 0.1-ionic strength NaCl-HCl solvent at pH 1.9. The apparent weight-average molecular weight obtained ($M \simeq \tau/Hc$) was 25,500 ± 1,300 from 2–6 g/liter of G-β, and lower concentration points were obtained which could be extrapolated to the vicinity of 20,000. Neglecting virial effects, these values are quite close to those given by β-A and β-B under dissociating conditions (3, 6) indicating that the goat β-lactoglobulin, like the bovine analogs, also dissociates reversibly into monomers of half the isoelectric molecular weight.

Since the G-β dissociates into subunits, and seems to have many physical properties similar to the bovine proteins, an experiment was carried out to determine if it could possibly combine with β-B to give a 35,500 dalton hybrid molecule composed of a subunit from each variety, such as can be made with hemoglobins from different species (10). A 15 g/liter solution of each protein was prepared in 0.1 M HCl (pH of solution 1.1). These were divided in half and one portion of each was mixed and allowed to stand 35 min. This mixture was neutralized to pH 6 with a drop of 5 M NH_4OH and dialyzed overnight *vs.* a large volume of pH 5.96, $\Gamma/2$ 0.1 Tris-acetate solvent. The remaining portions of β-B and G-β were neutralized and dialyzed separately *vs.* the same buffer, and subsequently mixed immediately before filling the electrophoresis cell. Patterns given after electrophoresis are shown in Fig. 2. It can be seen that no visible hybrids are formed. Such hybrid formation would hardly be expected, however, as the bovine A and B varieties, differing from each other by only two residues per chain in primary structure, likewise fail to form hybrids under these conditions (11). Other Tiselius electrophoresis experiments were carried out in 0.1 ionic strength acetate

[2] The mention of commercial items is for the reader's convenience and does not constitute an endorsement by the Department of Agriculture.

[3] These results are not inconsistent with the original work on this protein by Askonas (9), who found no significant $s_{20,w}$ difference from bovine β-lactoglobulin at a single pH. The experiments were done on an ultracentrifuge lacking close temperature control so this statement and the present data cannot be considered contradictory.

[4] The report of Phillips and Jenness (8) that G-β does not undergo the low temperature association, which is a characteristic property of bovine β-lactoglobulin A (3), is corroborated by the point shown as the square in Fig. 1A, taken at pH 4.65 and 2°.

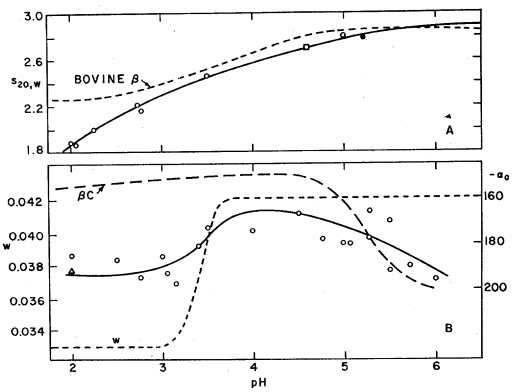

FIG. 1. A, Sedimentation coefficient of caprid β-lactoglobulin as a function of pH at 25°, 10 g/liter. Open circles: Γ/2 = 0.1 NaCl-HCl; filled circle: exposed to pH 2 for 3 hours; square: Γ/2 = 0.1 NaAc-HAc buffer, 2°; dashed line: βA-βB mixture (data from Townend *et al.* (6)). B, Circles and solid line: a_0 values on freshly made solutions; triangle: exposed to pH 2 for 72 hours; long dashes: equivalent values given by bovine β-C (4); short dashes and left hand ordinate: electrostatic interaction parameter w.

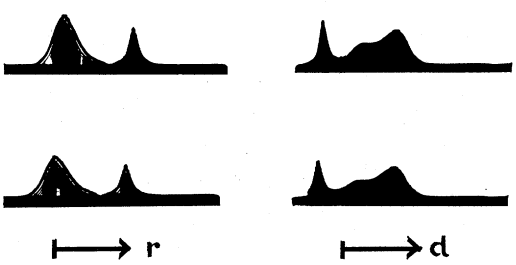

FIG. 2 Electrophoretic patterns, pH 5.96 Γ/2 = 0.01 Tris-acetate buffer. Upper pattern: acidified and mixed before neutralization; lower pattern: neutralized separately, mixed immediately before running. Time: 3.9 hours, 11.85 V/cm field strength. Starting positions of rising and descending boundaries indicated by vertical bars on the arrows.

buffers and the point of zero mobility was found to be pH 6.0 within experimental error, in agreement with the literature (8, 9). The bimodal, strikingly nonenantiographic patterns obtained in these weak-acid buffers are indistinguishable from those of bovine β-lactoglobulins above pH 5 and indicate that G-β, like the bovine proteins (12), interacts strongly with these weak-acid-containing buffers (13).

The Moffitt-Yang optical rotatory parameters of the three bovine β-lactoglobulin variants are known to reflect changes in aggregation state (4). In a given solvent system, the a_0 may be assumed to be dependent on the fraction of the native molecule which is "interior," that is, which is buried in hydrophobic regions and is inaccessible to solvent (14). Changes in this parameter may be expected to give independent information on the stability and compactness of the molecule. Figure 1B shows, as the solid line, the a_0 values obtained on G-β in one-tenth ionic strength NaCl-HCl solutions. Measurements were taken at mercury wavelengths between 335 and 578 mμ. The b_0 values remain at 72 ± 6 over the entire range of pH studied, the same values as were obtained on bovine β-lactoglobulins A, B, and C (β-A, β-B, and β-C) (4). Plotted for comparison, as the dashed line, are the β-C data. This bovine variety has the most negative a_0 values below pH 4.5 and these values undergo the same general decrease in magnitude as the others when the pH is lowered below this point (4). The G-β also seems to show a small decrease in a_0 as pH is lowered through this range but the change is much less pronounced than that given by any of the bovine variants. Below pH 4 the a_0 values of the G-β, while scattered, are significantly more negative than those given by any of the bovine lactoglobulins. This may indicate a greater unfolding than β-C and a greater exposure of interior regions at a given electrical charge. Such a deformation could also account for the lowered rate of sedimentation at these acid pH values due to an increased frictional coefficient relative to the bovine proteins.

The circular dichroic spectrum of the native G-β protein, as has been reported elsewhere (15), is almost identical with the B- variety of bovine β-lactoglobulin. Upon denaturation with acidic methanol of greater than 40% volume concentration, circular dichroic and optical rotatory dispersion spectra are obtained which are qualitatively like those of α-helical polypeptides and essentially indistinguishable from spectra given by the bovine proteins (15, 16) under the same conditions. The amino acid analysis of the G-β is presented in Table I (quadrupli-

TABLE I

AMINO ACID ANALYSIS OF GOAT β-LACTOGLOBULIN

	G-β	β-B[a]	Differences	
			This work	P. – J.[b]
Lys	16.0	14.8	+1	0
His	2.0	1.8		
Arg	3.0	2.9		
Asp	15.0[c]	15.0		
Thr[d]	8.3	8.1		
Ser[d]	6.7	6.9		
Glu	25.0	25.0		
Pro	8.1	8.2		
Gly	5.1	4.0	+1	+1
Ala	16.4	14.9	+1	0
½-Cys	3.9	3.7		
Val[d]	10.4	8.9	+1	+1
Met	3.8	3.7		
Ileu[d]	9.7	9.7		
Leu	21.1	21.9	−1	−2
Tyr	3.9	3.6		
Phe	4.0	3.8		
Trp[e]	2.0	2.0		

[a] Data of Piez *et al.* (17) as residues per 18,300 daltons.

[b] Data of Phillips and Jenness (8).

[c] Used as basis for residue ratios.

[d] Extrapolated, duplicate analyses only. All other amino acids represent average of at least four hydrolyses.

[e] Spectrophotometric; see text.

cate analyses for the neutral and acidic residues, sextuplicate for the basic residues). These are calculated as residues per 18,300 g protein, to facilitate comparison with the reported analytical data on the bovine lactoglobulins (17, 18), even though these have physically determined molecular weights near 35,500 (19, 20), i.e., a subunit weight of 17,750 daltons.

In calculating the data for a given analysis, the actual molar amount of each amino acid found was normalized, using 15 aspartic residues as a basis; this is the number present in the half-molecule of β-B (17, 18, 21). (All other amino acids give closest to whole numbers of residues when this basis is chosen.) The fourth and fifth columns of Table I give rounded amino acid differences (residues in G-β minus residues in β-B per 18,300 daltons) found here and the differences as reported by Phillips and Jenness (8). In disagreement with these authors, our analyses yield a difference in one basic amino acid, lysine, with the goat β lactoglobulin containing one residue more than the number in the bovine varieties.

The ultraviolet spectrum of G-β in the 250–300 mμ range is almost completely superimposable on that of β-A. This shows, once the tyrosine contents are known by analyses to be identical, that the tryptophan content is the same as in the bovine varieties, i.e., two residues per monomer (22).

The difference of two lysine side-chains per dimer molecule is not sufficient by itself to account for the large differences in isoelectric points between G-β and β-B (these proteins have zero mobility at pH 6 and pH 5.35, respectively, in 0.1 ionic strength solvents). Such a difference also seems rather large to be understood on the basis of conformational changes at these pH values, as has been suggested (8). Therefore, titration experiments were undertaken to determine if this difference could be explained on the basis of fewer titratable residues in the caprid β-lactoglobulin. The titrations were performed in a nitrogen atmosphere using 0.1 M NaCl as a supporting electrolyte. The reference point of zero net protons bound (isoionic) was determined to be pH 5.96, by deionizing the protein according to the method of Dintzis (23), although it was found necessary to pass the protein solution down the mixed-bed resin column (0.9 × 20 cm) twice to reach this final pH. The fact that G-β is a much more basic protein than the bovine β-lactoglobulins is in agreement with our electrophoretic experiments and with Askonas' report of an isoelectric point of very near 6 (9).

The titration data obtained are given by the open circles in Fig. 3. When net charge $\bar{Z}_H$, due to proton binding alone, is compared at pH 8 with the $\bar{Z}_H$ of bovine β-A (24) (shown as the crosses), the G-β is seen to have eight fewer negative charges. At this pH all normal carboxyl groups are ionized, only a small fraction of the lysines are unprotonated, and, as the histidine residues occur in the same number in both proteins, the entire difference in charge can be assumed to be due to a difference in the number of free carboxyl groups. β-B has two fewer negative charges at this pH than β-A (20, 24) due to

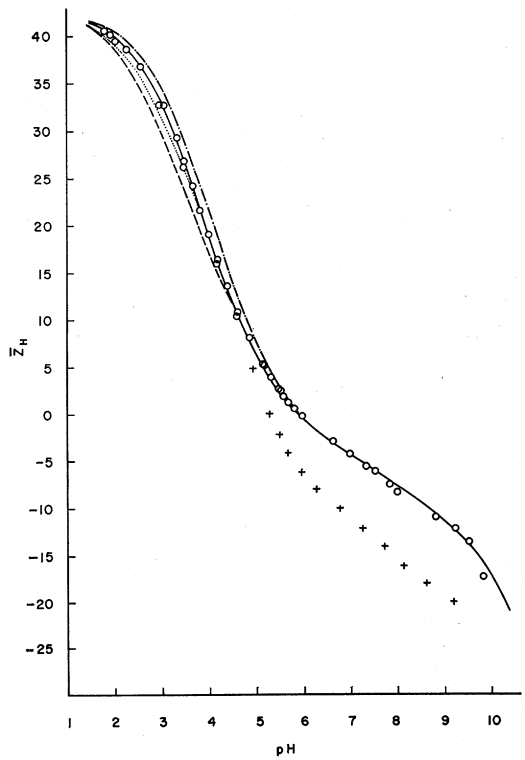

FIG. 3. Titration of G-β. Circles: experimental titration data at 0.1 ionic strength; dotted line: theoretical curve with single carboxyl pK and single valued w; dashed line: two pK values and single w; solid line: two pK values and pH-dependent w (see text); dot-dash line: single pK and varying w; crosses: data for bovine β-A above pH 4.3 (24).

its having two fewer aspartic acid residues (17, 18, 21) so that G-β has, at pH 8, six fewer negative charges than β-B. This indicates, since the total ASP and GLU found on analysis are the same for G-β and β-B (Table I), that six of the 40 residues are not available to titration, as they are in the equivalent protein of cow's milk.

It should be noted that this difference of six is independent of whether one calculates on a basis of 36,600 daltons (obtained on the bovine species (17, 18) by amino acid analysis) or 35,500 (obtained by physical measurements (19, 20)) as long as the reasonable assumption of essentially identical molecular weights for the bovine and caprid series is made. Likewise, the differences of two lysine residues per dimer molecule, and the identity between the species of the numbers of other residues found by amino acid analysis is not affected by choice of molecular weight, although the absolute numbers of residues occurring in large amounts may be.

Therefore, to enable direct comparison to be made with accurate titration work previously carried out on the β-lactoglobulins of the cow (20, 24, 25), the assumption was made that G-β has a weight of 35,500 daltons and contains 30 lysine residues (two more than the 28 groups titrating in the bovine β-lactoglobulins (24, 25)), and 42 "normal" carboxyl side chains (6 less than in β-B (20, 24)).

The titration data were analyzed in terms of pK (intrinsic) and w values reported for β-A, β-B, and β-C. These values are summarized in Table II.

Assuming that the electrostatic interaction parameter w has a value of 0.039 and that other titratable groups have the pK_{int} values given in Table II, the titration curve shown as the dotted line in Fig. 3 was calculated. This curve agrees quite well with the data (open circles) above pH 3.5, if pK_{int} = 7.0 is taken for histidines and if (and only if) two buried carboxyls (25) are included. Below this pH, however, the curve deviates downward from the measured data by as much as 1 unit of charge. Thus, it is obvious that some of the parameters picked do not fit the physical system. The fact that the deviation from the measured values occurs in the same pH range as the deviation of the $S_{20,w}$ and a_0 parameters from the data given by the bovine proteins suggests a change in conformation. Both an increase in a_0 and a decrease in sedimentation coefficient can be caused by a loosening of internal structure and a concomitant swelling or shape change.[5] Such a swelling of the molecule would also be reflected in a decrease in the interaction parameter w. If

[5] These differences could also occur if G-β should undergo a significantly higher degree of dissociation into monomers than β-A or β-B, but the light-scattering results at pH 2 show that it does not. Furthermore, at 0.1 ionic strength, dissociation is not significant at pH's above 2.5. Hence, these deviations are probably conformational in origin.

TABLE II

NUMBERS OF TITRATABLE SIDE CHAINS PER 35,500 DALTON β-LACTOGLOBULIN MOLECULE AND pK_{int} USED TO CALCULATE TITRATION CURVE[a]

Residue	G-β	β-B[b]	β-A[b]	-C[c]
Normal carboxyls	42[d]	48 (pK 4.7)	50 (pK 4.7)	48 (pK 4.7)
ϵ-Amino	30 (pK 9.9)	28 (pK 9.9)	28 (pK 9.9)	28 (pK 9.9)
Histidine	4 (pK 7.0)	4 (pK 7.25)	4 (pK 7.25)	4 (pK 7.25)
Arginine	All have 6 with $pK_{int} > 12$			
Tyrosine	All have 6 with pK_{int} of 9.9			
Terminal carboxyls	All have 2 with pK_{int} of 3.75			
Terminal aminos	All have 2 with pK_{int} of 7.80			
Abnormal carboxyls[b, c]	All have 2 with pK_{int} of 7.25			

[a] Molecular weights taken to be equal and the electrostatic interaction parameter is taken to be 0.039 for the bovine proteins.

[b] Data of Tanford *et al.* (20, 25).

[c] Data of Basch and Timasheff (24).

[d] More than one pK_{int} needed to fit data (see text).

the titration equation

$$pH = pK_{int} + \log \frac{\alpha}{1 - \alpha} - 0.868\, w\bar{Z}_H \qquad (1)$$

is solved for w by plotting pH $- \log \alpha/(1 - \alpha)$ *vs.* $\bar{Z}_H$, it does not give a straight line. The plot curves upward strongly near the point of zero charge, similar to plots given by bovine serum albumin in similar media (26). This could indicate that w may not be a single-valued function for this protein. If equation 1 is solved for w point-by-point, over the range of pH 4.7–1.7, where only carboxyl groups are titrating, it is found that this parameter is quite "normal" (0.040) below pH 3 but gives improbably large values ($\approx$0.055) in the range above pH 3.8. Hence, another approach was sought to fit the experimental data. In the pH range below 3.5, if the discrepancy between the data and the calculated curve is examined (Fig. 3, dotted line—single pK of 4.7 assumed for all side chain COOH groups), it appears as if a few carboxyl groups are becoming titrated with a slightly lower pK than the majority. In other words, the carboxyl groups seem to be divisible into two classes, titrating with formally different pK values, similar to findings of Tanford and Hauenstein (27) in the case of ribonuclease.

By trial and error it was found that a division of the 42 carboxyl groups into 32 with a pK of 4.7 and 10 with a pK of 4.3 gave a constant w value of 0.042 above pH 3.5, an unchanging value of 0.033 below pH 3.3, and a smooth variation between these points. These values are plotted in Fig. 1B as the short dashes. It can be seen in this figure that the change in the w value occurs at the same pH where the a_0 values of the G-β deviate most strongly from those of bovine β-C, and where the sedimentation coefficient likewise begins to be significantly lower than that of mixed β-A and β-B (6). Using these variable values of w and the two types of carboxyl groups resulted in a titration curve in quite good agreement with the data (solid line of Fig. 3).

Attempts were made at other fittings. If a curve is calculated with a single carboxyl pK of 4.7 and a variable w, the dot-dash line of Fig. 3 is obtained. Division of the carboxyls into 32 (pK 4.7) and 10 (pK 4.3) with a single w (0.039—that of the bovine β-lactoglobulins (20, 24)) results in the dashed line. Neither of these two approaches can by itself give a satisfactory representation of the titration data. The combined concept of two different classes of carboxyl groups, formally differing in pK, and an expandable molecule seems, then, to be the best compromise to explain the titration data obtained on this protein. The same conclusion has been reached independently by Ghose and Sen in the companion paper (28)

with only slightly different values of the parameters.

We had assumed tentatively that the results of identical numbers of Glu and Asp residues in β-B and G-β, and the differences between these proteins of six titratable groups was due simply to different numbers of amidated residues. Ghose and Sen (28) presented evidence that the difference is due to carboxyl groups buried in the interior which do not titrate in the native molecule; our data do not differentiate between these possibilities.

Under ideal conditions the following equation holds:

$$w = \frac{\epsilon^2}{2DkT}\left[\frac{1}{R} - \frac{\kappa}{1 + \kappa a}\right] \qquad (2)$$

where ϵ is the electronic charage (esu); D, the dielectric constant of the medium; κ the Debye-Hückel parameter, and a is the radius of exclusion of small ions, generally taken to be $R + 2$ Å. Using this equation and the above values of w, the radius of the equivalent sphere for G-β can be calculated to be 26.2 Å at all pH values above 3.6 and to increase from this value to 30.3 Å as pH goes from 3.6 down to 3.0. Again if we assume that the dimeric molecule behaves as a sphere, the sedimentation equation may be rearranged to give:

$$R = \frac{M(1 - \bar{V}\rho)}{6\pi\eta N s^0_{20,w}} \qquad (3)$$

This equation predicts an increase in radius from 26.1 Å to 34.8 Å as pH is lowered from 5 to 2, if we make the further assumptions that the G-β has the same slopes of $s_{20,w}$ *vs.* concentration at both pH values as the bovine protein (6) and that the pH 2 slope can be extrapolated through the region of dissociation to yield a meaningful $s^0_{20,w}$. Given these rather severe restrictions, the agreement is not unreasonable and the model of a molecule which changes its conformation to some larger or less symmetrical shape as pH falls below 3.5 seems to be believable.

Insofar as is presently known, no polymorphism has been detected in the β-lactoglobulin of the goat. However, such polymorphism may well exist, by analogy with that known in the major whey proteins of cows' milk (2, 29, 30) and ewe's milk (31); in view of the differences between the amino acid analyses reported here and those reported previously by Phillips and Jenness (8), such polymorphism seems highly likely.

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REFERENCES

1. Mawal, R. B., Barnabas, T., and Barnabas, J., *Nature*, **205**, 175 (1965).
2. Bhattacharya, S. D., Roychoudhury, A. K., Sinha, N. K., and Sen, A., *Nature*, **197**, 797 (1963).
3. Timasheff, S. N., and Townend, R., *J. Am. Chem. Soc.*, **83**, 464; 470 (1961).
4. Herskovits, T. T., Townend, R., and Timasheff, S. N., *J. Am. Chem. Soc.*, **86**, 4445 (1964).
5. Townend, R., Herskovits, T. T., Swaisgood, H. E., and Timasheff, S. N., *J. Biol. Chem.*, **239**, 4196 (1964).
6. Townend, R., Weinberger, L., and Timasheff, S. N., *J. Am. Chem. Soc.*, **82**, 3175 (1960).
7. Kalan, E. B., and Basch, J. J., *J. Dairy Soc.*, **49**, 406 (1966).
8. Phillips, N., and Jenness, R., *Biochem. Biophys. Res. Comm.*, **21**, 16 (1965).
9. Askonas, B. A., *Biochem. J.*, **58**, 332 (1954).
10. Singer, S. J., and Itano, H. A., *Proc. Natl. Acad. Sci. U.S.A.*, **45**, 174 (1959).
11. Townend, R., Kiddy, C. A., and Timasheff, S. N., *J. Am. Chem. Soc.*, **83**, 1419 (1961).
12. Timasheff, S. N., and Townend, R., *Arch. Biochem. Biophys.* in press (1968).
13. Cann, J. R., and Goad, W. B., *J. Biol. Chem.*, **240**, 148 (1965).
14. Tanford, C., De, P. K., and Taggart, V. G., *J. Am. Chem. Soc.*, **82**, 6028 (1960).
15. Townend, R., Kumosinski, T., and Timasheff, S. N., *J. Biol. Chem.*, **242**, 3755 (1967).
16. Timasheff, S. N., Townend, R., and Mescanti, L., *J. Biol. Chem.*, **241**, 1863 (1966).
17. Piez, K. A., Davie, E. W., Folk, J. E., and Gladner, J. A., *J. Biol. Chem.*, **236**, 2912 (1961).
18. Gordon, W. G., Basch, J. J., and Kalan, E. B., *J. Biol. Chem.*, **236**, 2908 (1961).
19. Senti, F. R., and Warner, R. C., *J. Am. Chem. Soc.*, **70**, 3318 (1948).

20. Tanford, C., and Nozaki, Y., *J. Biol. Chem.*, **234**, 2874 (1959).
21. Townend, R., Basch, J. J., and Kiddy, C. A., *Arch. Biochem. Biophys.*, **109**, 325 (1965).
22. Herskovits, T. T., *J. Biol. Chem.*, **240**, 628 (1965).
23. Dintzis, H. M., *Ph.D. Thesis*, Harvard Univ., 1952.
24. Basch, J. J., and Timasheff, S. N., *Arch. Biochem. Biophys.*, **118**, 37 (1967).
25. Tanford, C., Bunville, L. G., and Nozaki, Y., *J. Am. Chem. Soc.*, **81**, 4032 (1959).
26. Tanford, C., Swanson, S. A., and Shore, W. S., *J. Am. Chem. Soc.*, **77**, 6414 (1955).
27. Tanford, C., and Hauenstein, J. D., *J. Am. Chem. Soc.*, **78**, 5287 (1956).
28. Ghose, A. C., Chaudhuri, S., and Sen, A., *Arch. Biochem. Biophys.* **126**, 232 (1968).
29. Aschaffenburg, R., and Drewry, J., *Nature*, **176**, 218 (1955).
30. Blumberg, B. S., and Tombs, M. P., *Nature*, **181**, 683 (1958).
31. Bell, K., and McKenzie, H. A., *Biochim. Biophys. Acta*, **147**, 123 (1967).