Zonal Ultracentrifugation of β-Lactoglobulin and κ-Casein Complexes Induced by Heat [1]

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Abstract

A zonal ultracentrifuge was used to isolate the heat-induced complexes of β-lactoglobulin and κ-casein. The complexes formed at 80 C in sodium cacodylate buffer, pH 6.65, ionic strength 0.08, varied in size and composition, but moved as a single band in starch-gel electrophoresis. The maximum ratio in the complex was estimated to be 3 β-lactoglobulin :1 κ-casein. When a 1:1 mixture was heated at 110 C the major component banded in 6.9% sucrose, had a sedimentation coefficient of 2.35, and moved as a single band in starch gel. At 140 C, κ-casein was degraded extensively, and no complex could be detected. The complex formed at 90 C from a 1:1 mixture in a synthetic serum containing calcium had a sedimentation coefficient of 39. For other mixtures in the presence of calcium the unreacted proteins could not be quantitatively separated from the complex. Zonal ultracentrifugation of heated skimmilk and colloidal calcium phosphate free milk failed to show a discrete complex peak.

Formation of a heat-induced complex between β-lactoglobulin and α-casein was suggested by McGugan et al. (11), and other researchers, using moving-boundary electrophoresis (19, 24), zone electrophoresis (4, 5, 6, 16), polarization of fluorescence (12), and light scattering (9), have shown that a reaction occurs between β-lactoglobulin and κ-casein. The possible influence of such a complex on the heat stability of skimmilk (17, 18, 21) led us to study this protein interaction more fully.

Our paper reports work with the zonal ultracentrifugation technique of Anderson (1, 2). The objective was to isolate the complex and to calculate the complexing ratio of the two proteins involved. Some results for skimmilk are also given.

Materials and Methods

κ-Casein was prepared according to Zittle and Custer (23) and purified by the Sephadex method of Yaguchi et al. (22). β-Lactoglobulin (N.B. Co., 3 ×) was used without further purification. Solutions of β-lactoglobulin or κ-casein, and mixtures of the two proteins, were prepared in 0.02 M sodium cacodylate buffer, pH 6.65, ionic strength 0.08 (with NaCl), or in Jenness and Koops synthetic serum (7), pH 6.65. Protein concentration in the solution was usually 0.5%. Samples were deaerated at room temperature for 5 min and the vacuum broken with nitrogen, then heated in a thermostated water bath at 80 or 90 C for 10 min, or in sealed glass tubes in an oil bath at 110 or 140 C for 10 min.

A Beckman model ZU zonal ultracentrifuge with a B-IV rotor (3) was used. Sucrose solutions for the gradients were prepared with the same buffer as the sample. Conditions of zonal ultracentrifugation are indicated in the figure captions.

Twenty-milliliter fractions were collected manually from the rotor after centrifugation and the absorbance at 280 mμ determined. The relative protein content of each zone was then estimated from the areas under the zonal profile without allowance for differences in specific absorption. The fractions were clear, or very slightly opalescent, and a base-line correction was applied only for the absorption of the stock sucrose solutions. The gradient was determined with a hand refractometer calibrated in per cent sucrose.

Combined fractions were dialyzed for 24 hr against running tap water, followed by an additional 24 hr against distilled water, then concentrated by ultrafiltration (20), adjusted to pH 7.0, with 0.1 N HCl, and freeze dried. Urea starch gel electrophoresis, with or without 2-mercaptoethanol, was done as reported elsewhere (15).

Aliquots of the test solutions were also analyzed by a Model E ultracentrifuge and the sedimentation coefficients (S_{20}) calculated according to standard procedures.

Results

β-Lactoglobulin and κ-casein in sodium cacodylate buffer heated at 80 C, 10 min. The ultracentrifuge profiles for β-lactoglobulin and κ-casein, not heated and heated individually

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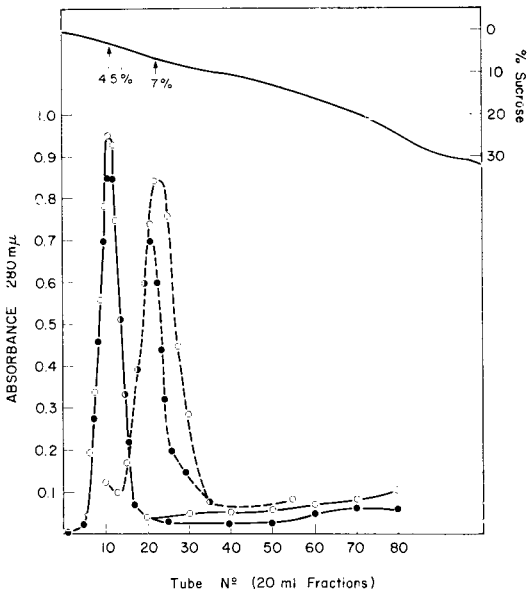

FIG. 1. Sedimentation profile of β-lactoglobulin ————, and κ-casein – – – –, in sodium cacodylate buffer, pH 6.65, unheated ○, and heated at 80 C, 10 min ●. A 25-ml sample was introduced above a 1,200 ml, 5 to 20% sucrose gradient with a cushion of 30% sucrose. The sample was overlaid with 200 ml of sodium cacodylate buffer pH 6.65 and centrifuged at 40,000 rpm for three hours at 5 C.

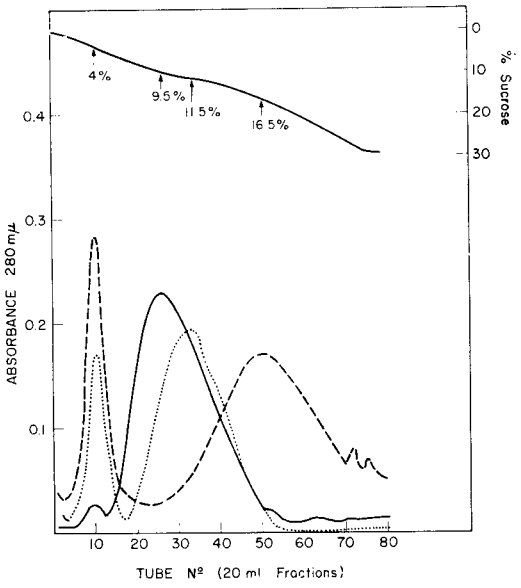

FIG. 2. Sedimentation profile of various mixtures of β-lactoglobulin and κ-casein heated at 80 C, 10 min in sodium cacodylate buffer, pH 6.65: Ratio of β-lactoglobulin to κ-casein in initial mixture: 1:1 ————, 3:1 ....., 4:1 – – – –. Zonal ultracentrifugation conditions were the same as in Fig. 1.

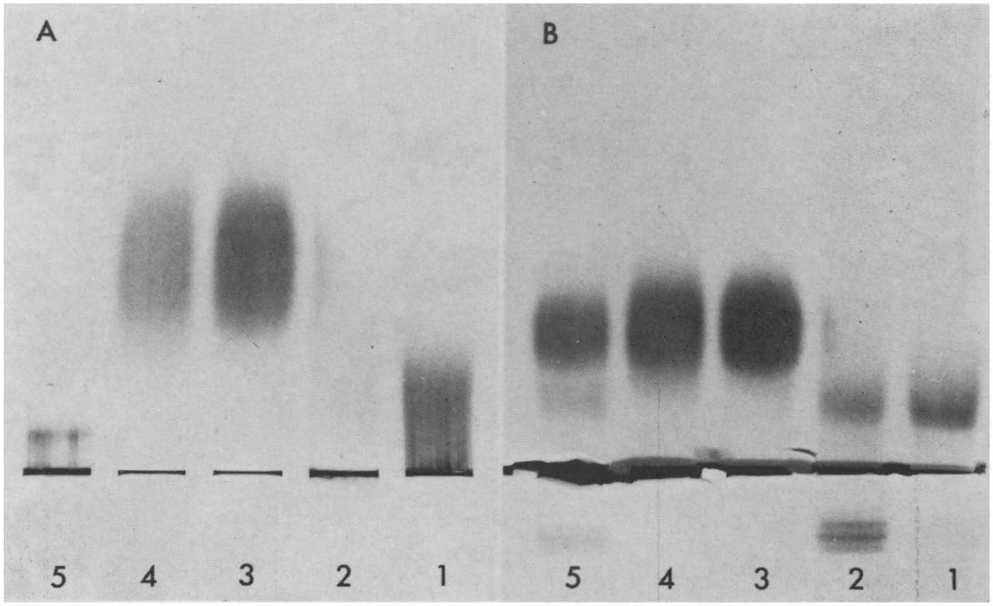

FIG. 3. Starch-gel electrophoretic patterns. A, without and B with 2-mercaptoethanol, of 1, unheated κ-casein, 2, κ-casein heated at 80 C, 10 min, 3, unheated β-lactoglobulin; 4, β-lactoglobulin heated at 80 C, 10 min, 5, β-lactoglobulin-κ-casein complex from the heated 1:1 mixture (of Fig. 2).

at 80 C for 10 min (Fig. 1) showed that both the nonheated and heated samples of β-lactoglobulin and of κ-casein banded in 4.5 and 6.5% sucrose, respectively. Heated mixtures of β-lactoglobulin and κ-casein (Fig. 2) banded into two groups of peaks. The light fractions banded in 4% sucrose, and the heavier components banded in 9.5, 11.5, or 16.5% sucrose, depending upon the β-lactoglobulin:κ-casein ratio in the initial mixture. Starch-gel electrophoresis of the light fraction showed that it consisted of uncomplexed β-lactoglobulin. The heavier components each moved in urea starch gel as a single zone (Fig. 3, A5), but in a gel containing 2-mercaptoethanol these components dissociated into zones corresponding to β-lactoglobulin and κ-casein (Fig. 3, B5), indicating that these components were heat-induced complexes.

The ratio of β-lactoglobulin to κ-casein in the complexes was calculated from the amounts present initially, minus the residual β-lactoglobulin. Data thus obtained indicated that all the β-lactoglobulin was complexed in mixtures containing up to 1.6 β-lactoglobulin:1 κ-casein. Solutions containing higher ratios of β-lactoglobulin to κ-casein contained residual uncomplexed β-lactoglobulin after heating and formed complexes in which the β-lactoglobulin:κ-casein ratios increased toward a maximum of 3:1.

β-lactoglobulin and κ-casein in sodium cacodylate heated at 140 and 110 C, 10 min. β-Lactoglobulin solutions remained clear when heated at 140 C for 10 min, and the zonal profile (Fig. 4) was similar to that of unheated β-lactoglobulin (Fig. 1). κ-Casein solutions remained clear when heated but became turbid on cooling. Centrifugation yielded only one component, which banded at 3.8% sucrose (Fig. 4A), but some of the κ-casein sedimented to the rotor wall. A loss of 52% of the κ-casein was estimated from the peak area.

The zonal profiles of 1:1 mixtures of β-lactoglobulin and κ-casein heated at 140 and 110 C showed only single peaks banding in 4.3 and 6.9% sucrose, respectively (Fig. 4B). No heavy components comparable to the complex formed at 80 C were present. The heated mixtures were slightly turbid and the protein losses to the rotor wall were 11.5 and 18.5% at 110 and 140 C, respectively.

Starch-gel electrophoresis of β-lactoglobulin heated at 110 C gave a zone similar to an unheated sample, but β-lactoglobulin heated at 140 C gave a more diffuse band that migrated further into the gel (Fig. 5). With 2-mercaptoethanol in the gel, both heated samples gave zones normal for β-lactoglobulin. κ-Casein

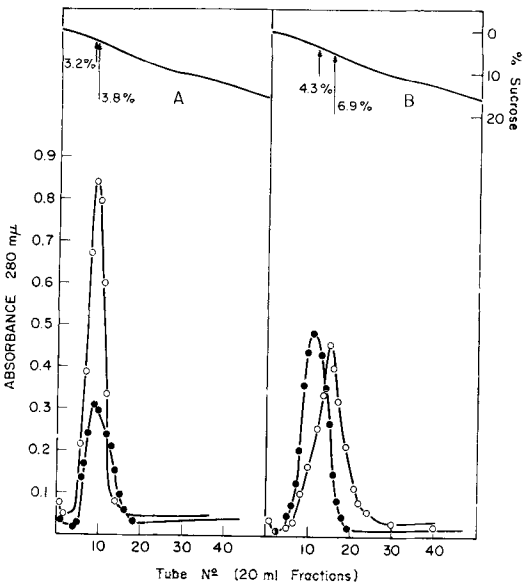

FIG. 4. Sedimentation profile. A, β-lactoglobulin—○, κ-casein—●, in sodium cacodylate buffer, pH 6.65, heated 140 C, 10 min, B, 1:1 mixture of β-lactoglobulin and κ-casein heated 110 C, 10 min and 140 C, 10 min—●. Zonal ultracentrifugation conditions same as Fig. 1.

heated at 110 C appeared unchanged in starch gel (Fig. 5) but, after heating to 140 C, κ-casein zones were very diffuse and stained poorly.

A 1:1 β-lactoglobulin:κ-casein mixture heated at 110 C contained only one electrophoretic component in the absence of 2-mercaptoethanol (Fig. 6). When 2-mercaptoethanol was added, this material dissociated into zones with the mobilities of β-lactoglobulin and κ-casein. Both the supernatant and precipitate from a mixture heated at 140 C showed only diffuse zones, with no clear evidence that any complex had formed (Fig. 6).

β-Lactoglobulin and κ-casein in a synthetic serum heated 90 C, 10 min. Unheated β-lactoglobulin and κ-casein in synthetic serum banded in 5 and 7.5% sucrose, respectively. κ-Casein heated at 90 C banded in 10% sucrose, and heated β-lactoglobulin gave a major peak at 43.5% sucrose (Fig. 7). The zonal profile for heated β-lactoglobulin between 15 and 43.5% sucrose suggested that aggregates of various sizes were formed. A heated 1:1 mixture of β-lactoglobulin and κ-casein separated in the zonal centrifuge into two definite components: the residual uncomplexed β-lactoglobulin banding in 4% sucrose and the complex banding in 22% sucrose (Fig. 7). Other mixtures gave less well-defined peaks (Fig. 8): a heated 0.4:1

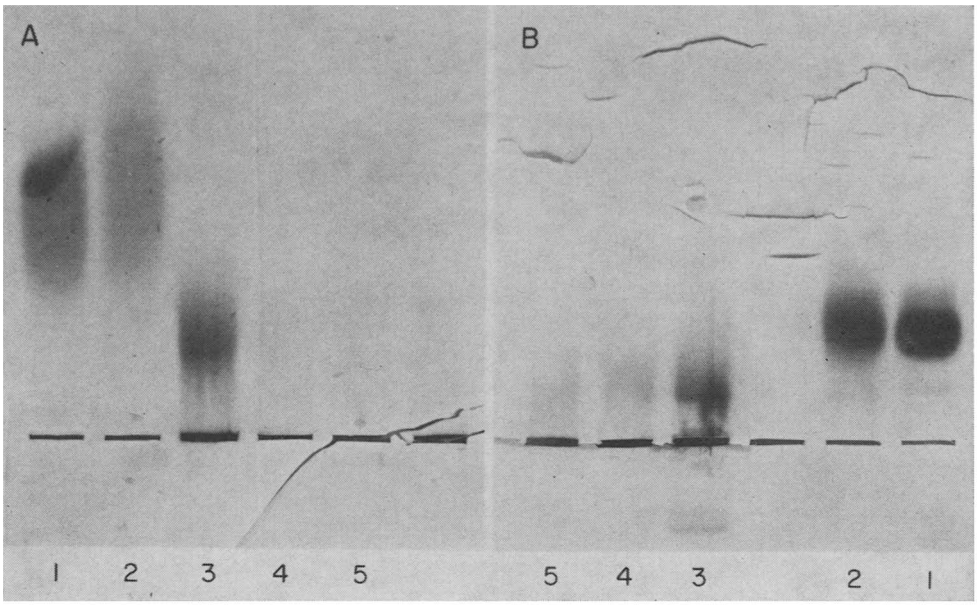

FIG. 5. Starch-gel electrophoretic patterns, A, without and B with 2-mercaptoethanol. 1, β-lactoglobulin heated 110 C, 2, β-lactoglobulin heated 140 C, 3, κ-casein heated 110 C, 4, supernatant of κ-casein heated 140 C, and centrifuged at 800 × *g*, 5, precipitate from 4.

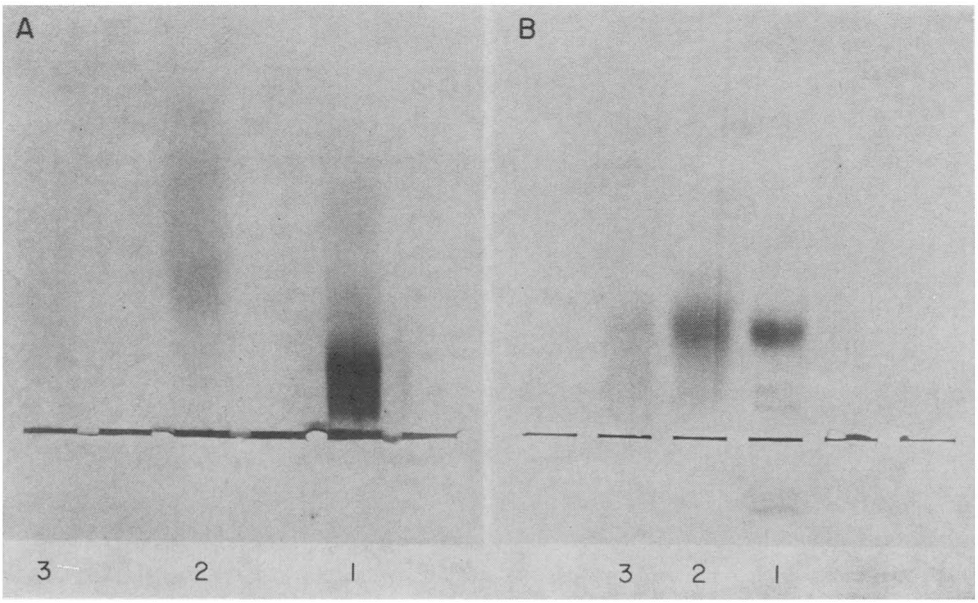

FIG. 6. Starch-gel electrophoretic patterns of 1:1 mixture of β-lactoglobulin and κ-casein, A, without and B, with 2-mercaptoethanol. 1, heated at 110 C, 2, supernatant of solution heated 140 C and centrifuged at 800 × *g*, 3, precipitate from 2.

mixture contained components banding in 4, 8, 15, and 55% sucrose, whereas in a 2.3:1 mixture the complex peak was broad and the peak in the curve at 36% sucrose suggested that some of the excess β-lactoglobulin had aggregated.

The complexes formed in synthetic serum did not move into urea starch gels. Addition of 2-mercaptoethanol dissociated some of the complex, but aggregated material also remained in the slots.

Solutions of β-lactoglobulin and κ-casein in synthetic serum precipitated when heated at 110 or 140 C. Zonal ultracentrifugation studies were, therefore, not possible.

Zonal ultracentrifugation of skimmilk. The zonal profiles for unheated and heated (90 C, 10 min) skimmilk (Fig. 9) showed the presence of soluble proteins and of micelles that banded in 5 and 55% sucrose, respectively. Heating decreased the area of the soluble protein peak by about 45%. No peak corresponding to a β-lactoglobulin-κ-casein complex was present, even when supplementary κ-casein had been added to the milk before heating. The small peak at Tube 37 was not present in all heated samples and did not yield sufficient sample for analysis. Nonheated and heated colloidal cal-

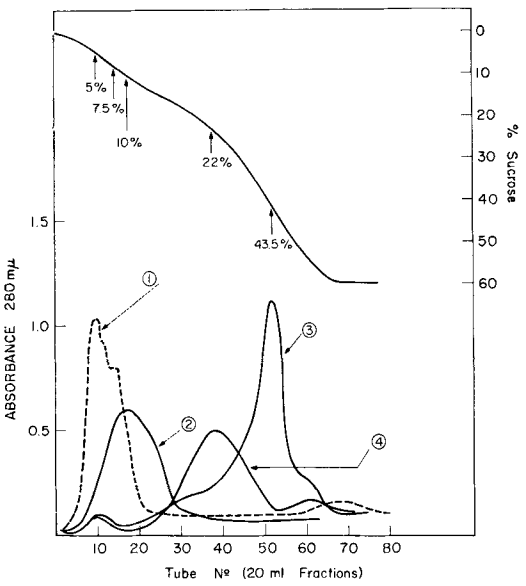

FIG. 7. Sedimentation profiles of β-lactoglobulin and κ-casein solutions in synthetic serum. 1, unheated 1:1 mixture; 2, heated κ-casein 90 C, 10 min, 3, heated β-lactoglobulin 90 C, 10 min, 4, heated 1:1 mixture. A 25-ml sample (1%) was introduced above a 500-ml, 5 to 30% and 500 ml, 30 to 60% sucrose gradient with a 60% sucrose cushion. The sample was overlaid with 200 ml of serum and centrifuged at 35,000 rpm for three hours at 5 C.

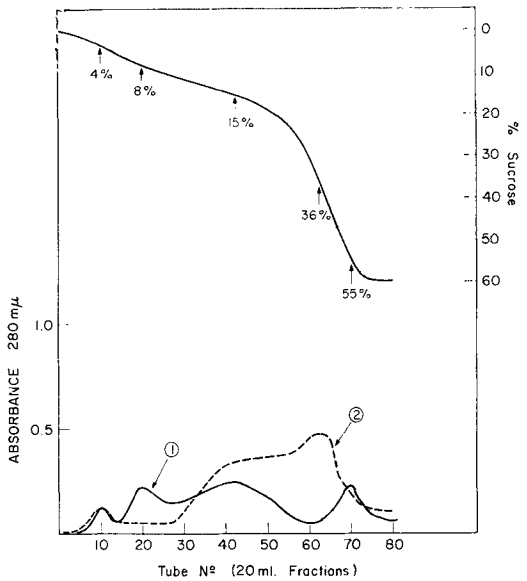

FIG. 8. Sedimentation profiles of mixtures of β-lactoglobulin and κ-casein in synthetic serum heated 90 C, 10 min. Ratio of β-lactoglobulin:κ-casein in initial mixture: 1, 0.4:1, 2, 2.3:1. A 25-ml sample was introduced above 1,200 ml of 5-30% sucrose gradient with a 60% sucrose cushion. The sample was overlaid with 200 ml of serum and centrifuged at 35,000 rpm for three hours at 5 C.

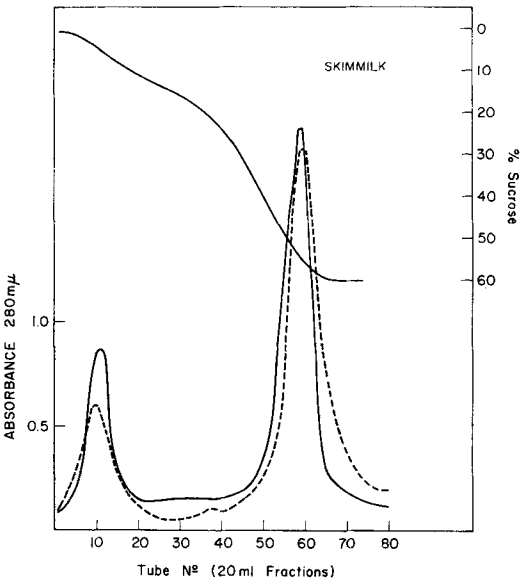

FIG. 9. Sedimentation profile of skimmilk: not heated, ————, heated 90 C, 10 min, – – – –. Zonal ultracentrifugation conditions same as Fig. 7.

TABLE 1. Sedimentation coefficients of β-lactoglobulin-κ-casein and the components of heated mixtures.

Samples	Na cacodylate buffer				Synthetic serum	
	Not heated	Heated 10 min			Not heated	Heated 90 C 10 min
		80 C	110 C	140 C		
	(S_{20})				(S_{20})	
β-Lactoglobulin	2.0	3.3	3.3	3.3	3.05	130[a]
κ-Casein	14.6	18.5	13.7	18.5	16.5	36.0
β-Lactoglobulin-κ-casein Complex						
Mixtures: 0.3:1		21.4				
1:1		26.8	2.35	1.36		39.0
3:1		28.3				
4:1		40.7				

[a] Component of Fig. 7.

cium phosphate free milks [Jenness et al. (8) procedure] gave profiles with only a single peak at about 4% sucrose.

Sedimentation coefficients (S_{20}). The sedimentation coefficients (Table 1) of the components identified as β-lactoglobulin-κ-casein complexes varied from 20 to 40 S, depending primarily on the proportion of β-lactoglobulin complexed per unit of κ-casein. Temperatures of 110 and 140 C apparently caused degradation of κ-casein, and even the component formed at 110 C in cacodylate buffer had an S value below that of β-lactoglobulin heated alone.

Discussion

It was possible by zonal ultracentrifugation to isolate the heat-induced complex of β-lactoglobulin and κ-casein from heated mixtures of these two proteins in sodium cacodylate buffer. In starch gel the complex moved as a single zone, but it was dissociated by 2-mercaptoethanol into zones corresponding to β-lactoglobulin and κ-casein. When the initial reaction mixture contained β-lactoglobulin and κ-casein in ratios up to 1.6:1, all the β-lactoglobulin was complexed. At higher initial ratios of β-lactoglobulin to κ-casein the maximum ratio in the complex approached 3:1. These results are in reasonable agreement with those of Long et al. (10), who obtained a maximum interaction ratio of 2.2:1 at 85 C for 20 min. The size of the complex, as judged from either gradient centrifugation or from a determination of the sedimentation coefficient, was related to the proportion of β-lactoglobulin in the mixture. The sedimentation coefficient values of 21.4-40.7 S reported in this paper agree with the range 25-48 S reported by others (10, 24).

There was no unequivocal evidence that a complex formed in β-lactoglobulin-κ-casein mixtures heated in cacodylate buffer at either 110 or 140 C. The major effect of these temperatures was apparently a degradation of κ-casein.

In artificial milk serum, both β-lactoglobulin and κ-casein aggregated when heated at 90 C, 10 min, but the effect was most marked for β-lactoglobulin, which formed a component banding in 43.5% sucrose (130 S). The heat-induced complex could be separated only from a 1:1 mixture of β-lactoglobulin and κ-casein; this was interpreted as being the ratio at which neither of the two proteins was present in excess. In other mixtures, the overlapping of the aggregated and nonaggregated protein peaks prevented an accurate interpretation of the zonal profile.

The zonal profiles of heated skim and colloidal calcium-phosphate free milk gave no evidence of a 25-48 S component. Morr et al. (13) detected a 48 S polydispersed component in oxalate solubilized casein micelles, and suggested that it was the heat-induced complex. However, dissociating the oxalate solubilized micelles further, by making the solution 6.6 M with urea, destroyed this component (14); therefore, it is unlikely that it was a β-lactoglobulin-κ-casein complex. In our opinion, formation in milk of a complex corresponding to that found in heated mixtures of β-lactoglobulin and κ-casein has not been demonstrated.

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