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# The Solution Properties of $\beta$ -Lactoglobulin C

Bovine  $\beta$ -lactoglobulin has been known since 1955 to exist in two genetically controlled variants (1), called  $\beta$ -lactoglobulin A ( $\beta$ -A) and  $\beta$ -lactoglobulin B ( $\beta$ -B) (2), in order of their decreasing electrophoretic mobilities on paper at pH 8.6. In aqueous solutions, both proteins exist at their isoelectric points ( $\beta$ -A, 5.09;  $\beta$ -B, 5.23) (3) as symmetrical (4) dimers of identical polypeptide chains (5). The mobility difference between  $\beta$ -A and  $\beta$ -B is due to the substitution of an aspartic acid residue in the  $\beta$ -A sequence by a glycine residue in  $\beta$ -B (6). A second known difference in composition is the substitution of a single  $\beta$ -A valine residue by an alanine in  $\beta$ -B (7-9).

Recently, a third genetic variant has been found by Bell in an Australian herd (10). This form of the protein moves somewhat more slowly than  $\beta$ -B in starch-gel electrophoresis at alkaline pH and has been designated as  $\beta$ -lactoglobulin C ( $\beta$ -C). This discovery has been confirmed by Aschaffenburg<sup>1</sup> as well as in our laboratory.<sup>2</sup> From its amino acid composition,  $\beta$ -C appears to be the product of a mutation of  $\beta$ -B, a glutamic acid (or glutamine) residue in the latter having been replaced by a histidine in the  $\beta$ -C primary structure (11).

Studies carried out in this laboratory over the last few years have revealed a distinct pattern of molecular associations in the  $\beta$ -lactoglobulins below the isoelectric point. Below pH 3.5, the dimeric  $\beta$ -A and  $\beta$ -B species dissociate reversibly into their component chains of 18,000 molecular weight (5, 12). Between pH 3.7 and 5.1 a specific aggregation to an octamer<sup>3</sup> of 144,000 molecular weight takes place, with  $\beta$ -A associating much more strongly than  $\beta$ -B (13). This octamer has a compact closed structure, best described by a symmetrical array of eight spheres (14, 15). The reaction is maximal at pH 4.4 to 4.65 and is characterized by a negative enthalpy of -55 kcal per mole in this pH range (16).

It is the purpose of this paper to report on the behavior of  $\beta$ -C under comparable conditions and on the effect of the addi-

tional amino acid substitution on the physical properties of the protein.

## EXPERIMENTAL PROCEDURE

The  $\beta$ -C was prepared from the milk of a homozygous animal of the Jersey breed, kindly supplied to us by Dr. Murray Brown, Department of Dairy Science, Texas A and M College, College Station, Texas. The protein was prepared by the Aschaffenburg technique (2) and recrystallized three times. The crystallization of the first crop was found to occur much more slowly than in the case of  $\beta$ -A or  $\beta$ -B. It was identified by comparison with an authentic sample furnished us through the courtesy of Dr. K. Bell.  $\beta$ -A and  $\beta$ -B preparations have been described elsewhere (13).

Ultracentrifugal experiments were performed with a Spinco model E instrument,<sup>4</sup> Tiselius electrophoresis in a Spinco model H apparatus,<sup>4</sup> optical rotatory dispersion measurements in a Rudolph model 200S spectropolarimeter<sup>4</sup> with rocking polarizer, and direct spectra and difference spectra were recorded on a Cary model 14 spectrophotometer.<sup>4</sup> Concentration measurements were made spectrophotometrically in a Zeiss model PMQ II instrument<sup>4</sup> at 278 m $\mu$ . For the determination of the absorptivity of the  $\beta$ -C, samples of the three variants were kept in a vessel at 100% humidity. Concentrations were then determined gravimetrically with the known absorptivity values of 9.6 dl per g at 278 m $\mu$  for  $\beta$ -A and  $\beta$ -B to establish the moisture content of all three samples. The solvent perturbation techniques employed in obtaining difference spectra have been described elsewhere (17). These measurements were carried out in 1.0-cm tandem cells, to subtract the contributions of solvent and perturbant to the difference spectra.

## RESULTS

*Optical Measurements*—The values of the absorptivity, the solvent perturbation ratio at 292 and 278 m $\mu$ , and the Moffitt-Yang (18) rotatory dispersion parameters of the three genetic variants are given in Table I. The absorptivities and the solvent perturbation ratios,  $\Delta\epsilon/\epsilon$ , are seen to be identical for the three proteins within experimental error. These similarities are strong indications of the essential identity of the location and interactions of tryptophan, tyrosine, and phenylalanine in these proteins.

Fig. 1 shows the direct spectra and the difference spectra in aqueous solution with 20% sucrose as a perturbant. Compari-

<sup>4</sup> It is not implied the United States Department of Agriculture recommends the above company or its products to the possible exclusion of others in the same business.

<sup>1</sup> R. Aschaffenburg, personal communication.

<sup>2</sup> M. P. Thompson, unpublished experiments.

<sup>3</sup> In previous publications the kinetic unit in this state of aggregation was referred to as a lactoglobulin tetramer (13-16), since the described equilibrium exists between the isoelectric 36,000 and the low temperature 144,000 molecular weight species. It is known, however, that the 36,000 molecular weight species is a dimer of two identical 18,000 molecular weight chains. Therefore, it seems appropriate to refer to it as a dimer and to the higher low temperature aggregate as an octamer. The reactions may thus be summarized by the scheme:

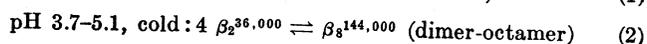
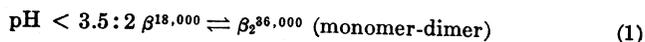


TABLE I  
Comparison of spectral and rotatory dispersion parameters of  $\beta$ -lactoglobulins

Genetic variant	Spectral parameters		Rotatory parameters							
	$\epsilon_{1\text{cm}}^{1\%}$ (278 m $\mu$ )*	$(\Delta\epsilon_{292} - \epsilon_{278})^\dagger$	pH 4.56, $\Gamma/2 = 0.1$ acetate				pH 2.7, 5 g/liter, 25°, NaCl-HCl			
			Concentration extrapolation	2°		30°				
				$-a_0$	$-b_0$	$-a_0$	$-b_0$	$\Gamma/2$	$-a_0$	$-b_0$
$\beta$ -A	9.6 $\pm$ 0.1	0.016 $\pm$ 0.002	C $\rightarrow$ 0	132	76	148	75	$\rightarrow$ 0	162	83
			C $\rightarrow$ $\infty$	63	76	148	75	= 1	132	83
$\beta$ -B	9.6	0.014	C $\rightarrow$ 0	143	75	157	73	$\rightarrow$ 0	162	87
			C $\rightarrow$ $\infty$	74	75	157	73	= 1	132	87
$\beta$ -C	9.5	0.015	C $\rightarrow$ 0	156	73	177	72	$\rightarrow$ 0	182	81
			C $\rightarrow$ $\infty$	156	73	177	72	= 1	(152)	81

\* Based on  $\epsilon_{1\text{cm}}^{1\%}$  (278 m $\mu$ ) = 9.6 for  $\beta$ -A and gravimetric protein concentration determination, assuming the same moisture content.  
 † Obtained with 20% sucrose as perturbant,  $\Gamma/2 = 0.03$ , pH = 2 to 5.5, 25°.

son of the perturbed difference spectra of the native proteins (solid and dashed lower lines) with the difference spectrum of  $\beta$ -A reduced by thioglycolate and fully unfolded by 8 M urea (dotted line) indicates that in all three variants more than half of the tryptophyl and tyrosyl residues of the native proteins are buried in the solvent-free interior of the molecules.

Comparison of the  $a_0$  and  $b_0$  optical rotatory dispersion parameters, shown in Table I, suggests that the secondary structures of the three genetic variants do not differ significantly from each other. The low  $b_0$  values indicate the presence of little or no helix, although the abnormally low  $a_0$  values suggest a fair amount of order in the folded structure, as well as the burial of a major fraction of the peptide chain in the low dielectric constant interior. Such a situation is perfectly consistent with the known compact structures of  $\beta$ -A and  $\beta$ -B (4, 15, 16).

**Electrophoretic Behavior**—In Tiselius electrophoresis experiments in 0.1 ionic strength acetate buffers near the isoelectric pH,  $\beta$ -C gives patterns similar to those obtained with the B species of  $\beta$ -lactoglobulin (19). These patterns, shown in Fig. 2, Patterns I and II, are complex, with leading shoulders in the descending limbs and trailing shoulders in the rising limbs. Like  $\beta$ -B, the shapes of the patterns are functions of ionic strength, nature of supporting electrolyte, and field strength,<sup>5</sup> indicating interactions with buffer components and possibly an isomerization, similar to effects observed by Cann (20) for serum albumin. The possible occurrence of an isomerization is indicated also by optical rotation dispersion experiments. In this pH range, the  $a_0$  parameter is found to undergo a gradual change toward more negative values, with increase in pH, as shown in Fig. 3I. Although the absolute values of  $a_0$  differ for the three variants, a similar change is observed for all three in this pH range (21).

The mobilities of the major peak in 0.1 ionic strength acetate buffers are listed in Table II. Interpolation shows the isoelectric point of  $\beta$ -C to be between pH 5.32 and 5.34 in this solvent. This is slightly higher than the value of 5.23 found for  $\beta$ -B (3), as would be expected from the nature of the amino acid substitution. Electrophoretic separation from  $\beta$ -B, although possible in fixed media (10), does not seem to be possible by Tiselius electrophoresis. Experiments, carried out between

<sup>5</sup> S. N. Timasheff and R. Townend, paper in preparation.

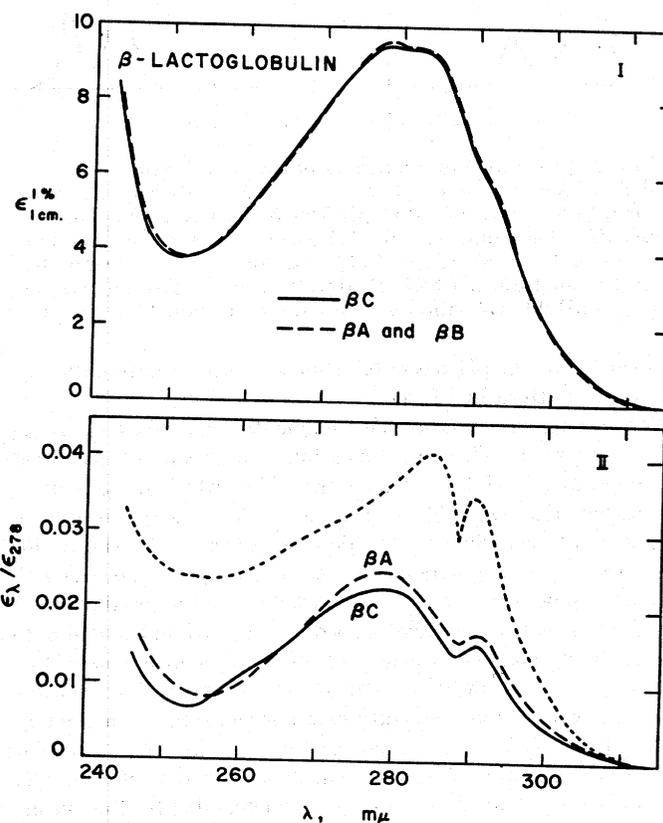


FIG. 1. Spectral data on the  $\beta$ -lactoglobulins. I, direct spectra at isoelectric pH (solid line,  $\beta$ -C; dashed line,  $\beta$ -A and  $\beta$ -B),  $\Gamma/2 = 0.15$ . II, solvent perturbation difference spectra with 20% sucrose as perturbant; solid line,  $\beta$ -C, pH 4.0,  $\Gamma/2 = 0.03$ ; dashed line,  $\beta$ -A, pH 3.3,  $\Gamma/2 = 0.03$ ; dotted line, thioglycolic acid reduced  $\beta$ -A, 8 M urea, pH 3.4, 0.02 M thioglycolate. Data on  $\beta$ -B essentially superimpose on those of  $\beta$ -C and are omitted for reasons of clarity.

pH 5.2 and 5.4, where the effect of the charge difference should be maximal, resulted invariably in schlieren patterns consisting of a single, highly skewed boundary, as shown in Fig. 2, Pattern III. Separation from  $\beta$ -A, on the other hand, can be accom-

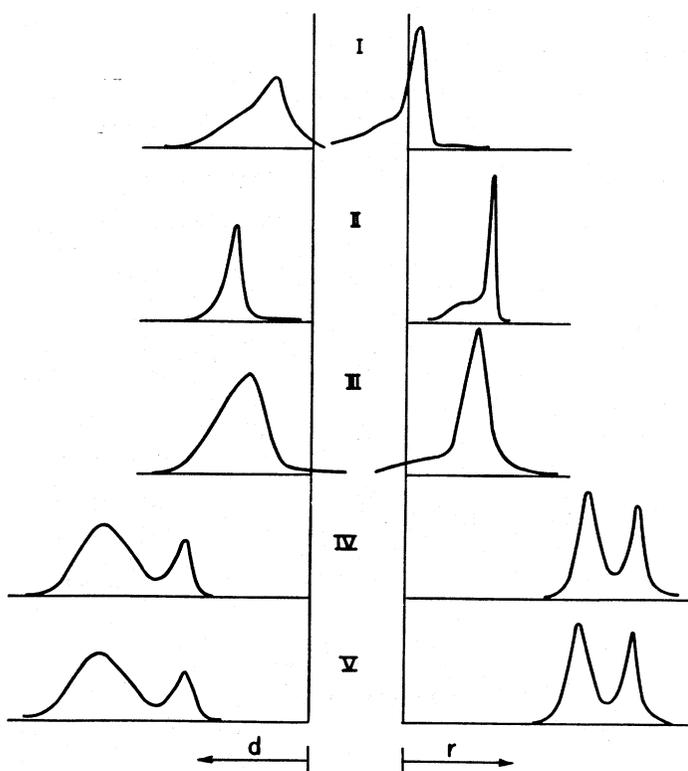


Fig. 2. Electrophoretic patterns of  $\beta$ -C and mixtures with  $\beta$ -A and  $\beta$ -B. Acetate buffers,  $\Gamma/2 = 0.1$ . I,  $\beta$ -C, pH 5.31, 5.59 volts per cm, 1319 minutes; II,  $\beta$ -C, pH 5.09, 5.61 volts per cm, 329 minutes; III,  $\beta$ -B-C mixture, pH 5.29, 5.03 volts per cm, 1255 minutes; IV,  $\beta$ -A-C mixture, pH 5.57, 11.64 volts per cm, 470 minutes; V,  $\beta$ -A-C mixture, pH 5.57, 11.64 volts per cm, 470 minutes, exposed to pH 2 for 55 minutes before dialysis against the buffer.

plished cleanly at pH 5.6 in 0.1 ionic strength acetate buffer, as shown by Pattern IV of Fig. 2.

**Octamerization Reaction**—The sedimentation coefficients ( $s_{20,w}$ ) of  $\beta$ -C are given in Table III as a function of concentration and temperature at pH 4.6. This is the pH at which the  $\beta$ -A octamerization reaction (13) is maximal. This reaction results in bimodal ultracentrifugal “reaction boundaries,” as shown for  $\beta$ -A by the inner diagrams of Fig. 4, the two “components” having sedimentation coefficients of 3.05 S and approximately 5 S. Experiments were also carried out at pH 4.3 and 4.8 to check on any possible displacement of the pH dependence of this reaction. The results show that  $\beta$ -C sediments at all concentrations, pH values, and temperatures studied as a single, completely symmetrical boundary. Examination of the sedimentation coefficients in Table III shows that octamerization, if occurring at all, is present at 2° to an extent undetectable by this means at protein concentrations as high as 50 g per liter. The slope of the best  $s_{20,w}$  against  $c$  plot through the data points has the value  $s_{20,w} = (2.97 - 0.011c) \times 10^{-13}$ , for both the 2° and 25° data. Short column equilibrium ultracentrifugation was carried out at pH 4.6,  $\Gamma/2 = 0.1$  on 10 g per liter of  $\beta$ -C solutions. Application of the calculation method of Yphantis (22) yielded weight average molecular weights of 37,400 at 25° and 37,800 at 2° and  $z$ -average molecular weights at the two temperatures of 37,800 and 36,900, respectively, showing essentially complete absence of aggregates.

By the criteria applied so far,  $\beta$ -C can be considered as not

capable of octamerization at pH 4.6. Since in the case of  $\beta$ -B, its ability to associate was established in experiments with  $\beta$ -A in which mixed octamer formation was evident (13), it was of interest to determine if  $\beta$ -C, like  $\beta$ -B, can form mixed octamers with  $\beta$ -A. In Fig. 4 the ultracentrifugal patterns obtained with  $\beta$ -A- $\beta$ -B and  $\beta$ -A- $\beta$ -C mixtures under octamerizing conditions are compared with each other, as well as with  $\beta$ -A at the same concentration as used in the mixtures. In the *left-hand figure*,

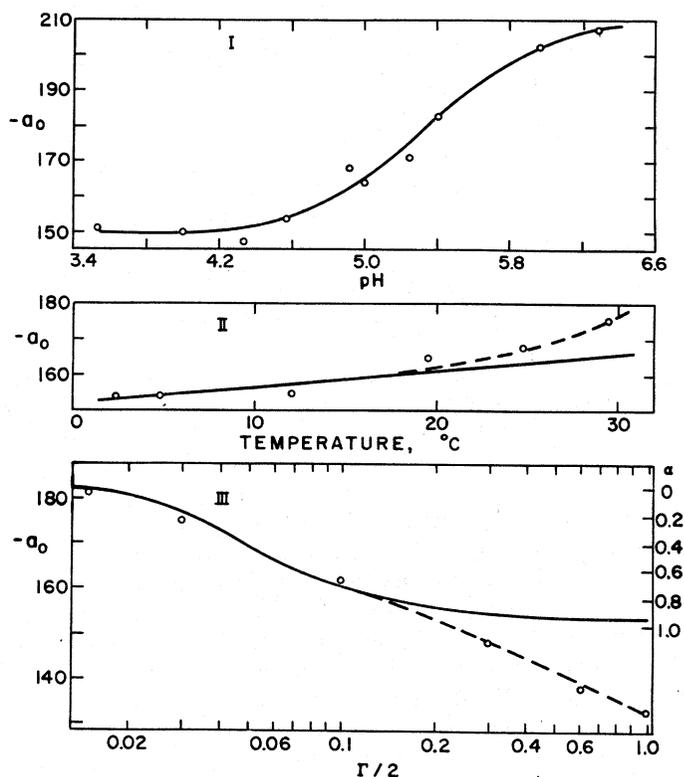


FIG. 3. Optical rotatory parameter  $\alpha_0$  of  $\beta$ -C. I, 30.0 g per liter of protein in  $\Gamma/2 = 0.1$  acetate buffers, 2°. II, 30.0 g per liter of protein in pH 4.57,  $\Gamma/2 = 0.1$  acetate buffer. III, 5.2 g per liter of protein in NaCl-HCl of pH 2.70 at 25°; the degree of association,  $\alpha$ , is shown on the *right-hand ordinate*.

TABLE II  
Electrophoretic mobilities of  $\beta$ -lactoglobulin C

Buffer	Electrophoretic mobility	
	Descending	Ascending
	$\times 10^6 \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$	
Acetate*		
pH = 5.09	1.79	2.09
pH = 5.19	1.43	1.64
pH = 5.31	0.17	0.05
pH = 5.40	-0.25	-0.43
pH = 5.51	-0.62	-0.74
pH = 5.57	-0.95	-1.45
pH = 5.80	-1.02	-1.56
NaCl-HCl†		
$\Gamma/2 = 0.02$	11.89	15.44
$\Gamma/2 = 0.30$	6.17	6.52

\* Protein concentration, 10 g per liter;  $\Gamma/2 = 0.1$ .

† Protein concentration, 5 g per liter; pH = 2.75.

a 1:1 mixture of  $\beta$ -A and  $\beta$ -B is shown as the *outer curve*, superimposed on a pattern given by  $\beta$ -A alone at one-half the total protein concentration. In the *right-hand figure* the same is shown for a  $\beta$ -A- $\beta$ -C mixture. It can be seen that in the A-C pattern much less of the total area sediments under the rapid peak than in the A-B mixture. The Gilbert theory (23) of moving boundaries in associating systems was applied to these patterns with the assumption that only  $\beta$ -A can octamerize. The expected area distributions in this case are found to be, after correction for the Johnston-Ogston anomaly (24), 35% fast for the A-B mixture and 37% fast for the A-C mixture. These are to be compared with the experimental values of 57% fast in A-B and 42% in A-C. Thus, while in the  $\beta$ -A- $\beta$ -B mixture it is evident that  $\beta$ -B interacts with  $\beta$ -A to form mixed octamers with a large fraction of the  $\beta$ -B sedimenting under the rapid peak, no effect of comparable magnitude is found with  $\beta$ -C. These data indicate that tendency to form any  $\beta$ -A- $\beta$ -C mixed octamers is exceedingly weak, if present at all.

It has been found previously that the optical rotatory dispersion parameter,  $a_0$ , is a sensitive indicator of octamer formation in the  $\beta$ -lactoglobulin system (21), this parameter assuming less negative values with increasing aggregation. By this criterion, as well,  $\beta$ -C undergoes no changes under optimal conditions of octamerization, as shown in Table I.  $a_0$  is independent of concentration at 2° for  $\beta$ -C, although a strong change is observed with  $\beta$ -A. Typical data for  $\beta$ -C as a function of temperature are shown in Fig. 3II. The *solid line* is the temperature dependence of  $a_0$  for monomeric  $\beta$ -A and  $\beta$ -B (21). The  $\beta$ -C experimental points fall on this line in the low temperature range, where octamerization is maximal and large downward

TABLE III

Sedimentation coefficients of  $\beta$ -lactoglobulin C at various conditions

pH	Medium	$\Gamma/2$	Concentration g/liter	Temperature	$s_{20,w}$
4.63	Acetate	0.1	9.0	2.0°	2.84
4.63	Acetate	0.1	9.0	25.0	2.86
4.63	Acetate	0.1	18.0	2.0	2.78
4.63	Acetate	0.1	18.0	25.0	2.76
4.62	Acetate	0.1	50.0	2.0	2.44
4.60	Acetate	0.1	46.4	25.0	2.42
4.30	Acetate	0.1	26.5	2.0	2.40
4.80	Acetate	0.1	27.1	2.0	2.63
2.10	NaCl-HCl	0.1	2.0	25.0	2.30
2.10	NaCl-HCl	0.1	5.0	25.0	2.25
2.08	NaCl-HCl	0.1	11.6	25.0	2.57

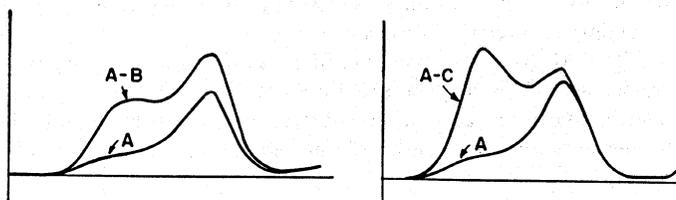


FIG. 4. Ultracentrifugal patterns at pH 4.6,  $\Gamma/2 = 0.1$  acetate, 2°. Left: upper diagram, A-B mixture, 10 g per liter of each (57% fast), 160 minutes at 59,780 r.p.m.; lower diagram, 10 g per liter of B-A, at 128 minutes. Right: upper diagram, A-C mixture, 11.2 g per liter of each (42% fast); lower diagram, 11.2 g per liter of  $\beta$ -A; conditions same as left patterns.

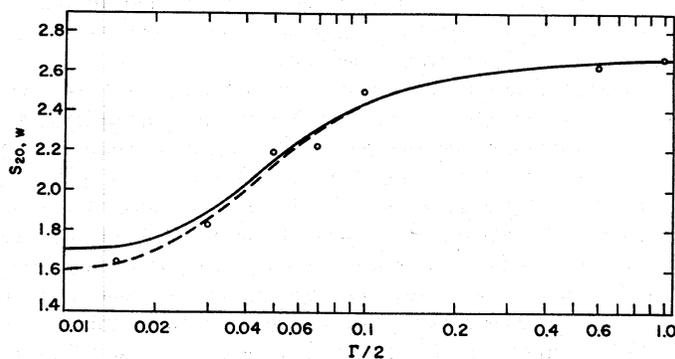


FIG. 5. Ionic strength dependence of sedimentation coefficient of  $\beta$ -C at pH 2.70, 25°, 5 g per liter of protein. Solid line, calculated from equilibrium constants of dissociation; dashed line, solid line corrected for primary charge effect.

deviations are observed with A and B. The upward deviation at higher temperatures can not be related to this reaction and might indicate incipient denaturation of the C-protein.

*Low pH Dissociation*—Equilibrium ultracentrifugation experiments were carried out with a 2-mm column of solution (22) of 3 g per liter of  $\beta$ -C in a pH 2.02 NaCl-HCl solvent (0.1 ionic strength). The apparent weight average and z-average molecular weights<sup>6</sup> obtained were  $21,500 \pm 200$  and  $27,150 \pm 200$ . With the light scattering equilibrium constants for the other variants (5) at the same conditions, the values of these parameters can be calculated to be 24,300 and 27,300, indicating that  $\beta$ -C dissociates at low pH in a similar manner. This is further supported by the concentration dependence of the sedimentation coefficient measured at pH 2.1, shown in Table III, which is similar to that previously observed for pooled lactoglobulin under similar conditions (13).

Fig. 5 shows the ionic strength dependence of the sedimentation coefficient of a solution of 5 g per liter of  $\beta$ -C at pH 2.7. Like the other two variants, the lowering of the sedimentation coefficient from the isoelectric value of 2.85 is almost completely reversed in environments of ionic strength greater than 0.1. Light scattering studies (25) have shown that in the case of  $\beta$ -A and  $\beta$ -B this phenomenon is due to a reversible dissociation of the 36,000 molecular weight unit. This dissociation is driven by nonspecific electrostatic repulsion as the molecule becomes progressively more positively charged and the screening of the supporting electrolyte is diminished. This gives rise to kinetic units of 18,000 molecular weight in equilibrium with undissociated protein. With the  $\beta$ -A and  $\beta$ -B dissociation equilibrium constants as a function of ionic strength at this pH (25), the weight average sedimentation coefficients (23, 26) were calculated. In this calculation, it was assumed that the difference in  $s_{20,w}$  of 1 S between the monomer and dimer found for the other two variants persists in  $\beta$ -C; knowledge that, at  $\Gamma/2 = 1.0$ , 97% of the protein is in the undissociated form, led to an  $s_{20,w}$  value for it of 2.7 S. The resulting theoretical curve is shown in Fig. 5 as the *solid line*. Correction at lower ionic strengths for the primary charge effect (26), with the electrophoretic mobilities of Table II at pH 2.75, lowers the calculated curve to the *dashed line*. This agrees very well with the experimental data, leading to the conclusion that the low pH dissociation of

<sup>6</sup> These values are not corrected for multicomponent and virial effects.

$\beta$ -C has thermodynamic parameters very similar to those of  $\beta$ -A and  $\beta$ -B, *i.e.* the free energy of attraction between the two subunits,  $\Delta F^a$ , is  $-9.8$  kcal per mole.

Optical rotation measurements on  $\beta$ -A and  $\beta$ -B have shown that  $a_0$  may be also used as a quantitative measure of the degree of dissociation at low pH (21). The theoretical curve obtained for  $\beta$ -A and  $\beta$ -B as a function of ionic strength at pH 2.7 is shown by the *solid line* of Fig. 3III, after translation to the extrapolated value at zero ionic strength (see Table I). The experimental  $\beta$ -C points are seen to fall on this curve below  $\Gamma/2 = 0.1$ . Above this ionic strength, the  $a_0$  values continue to become progressively less negative, indicating a minor structural readjustment or change upon association which is not observed in the other two variants. That this process does not involve a change in molecular weight or a gross alteration in shape, is evidenced by the sedimentation data of Fig. 5 which are accounted for quantitatively in terms of the low pH dissociation up to  $\Gamma/2 = 1.0$ . In this connection, it is interesting to note that  $\beta$ -C appears to be much less stable in general than the other two variants. Thus, at pH 5.7, where both  $\beta$ -A and  $\beta$ -B are indefinitely stable,  $\beta$ -C shows a slow increase in both  $a_0$  and  $b_0$ , and the solutions become turbid in a few hours, indicating a slow irreversible denaturation.

**Hybridization Experiments**—Experiments with radioactively labeled  $\beta$ -A have shown that if dissociation by low pH in the presence of unlabeled  $\beta$ -B is followed by reassociation at neutrality and electrophoretic separation, all the radioactivity remains associated with the  $\beta$ -A (27). The same experiment was carried out with  $\beta$ -C as follows: 2 ml of a solution of 24 g per liter of  $\beta$ -C were mixed with 2 ml of a solution of 24 g per liter of  $\beta$ -A, labeled *in vivo* with  $^{14}\text{C}$ -valine. The mixture was acidified to pH 2.0 with concentrated HCl and allowed to stand 55 minutes at room temperature. Following neutralization with  $\text{NH}_4\text{OH}$ , the solution was dialyzed for 8 hours against pH 5.57 NaOAc buffer of 0.1 ionic strength. The resulting solution gave an electrophoretic pattern identical with that of an A-C mixture that had not been acidified (compare Fig. 2, Patterns IV and V). Separation of the components was effected by 8 hours of electrophoresis at a field strength of 9.7 volts per cm.  $\beta$ -C was recovered by sampling from the trailing peak in the descending limb,  $\beta$ -A by sampling from the leading peak in the rising limb. Radioactivity was measured by evaporating the sampled material onto a planchet and counting at 50% relative humidity. The results were, after subtracting background:  $\beta$ -C, 1.31 c.p.m.;  $\beta$ -A, 21.59 c.p.m. It is evident that essentially all the radioactivity has remained with the  $\beta$ -A component in the acidified mixture. These experiments indicate that, in  $\beta$ -C, which differs from  $\beta$ -A by only three amino acids, the sites of contact between the subunits are species-specific just as with the B-variant.

#### DISCUSSION

The physicochemical characterization of the third known genetic variant of  $\beta$ -lactoglobulin,  $\beta$ -C, reveals few differences in the gross properties of this protein when compared with the better known variants. Where  $\beta$ -A and  $\beta$ -B differ,  $\beta$ -C resembles the latter, the progression of properties following the increase in isoelectric points.

As examples of this variation,  $\beta$ -C is highly soluble in water at the isoelectric point, as evidenced by the extensive pervaporation needed (11) to cause crystallization during the preparative pro-

cedure.  $\beta$ -B is likewise more soluble than  $\beta$ -A at isoionic conditions (28). Furthermore,  $\beta$ -A is more resistant to heat denaturation than  $\beta$ -B (29), and  $\beta$ -C has been observed to become visibly turbid overnight at refrigerator temperature and pH 5.7, conditions at which the A and B variants seem to be stable indefinitely.

The major difference found is the essential absence, in  $\beta$ -C, of the characteristic octamer given by  $\beta$ -A and  $\beta$ -B (see Fig. 4). Other than this, we have been unable to devise experiments that would demonstrate quantitatively further physicochemical differences in the properties of the three genetic variants. Neither the molecular weight, the hydrodynamic behavior, nor the spectral or the optical rotatory properties are sufficiently different to permit any structural assignment to the difference in the aggregation properties of  $\beta$ -lactoglobulin C. cursory observation indicates that the replacement of aspartic, valine and glutamic residues in  $\beta$ -A by glycine, alanine, and histidine, respectively, makes  $\beta$ -C somewhat more heat labile and also more soluble in water than the two other variants. We believe that steric differences not detectable by the above methods must be produced in the surface site of octamerization of  $\beta$ -lactoglobulin to account for the lack of octamer formation.

This study also serves to demonstrate further how the replacement of a few amino acids by genetic factors may alter physical properties which in turn may radically alter ability to carry out physiological function properly. The relative rarity of the C gene might indicate that the increased solubility and lower degree of conformational stability are detrimental to the bovine species, which is opposite to the solubility properties of the aberrant human hemoglobins which cause sickle cell anemia (30). Genetic changes, however, may equally well bring about the production of proteins or enzymes which enable the organism to withstand adverse environmental conditions. Such a case is the mutant tryptophan synthetase which has been found in heat and acid resistant *Escherichia coli* by Maling and Yanofsky (31) and Yanofsky (32).

The  $\beta$ -lactoglobulin C gene never occurs, as far as is known, in the Holstein Friesian breed,<sup>7</sup> and further experiments to determine other bovine characteristics which may be linked to the  $\beta$ -lactoglobulin type will be of great interest.

#### SUMMARY

The recently discovered C variant of bovine  $\beta$ -lactoglobulin has been confirmed as a  $\beta$ -lactoglobulin by physicochemical criteria. Comparison with the A and B variants was carried out by ultracentrifugation, electrophoresis, difference spectral analysis, and optical rotatory dispersion. The over-all properties of  $\beta$ -C lactoglobulin are found to be very similar to those of the other two variants, suggesting an essentially identical secondary and tertiary structure.

Like  $\beta$ -B,  $\beta$ -C was found to differ from  $\beta$ -A in its aggregation properties. This may be attributed to the difference in amino acid composition, and, where differences have been found, the sequence is always in order of the increasing isoelectric points, *i.e.* A, B, C.

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<sup>7</sup> K. Bell, personal communication.

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