

**ABSTRACT**

Gel chromatography was employed in studies of interactions of soluble whole casein that was prepared by dissociation of casein micelles with ethylenediaminetetraacetate. With increasing protein concentration at pH 6.6 and 37°C, components of whole casein associate to polymers that approach molecular radii with apparent upper limit of  $9.4 \pm .4$  nm. With decreasing protein concentration,  $\kappa$ -casein dissociates from the other casein components. This was shown by analysis of the eluted protein boundary by gel electrophoresis and radial immunodiffusion. The peak maximum elution volume and the broad, skewed character of the separated  $\kappa$ -casein peak indicate that in whole casein  $\kappa$ -casein exists in a size distribution of disulfide bonded polymers. This apparently suggests that SH- $\kappa$ -casein monomers aggregate independently of the other casein components in the growth of casein submicelles, and additional studies with the purified casein components support this concept. However, after disulfide bond reduction with dithiothreitol, chromatography of whole casein over the same concentration range did not result in separation of SH- $\kappa$ -casein polymers, because all of the casein was eluted under one peak. These findings show that, in vivo, casein submicelles could be formed by interaction of SH- $\kappa$ -casein monomers with those of  $\alpha_s$ - and  $\beta$ -casein, followed by S-S- $\kappa$ -casein polymer formation through oxidation after milking.

**INTRODUCTION**

Bovine casein micelle formation in vivo has been postulated by Slattery (11) to be a two-step process in which casein monomers of  $\alpha_s$ -,  $\beta$ -, and  $\kappa$ -caseins aggregate first to form submicelles of approximately uniform size and variable composition, followed by coalescence of the submicelles to the complete micelle structure. In this view  $\kappa$ -casein polymerization and  $\alpha_s$ : $\beta$ -casein aggregation are not considered to be separate processes in vivo.

A contrasting hypothesis by Talbot and Waugh (13) suggests that, in vivo, casein submicelles, composed of  $\alpha_s$ - and  $\beta$ -caseins, coalesce to particles with colloidal dimensions that are stabilized by interaction at their surfaces with independently formed SH- $\kappa$ -casein polymers.

Slattery's hypothesis was based on interactions of  $\alpha_s$ -,  $\beta$ -, and SH- $\kappa$ -caseins in reconstitution experiments (12) and the relationship between  $\kappa$ -casein glycosylation and micelle size (11). Talbot and Waugh derived their ideas from relationships of  $\kappa$ -casein polymer size to micelle stabilizing and size-transforming capacity (13). Both views ascribe the existence of  $\kappa$ -casein, as disulfide bonded polymers in a distribution of sizes, to oxidation on exposure to air after milking or to the action of a sulfhydryl oxidase. Such an enzyme has been purified from milk (5).

In our opinion, one approach to clarification of the primary phase of casein micelle formation is to study the association reactions in soluble whole casein in the presence and absence of disulfide reducing reagents employing the techniques of zonal gel chromatography and radial immunodiffusion.

**MATERIALS AND METHODS**

Fresh warm milk was obtained immediately after milking at a local dairy farm. The milk was skimmed at room temperature by centrifugation at  $4000 \times g$  for 10 min. Soluble

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whole casein was prepared by dispersal of the centrifuged casein micelles in buffer containing 50 mM ethylenediaminetetraacetate (EDTA). The casein solutions then were dialyzed exhaustively against Buffer A containing 5 mM EDTA so that the last vestiges of calcium were removed. Casein concentrations were determined spectrophotometrically at 280 nm by absorptivity of  $.85 \text{ ml mg}^{-1} \text{ cm}^{-1}$ . In the experiments in which the  $\kappa$ -casein component of the whole casein was reduced to SH- $\kappa$ -casein, whole casein solutions were adjusted to 7 M guanidine  $\cdot$ HCl and 1 mM dithiothreitol (DTT) (15). The guanidine  $\cdot$ HCl, a strong denaturing reagent, serves to dissociate the casein aggregates so that  $\kappa$ -casein is more readily converted to the SH-form. The guanidine  $\cdot$ HCl then was removed by dialysis after reduction of 5 days.

All experiments in Buffer A with the following composition: .05 M  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , .5 M NaCl, 1 mM EDTA,  $10^{-4}$  M  $\epsilon$ -amino caproic acid (a casein protease inhibitor (4)), .02%  $\text{NaN}_3$ , pH 6.6; 1 mM DTT was added for the experiments with SH- $\kappa$ -casein.

Gel filtration was on water jacketed columns of Sepharose CL-6B from Pharmacia,<sup>2</sup> maintained at 37°C. Because columns with varying dimensions were used for these experiments, elution positions of the eluted boundaries are reported as partition coefficients ( $K_{av}$ ) as defined by Laurent and Killander (6). Columns were calibrated as described (9). Sample volumes of .2 to .6 ml were applied to the top of the gel through a syringe. Samples were increased in density by addition of 10% sucrose to form a sharp boundary at the top of the gel bed. Elution of solute was commenced with a peristaltic pump supplied by Buchler Company.

Absorbance of the column effluent was monitored at 206 and 280 nm by an LKB Uvicord III dual beam, automatic recording spectrophotometer. Flow-through curvettes of 10 and 3 mm pathlength were used.

Composition of the casein eluted at definite positions in the boundaries was determined by polyacrylamide gel electrophoresis (14) and radial immunodiffusion (7).

<sup>2</sup>Reference to brand or firm name does not constitute endorsement by the U.S., Department of Agriculture over others of a similar nature not mentioned.

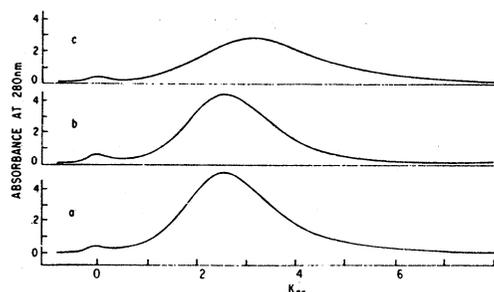


Figure 1. Elution profiles resulting from chromatography at the higher range of loading concentrations. a) 36.6 mg/ml, b) 28.0 mg/ml, c) 18.3 mg/ml. Sample volumes were .6 ml. Column size was  $.8 \times 36$  cm. The solvent was Buffer A at 37°C.

Gel electrophoresis was applied to the casein recovered from 5-ml fractions of the eluted boundaries after dialysis and lyophilization. For the immunodiffusion work it was necessary to obtain antibodies to  $\kappa$ -casein.  $\kappa$ -Casein was purified by the method of McKenzie and Wake (8). Antibodies to  $\kappa$ -casein were produced in rabbits by Cappel Laboratories, Cochranville, PA. The antiserum produced was highly specific for  $\kappa$ -casein. It gave no cross-reactions to purified  $\alpha_{s1}$ -,  $\beta$ -, or  $\gamma$ -caseins by either Ouchterlony diffusion or immunoelectrophoresis (3). The column effluent was collected in 5-ml fractions. A 1-ml sample of each fraction was placed in a plastic centrifuge tube and concentrated to 120  $\mu\text{l}$  in a Savant Speedvac Concentrator. To each sample, 2  $\mu\text{l}$  of 2-mercaptoethanol were added. A 3- $\mu\text{l}$  aliquot was withdrawn to assay for  $\kappa$ -casein content by the method of Mancini et al. (7) with  $\kappa$ -casein in 2-mercaptoethanol as the standard. A 100- $\mu\text{l}$  aliquot was taken for estimation of the protein content of each fraction by the Coomassie dye binding method (1) with whole casein as the standard.

## RESULTS AND DISCUSSION

Gel chromatography of soluble whole casein resulted in the elution profiles in Figure 1. Samples were applied at a constant volume of .6 ml; only profiles for the experiments at the highest concentrations are shown. Essentially a single peak was obtained because the small peak at the void volume ( $K_{av} = 0$ ) was collected and consisted of nonproteinaceous material.

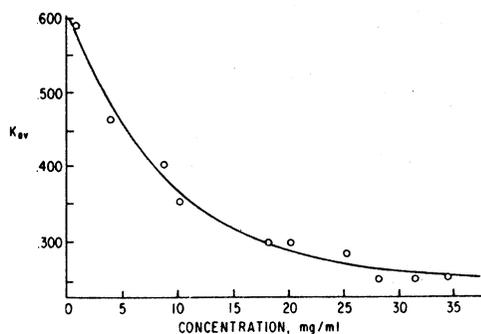


Figure 2. Concentration dependence of peak elution volume for gel chromatography of whole soluble casein illustrating the approach to a limiting  $K_{av}$ . Sample volumes were .6 ml. Column size was .8 X 36 cm. The solvent was Buffer A.

Peaks have trailing edges relatively more diffuse than their leading edges. In addition, peak elution positions show concentration dependence in that  $K_{av}$ , the solute partition coefficient, and solute concentration are inversely related. Both of these observations are characteristic of an equilibrium mixture of associating macromolecules. A plot is in Figure 2 of the relationship between  $K_{av}$  and whole casein concentration. The data given in Figure 2 were analyzed and fitted to the equation:  $K_{av} = .247 + .361e^{-.109c}$ . This relationship between solute concentration ( $c$ ) and solute partition coefficient ( $K_{av}$ ) means that  $K_{av}$ , with increasing concentration, approaches a lower limit of .247. No theoretical significance is attached to this relationship, which is employed only to derive a more precise limiting  $K_{av}$ . Because a function of  $K_{av}$  is inversely related to the Stokes radius of a molecule (6), it appears that increasing concentration favors formation of a casein oligomer that attains an upper limit in size. The Stokes radius of the oligomer corresponding to  $K_{av} = .247 \pm .010$  was obtained from a calibration plot expressing the relationship between these variables. The Stokes radius was  $9.4 \pm .4$  nm. This is close to the radius found by others (2, 12). It is possible that the casein oligomer observed in these studies in the absence of  $Ca^{2+}$  is identical to the submicellar particles observed in electron microscope studies of casein micelle structure (2). If this is the case, then gel chromatography of soluble whole casein at low concentration, which

promotes dissociation to smaller oligomeric species, would elucidate the association reactions leading to casein submicelle formation.

Figure 3 shows the elution profiles for gel chromatographic experiments in which sample volumes were reduced to .2 ml. The elution profiles for loading concentrations of 29.4 and 20.0 mg/ml gave single peaks with skewed trailing edges. When the loading concentration was lowered to 10 mg/ml (Figure 3c), a shoulder appeared at the leading edges of the boundary. Further reduction of the loading concentration to 1 mg/ml gave an elution profile showing two peaks (I and II, Figure 3d). The increase in elution volume of the peak maximum with decreasing concentration and the eventual splitting of the boundary indicated the dissociation of casein components. Identification of the nature of these components at successive elution positions was expected to clarify some aspects of their interactions.

Gel electrophoretic analyses were on fractions of the eluted boundaries as a function of elution position. Figure 4 shows the electrophoretic patterns for fractions of the boundary Figure 3a. The leading edge of the boundary, as shown in lane 6, is relatively rich in  $\kappa$ - and  $\beta$ -caseins compared to the control and other fractions. The composition of the fractions comprising the center of the peak, shown in lanes 7 and 8, appears similar to that of the control (C). The fractions making up the

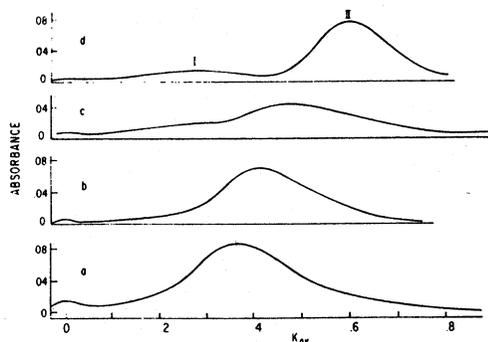


Figure 3. Elution profiles resulting from chromatography with lower loading concentrations and volumes illustrating dissociation of casein components. a) 29.4 mg/ml, b) 20.0 mg/ml, c) 10.0 mg/ml, d) 1.0 mg/ml. Sample volumes were .2 ml. The column effluent represented by profile d) was monitored at 206 nm. The others were monitored at 280 nm.

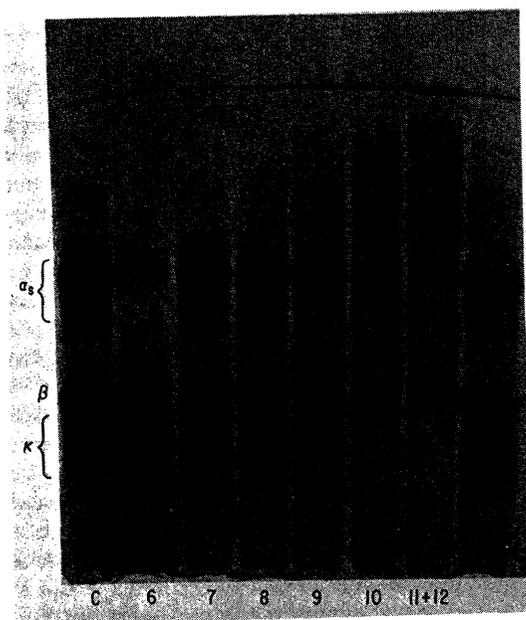


Figure 4. Gel electrophoresis of fractions taken from the boundary in Figure 3(a). The lanes marked 6, 7, 8, 9, 10, and 11 + 12 represent fractions of 4.6 ml each of the eluted boundary; fraction 6 begins at  $K_{av} = .074$ ; and each fraction represents a  $\Delta K_{av}$  of .110.

trailing edge of the boundary, shown in lanes 9, 10, and 11 plus 12, appear relatively deficient in  $\kappa$ -casein compared to the control.

Gel electrophoresis patterns of zones comprising the boundary in Figure 3c are in Figure 5. At the leading edge of the boundary where a shoulder was developing (lane 7), enrichment of  $\kappa$ - and  $\beta$ -caseins was indicated. The fractions eluted after this portion were depleted in  $\kappa$ -casein compared to the control (C) and contained predominantly  $\alpha_s$ - and  $\beta$ -caseins.

The gel electrophoretic patterns of the fractions constituting the boundary in Figure 3d were indistinct. Apparently not enough casein could be recovered from the column, at a loading concentration of 1 mg/ml, to give a clear pattern for the material in the fast peak of this boundary.

The results of estimating  $\kappa$ -casein distribution in the boundary of Figure 3c, by the more sensitive method of radial immunodiffusion, are given in Figure 6. This experiment was to test the feasibility of applying the method to estimation of  $\kappa$ -casein in the fractions obtained

from the boundary shown in Figure 3d, for which the gel pattern of the leading peak was indistinct. Because a clear gel pattern was obtained (Figure 5) for fractions of the boundary in Figure 3c, correspondence of the radial immunodiffusion assay with the former method would lend more confidence in its application to the boundary in Figure 3d. Figure 6 shows that  $\kappa$ -casein content is greatest at the leading edge of the boundary and decreases toward the trailing edge. This result corresponds with the qualitative analysis from the gel electrophoretogram (Figure 5) of the fractions from this boundary.

Proceeding to the radial immunodiffusion assay of the fractions from the boundary in Figure 3d we found, as in Figure 7, that the leading peak was predominantly  $\kappa$ -casein, whereas the major trailing peak contained little of this component. This series of experiments showed that gel chromatography of whole casein, at progressively lower concentrations, succeeds in separating  $\kappa$ -casein polymers from the  $\alpha_s$ : $\beta$ -casein complexes.

The data here concerning size of the casein

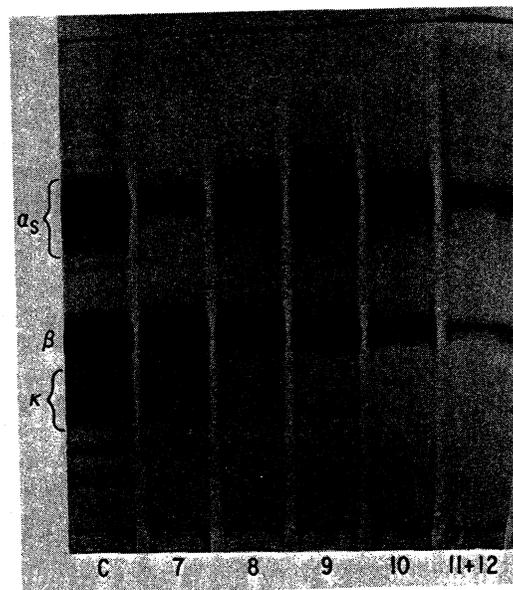


Figure 5. Gel electrophoresis of fractions taken from the boundary in Figure 3(c). The lanes 7, 8, 9, 10, and 11 + 12 represent 4.8 ml fractions of the eluted boundary with fraction 7 beginning at  $K_{av} = .103$  and successive fractions collected in  $\Delta K_{av}$  intervals of .105.

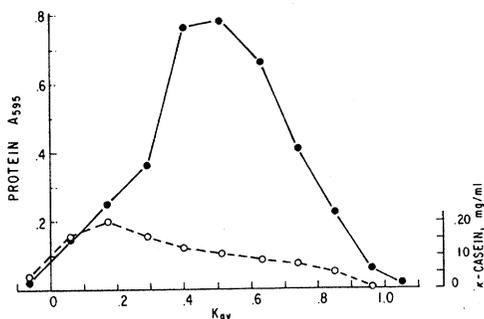


Figure 6. Estimation by radial immunodiffusion assay of the  $\kappa$ -casein distribution across the boundary represented by Figure 3c.

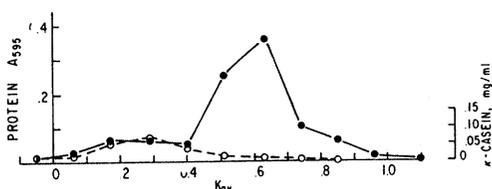


Figure 7. Estimation by radial immunodiffusion assay of the  $\kappa$ -casein distribution across the boundary represented by Figure 3d.

submicelles and properties of the chromatographic elution boundaries for whole casein do not correspond with (9, 10). In (9), zonal gel chromatography was in a low range of loading concentration on a much larger column than we used. This led to greater dilution of the protein zone as it migrated through the gel column. Under those conditions casein aggregation approaching an upper limit was not observed. Instead, a lower limit for the Stokes radius of 5 nm was reported. The limiting oligomer was assumed to represent the aggregating submicelle from which casein micelles are built. This interpretation followed from the elution of only single boundaries at all loading concentrations under the previous conditions. Splitting of the boundary, as in Figure 3d, was not observed because the low broad leading peak in the previous work would be eluted in a much larger volume and, therefore, would be more difficult to detect. Detection of the leading peak was facilitated by our monitoring the column effluent with an ultraviolet source at 206 nm.

In the second study (10), glutaraldehyde fixation of casein structure was tried in solution at a concentration where casein would be dissociated considerably. Consequently, a Stokes radius of 5.5 nm for the casein submicelle was found by gel chromatography. This corresponds closely with that in (9). The casein eluted at a position consistent with such a molecular radius was, by gel electrophoresis, depleted of  $\kappa$ -casein and primarily composed of  $\alpha_s$ - and  $\beta$ -caseins (10). These results are consistent with the present study, because the leading peak in Figure 3d consisted of  $\kappa$ -casein, which dissociated from the casein submicelles. One might infer from the data thus far that  $\kappa$ -casein self-association is stronger than  $\kappa$ - $\beta$  or  $\kappa$ - $\alpha_s$  interactions.

Gel chromatography was done with purified  $\kappa$ -casein and weight ratio mixtures of 1:1 of  $\alpha_{s1}$ - and  $\beta$ -caseins to support our interpretation of interactions with whole casein. As in Table 1, the  $\alpha_{s1}$ : $\beta$ -casein complex displays concentration-dependent elution behavior. The column partition coefficient for a .6-ml sample with a concentration of 36 mg/ml was .325. The corresponding  $K_{av}$  for whole casein with the same loading volume and concentration was .247. The difference probably reflects the interaction of  $\alpha_{s1}$ - and  $\beta$ -caseins with  $\kappa$ -casein for whole casein.

The  $\alpha_{s1}$ : $\beta$ -casein complex was eluted at the same volume ( $K_{av} = .600$ ) as the trailing peak in Figure 3d for whole casein when both samples were applied to the column with .2 ml of 1 mg/ml solutions. This shows that the  $\alpha_{s1}$ - and  $\beta$ -caseins in whole casein, under these conditions of loading volume and concentration, are probably moving down the column free from interaction with  $\kappa$ -casein. The data correspond to evidence from radial immunodiffusion assay that showed the trailing peak in Figure 3d was essentially free of  $\kappa$ -casein.

Chromatography of purified  $\kappa$ -casein (Table 1) resulted in a skewed peak with a  $K_{av} = .200$  for the maximum. Concentration dependent elution behavior was not observed for purified  $\kappa$ -casein. The purified  $\kappa$ -casein was eluted earlier than the leading peak ( $K_{av} = .290$ ) in Figure 3d, which contained predominantly  $\kappa$ -casein. Evidently  $\kappa$ -casein, when purified by chemical fractionation and isolated in concentrated solution, is aggregated to a greater extent than the  $\kappa$ -casein, which is separated from whole casein on a gel column at low

concentration. These results are consistent with investigations that showed purified  $\kappa$ -casein is highly aggregated in solution and dissociates upon interaction with other casein components (9). Purified, aggregated  $\kappa$ -casein also stabilized calcium sensitive caseins. Its capacity for stabilization in vitro is equal to that of SH- $\kappa$ -casein as found by Talbot and Waugh (13). Based on this kind of evidence, these researchers postulated that  $\kappa$ -casein in the mammary gland aggregates independently of the other casein components to SH- $\kappa$ -casein polymers, which occur in a size distribution and are converted to S-S- $\kappa$ -casein polymers by oxidation after insertion in the micelle surface.

The results with whole casein apparently support the ideas of Talbot and Waugh. To test these concepts further, the self-association of SH- $\kappa$ -casein was studied by gel chromatography. The purified  $\kappa$ -casein preparation was reduced with DTT, and peak elution volumes were determined for a series of concentrations. The partition coefficients ( $K_{av}$ ) for SH- $\kappa$ -casein varied with loading concentration as in Table 1 in the last column. Thus SH- $\kappa$ -casein is a self-associating protein that attains a polymer size equal to that of whole casein as seen by a  $K_{av} = .246$  for a .2-ml sample containing 22 mg/ml. The SH- $\kappa$ -casein elution rate displays a much sharper concentration dependence than the  $\alpha_{s1}:\beta$ -casein complex, because the  $K_{av}$  for the complex at the higher loading concentration

of 36 mg/ml results in a  $K_{av} = .325$ . This indicates that SH- $\kappa$ -casein polymerizes to a greater extent than does the  $\alpha_{s1}:\beta$ -casein complex at a given concentration. These data also support the idea that SH- $\kappa$ -casein polymerizes independently of other casein components during formation of casein micelles in the mammary gland.

In gel chromatographic studies with whole casein in which the  $\kappa$ -casein component had been converted to the SH-form by the method of Vreeman (15), elution profiles for loading volumes and concentrations corresponding to those used to obtain Figure 3b,c,d for unreduced whole casein, gave only single peaks with relatively diffuse trailing edges. The leading edges of the boundaries corresponding to Figure 3a,b were sharper for reduced whole casein. Furthermore, the pronounced shoulder on the leading edge of Figure 3c and the development of the broad, shallow leading peak of Figure 3d were not in evidence in the boundaries obtained with reduced whole casein. In addition, reduced whole casein was chromatographed at a loading volume of .2 ml and a concentration of 20 mg/ml, and a single peak with a  $K_{av} = .362$  was obtained. Because  $\kappa$ -casein is approximately 15% of whole casein, the 20 mg/ml whole casein solution contains 3 mg/ml of  $\kappa$ -casein. From interpolation of Table 1, if .2 ml of a 3 mg/ml SH- $\kappa$ -casein solution were chromatographed, a peak with a  $K_{av}$

TABLE 1. Concentration dependence of column partition coefficient ( $K_{av}$ ) for  $\kappa$ -casein, SH- $\kappa$ -casein,<sup>a</sup> and weight ratio mixture of 1:1 of  $\alpha_{s1}$ - and  $\beta$ -caseins.

Sample	Loading volume (ml)	Concentration (mg/ml)	$K_{av}$ <sup>b</sup>	SD
$\kappa$ -casein	.20	7.4	.190	.013
	.30	2.5	.210	
	.10	.10	.200	
SH- $\kappa$ -casein	.20	22.0	.246	.013
	.20	7.4	.500	.017
	.30	2.5	.610	
	.10	.10	.600	
$\alpha_{s1}:\beta$ -complex	.60	36.0	.325	.007
	.20	10.0	.400	.007
	.20	1.0	.597	.011

<sup>a</sup>SH- $\kappa$ -casein was made by introducing 40 mM DTT into the  $\kappa$ -casein solutions.

<sup>b</sup>Where standard deviations are given the results are averages of three or more experiments.

TABLE 2. Effect of reduction and reoxidation on elution positions of whole casein.

	$K_{av}^a$			
	Peak I <sup>b</sup>	SD	Peak II <sup>b</sup>	SD
Whole casein	.296	.016	.605	.014
Whole casein + 1 mM DTT	not detected		.610	.010
Reoxidized <sup>c</sup> 6 h at 5°C	.263		.575	
Reoxidized 4 days at 5°C	.280		.585	

<sup>a</sup> Average of three or more runs where SD is given, otherwise two runs.

<sup>b</sup> Peak I and II as designated in Figure 3d with same column and sample loading condition.

<sup>c</sup> Samples reoxidized by gentle exposure to air at 20.0 mg/ml, then diluted with buffer with no DTT.

between .5 and .6 would be expected. Therefore, if SH- $\kappa$ -casein were not interacting with the other casein components, such a peak would be observed in the elution profile in the above experiment with whole casein.

Reoxidation of the SH- $\kappa$ -casein in whole casein was to determine whether structural changes had been induced in the casein complexes by reduction with DTT so that  $\kappa$ -casein polymers could not be reformed. The results of chromatographing the reoxidized casein are in Table 2. Apparently the  $\kappa$ -casein polymers were reformed because the elution profiles regained the bi-modal character of the original whole casein solution shown in Figure 3d. The relative areas under peaks I and II were similar to the corresponding peaks in the elution profiles for the reoxidized casein complexes; the elution positions were also close in magnitude (Table 2). The small differences in these measures for the reoxidized casein probably result from different conditions with respect to the system as it is formed *in vivo*.

Experiments showing that SH- $\kappa$ -casein is not separated from the other components in reduced whole casein during gel chromatography at low concentration, and the evidence for the interaction of SH- $\kappa$ -casein with the other casein components suggests that the hypothesis of Talbot and Waugh (13) concerning the mechanism of casein association in the mammary gland is not plausible. The work in this paper supports the ideas of Slattery (11) in which  $\kappa$ -,  $\alpha_{s1}$ -, and  $\beta$ -casein monomers coalesce to submicelles of uniform size and variable composition as the first step in the aggregation

of the caseins to the colloidal casein particles which exist in milk. The existence of  $\kappa$ -casein in a size distribution of disulfide bonded polymers is consistent with this type of association mechanism in which insertion of SH- $\kappa$ -casein monomers into submicelles would be a random process. Therefore,  $\kappa$ - $\kappa$ -casein contacts in the submicelles reflect this random variation, and S-S- $\kappa$ -casein cross-linking through either oxidation upon exposure to air or the action of a sulfhydryl oxidase, results in the molecular state of  $\kappa$ -casein that is observed when this casein component is isolated by chemical fractionation or dissociated from other components of the casein micelle by dilution to low concentration.

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