STUDIES ON THE STRUCTURE AND INTERACTION
OF BOVINE κ-CASEIN, USING
FLUOROMETRIC TECHNIQUES

by
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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENT FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in the Department
of
Food Science

We accept this thesis as conforming to the
required standard.

THE UNIVERSITY OF BRITISH COLUMBIA
June, 1971
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ABSTRACT

The structure and interactions of \( \kappa \)-casein were studied using spectrofluorometric techniques including, fluorescent yield, emission wavelength, energy transfer, and fluorescence depolarisation. The effect of denaturing factors, temperature, sodium dodecyl sulfate, ionic strength, urea and pH, on the fluorescent characteristics of a conjugate of \( \kappa \)-casein and 8-anilinonaphthalene-1-sulfonate are indicative of structural changes in the protein. The importance of hydrogen bonding and hydrophobic interactions for the maintenance of the structural integrity of the molecule have been indicated. The structural changes are effected without the use of disulfide reducing reagents.

The dissociating agents, urea and sodium dodecyl sulfate, had very significant effects upon the conformation of the protein. Temperature induced changes were related to the bonding which maintained the conformation. Completely dissociated or random structures did not exist except at high pH, in 8M urea, or in 0.005M sodium dodecyl sulfate.
The interaction between $\alpha_s\;1^-$ and $\kappa$-casein was studied by the fluorescence polarisation technique. The thermodynamic parameters $\Delta S$ and $\Delta H$ increased with temperature. $\Delta S$ and $\Delta H$ were positive. $\Delta F$ was negative and decreased further with increasing temperature. These data suggest that the ease or spontaneity of interaction increases with temperature, and that the interaction is hydrophobic in nature. The results of the interaction studies at 40 and 50°C demonstrated a 1:1 mole ratio for the interacting proteins.

Blocking of the sites for electrostatic interaction, the negative charges on the $\alpha_s\;1^-$-casein, by polyethylimine did not inhibit the reaction or affect the interaction ratio.

It was therefore concluded that $\alpha_s\;1^-$ and $\kappa$-caseins interact through hydrophobic interactions in a 1:1 mole ratio.

Kappa-casein was modified in a stepwise manner by (1) carbamylation of the $\varepsilon$-amino groups of lysine with potassium isocyanate (2) esterification of the free carboxylic groups using the carbodiimide method and glycine methyl ester. Structural changes in the $\kappa$-casein,
thus modified, were followed by measuring changes in fluorescent properties of a conjugate of the proteins with 8-anilino-naphthalene-1-sulfonate. These structural changes were indicative of decreasing hydrophobicity of the caseins and loss of stabilising ability of $\alpha_s$-casein against calcium ion precipitation. It is concluded that the charged groups are responsible for the maintenance of the protein structure, but may not be directly related to stabilising ability or interaction with $\alpha_s$-casein.
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ACKNOWLEDGEMENTS

The author wishes to express his deepest gratitude to Dr. S. Nakai, Associate Professor, Department of Food Science, University of British Columbia, under whose supervision this project was undertaken, and for his guidance in the preparation of this thesis.

Appreciation is also extended to Dr. W.D. Powrie, Head of the Department of Food Science; Drs. J.F. Richards and M. Tung, Department of Food Science and Dr. R. Bose, Canada Department of Fisheries Research; for their discussion and criticism during the period of research and in the preparation of this thesis.
INTRODUCTION

The caseins in milk exist predominantly in a micellar structure, which also involves the inorganic ions phosphate and calcium. Gelation of milk products or precipitation of the caseins are associated with disruption of this structure. Kappa-casein, which represents 10-15% of the total casein in milk is responsible for the insensitisation of $\alpha_s$ - and $\beta$ -caseins to calcium ion precipitation. Because of this characteristic, $\kappa$ -casein is a very important constituent of the milk, highly related to the total casein stability; since the natural levels of calcium ion present could normally lead to the precipitation of $\alpha_s$ - and $\beta$ -caseins. Because of this importance to the stability of milk and milk products, the structure and mode of interaction of the caseins, particularly $\kappa$ -casein, have been areas of intense research for some time. Initially the concept of structureless caseins prevailed. However, in recent years, there have been suggestions of structure associated with the caseins, although there is still no unanimity regarding this or the type of interaction or even the interaction ratio among the caseins. The reaction specificity of $\kappa$ -casein insensitivity to calcium ion precipitation and insensitisation of $\alpha_s$ - and $\beta$ -casein to calcium ion would
suggest that there is some structure associated with the \( \kappa \)-casein. For these reasons it was decided to investigate the structure of \( \kappa \)-casein; its mode of interaction with \( \alpha_s1 \)-casein and the interaction ratio; and finally the effect of charged groups of the \( \kappa \)-casein on its structure and interaction with \( \alpha_s1 \)-casein.

The fluorescent techniques, polarisation, fluorescent yield, wavelength of maximum emission and energy transfer all lend themselves readily to a study of structural changes in a macromolecule. Polarisation is a property of a macromolecule that is dependent upon the molecular volume, rigidity and shape of the molecule. Fluorescent yield and wavelength of maximum emission of a macromolecule-fluorescent dye conjugate are properties associated with the polarity of the region where the dye is bound. Conformational changes will usually effect the level of penetration of the aqueous phase to this region, or the distribution of charged groups in the vicinity; associated with these would be changes in the solvation character of the region, hence the polarity and fluorescent parameters. The energy transfer is a measure of the efficiency of the absorbing chromophore (in the case of proteins, tryptophan or tyrosine) to give up its energy, to the emitting conjugated fluorescent dye. This efficiency
depends not only upon the distance of separation of the absorber and emitter (more commonly referred to as the donor-acceptor) but also the orientation of the donor-acceptor.

Association, dissociation or conformational changes in the protein molecule, should result in changes of some or all the fluorescent parameters listed. The structure and interaction of κ-casein were studied using the above mentioned techniques. 8-anilino-naphthalene-1-sulfonate and 1-dimethyl-amino naphthalene-5-sulfonyl chloride were used as fluorescent conjugates.
The principle proteins in milk, the caseins, exist predominantly in an aggregated state with calcium and phosphate in the form of micelles. Methods for separation and purification of the individual caseins involve the acid insolubility of the casein fractions, the variation of solubility at reduced temperatures, or the effect of calcium ions (36, 73, 85). It is this variation in the extractability at low temperatures, and the calcium sensitivity, which have been the most intriguing problems relating to the study and interaction of the proteins in milk.

Linderstrom-Lang (33) demonstrated the heterogeneity of the caseins, and although he did not separate the fractions at that time, he postulated the existence of a fraction which would be insensitive to calcium ion, but susceptible to the action of rennin, after which the protein is precipitated by calcium ion. His thesis was, that although independently one component would be susceptible to the calcium ion concentration, the interaction between the components would lead to insensitivity.

In 1956, Waugh and von Hippel (77) identified a casein fraction which fitted the definition of the
protein described by Linderstrom-Lang. It showed calcium insensitivity, was attacked by rennin, and interacted with $\alpha_s$ – and $\beta$ -caseins, rendering these proteins soluble in the presence of calcium ion.

During the action of rennin, a glycomacropeptide is released from $\kappa$ -casein. The remaining protein para-casein does not possess the ability to stabilise $\alpha_s$ – or $\beta$ -casein against calcium precipitation, and furthermore, is precipitated by calcium ions also (77).

Extensive studies have been performed using sedimentation analysis, gel filtration, difference spectra, electrophoresis and light scattering techniques, with a purpose of understanding the structure and therefore the interactions of the caseins. Herskovits (17) and Noelken and Reibstein (49) used optical rotary dispersion during their studies on the structure of the caseins, and found a low level of helical content, which is a usual index of order or structure in a protein. Viscosity and optical rotation measurement indicated that the caseins had properties typical of denatured proteins (22).

The above facts are difficult to reconcile with the properties of $\kappa$ -casein, namely calcium insensitivity
and rennin sensitivity. Recently, however, Leslie et al (32) have demonstrated by the use of nuclear magnetic resonance that $\kappa$-casein possesses a rigidity which is apparently absent in the other caseins. This possibility of structure in $\kappa$-casein has been confirmed by other workers (5, 6) and also has been suggested by Zittle (84).

Hill and Wake (21) have suggested a special role for the carbohydrate moiety in $\kappa$-casein in their discussion on the amphiphile nature of $\kappa$-casein. However, Mackinlay and Wake (38) have demonstrated that S-carboxymethyl $\kappa$-casein has full stabilising ability for $\alpha$- and $\beta$-caseins against calcium ion precipitation, although the carbohydrate content is very low. These authors concluded that the carbohydrate is not necessary for the stabilising ability, nor for the interaction between $\alpha$- or $\beta$-casein and $\kappa$-casein.

The fact that calcium ions and phosphate are included in the micelle structure, has led many authors to the conclusion that the interactions of the caseins involve partially, or exclusively, calcium and/or, phosphate bridges.
Removal of colloidal calcium phosphate by adjusting the pH of chilled milk to a pH of 4.8 to 5.0 and dialysing against a large excess of milk (34) leads to instability and disruption of the micelle structure.

Schmidt and Buchheim (59) found that calcium was responsible for large micelle formation by bridging smaller casein micelles together; this is illustrated by replicates of their electronmicrographs of micelles of milk and calcium free milk respectively (Plates 1 and 2).

Garnier and Ribadeau-Dumas (15) suggested that the micelle was composed of a $\kappa$-casein core interacted with $\alpha_s$-casein directly, which was interacted further with $\beta$-casein, all through hydrophobic bonds. These authors suggested that the calcium phosphate formed bridges with the hydrophobically interacted caseins leading to the stable micelle structure. Parry and Carroll (50) also suggested that the $\kappa$-casein was in the interior of the micelle.

Fox (11) found that ionic calcium could easily replace colloidal phosphate leading to a stable micelle structure, which suggested the possibility of non-specific electrostatic interaction. Nakai et al (42) found that
PLATE I. REPLICATE OF ELECTRONMICROGRAPH OF CASEIN MICELLE IN RAW MILK

PLATE II. REPLICATE OF ELECTRONMICROGRAPH OF CASEIN MICELLE IN CALCIUM FREE MILK
calcium bridges alone could not be responsible for the maintenance of micelle structure. Rose and Colvin (56) suggested that the calcium and phosphate were instrumental in maintaining the structure of the micelle. These authors also suggested that the calcium and phosphate were probably linked with the ε-amino group of lysine from the αs- and β-caseins. Although the importance of calcium and phosphate in the micelle stability had been demonstrated, certain temperature effects were difficult to explain if only salt bridges were involved. Fox (11) found the β-casein easily separates from the micellar structure on cooling, and that the structure was not dependent on the β-casein. Downey and Murphy (8) found that as much as 50% of the β-casein entered the serum on cooling, just above freezing. Rose and Colvin (57) found that β-casein which separated from the micellar structure on cooling, did not re-enter the micelle on subsequent warming.

Rose (55) suggested that the interaction between αs- and β-casein was principally hydrophobic in nature. This interaction product was proposed to be in the core of the micelle, covered by a layer of κ-casein. This hypothesis is similar to that of Talbot and Waugh (68), who envisaged a core of αs-casein surrounded by a layer of αs-κ-casein. This model is similar to that of Payens (52)
who suggested that $\alpha_s^1$- and $\beta$ -caseins were surrounded by $\kappa$ -casein, and that the structure was cemented by calcium phosphate bridges. Payens (52) suggested that the internal structure was maintained by hydrophobic interaction, based on the temperature dependence of aggregation of the individual caseins (60, 65, 70). This hypothesis is supported by Noelken (48) in his study using hydrophobic detergents.

Recently, the concept of non-specific electrostatic interactions between casein components has been suggested as the mechanism responsible for the stabilising ability of $\kappa$ -casein.

On photo-oxidation, $\kappa$ -casein loses its stabilising ability for $\alpha_s^1$ - against calcium ion precipitation (20, 83). The principal amino-acids destroyed by this process, as reported by Zittle (83) and Hill and Laing (20) were histidine and tryptophan. These authors proposed a role for histidine in the stabilising ability of $\kappa$ -casein, based on their results. Nakai et al (43), however, found that after modification of 1.5 residues of histidine in $\kappa$ -casein, there was no variation in its stabilising ability for $\alpha_s^1$ -casein. Hill and Craker (19), Woychik (81), Pepper et al (53), Talbot and Waugh (69) have
all suggested that lysine residues in κ-casein are related to its ability to stabilise αs1-casein against calcium ion precipitation. However, there was some variation in the extent of lysine modification necessary to produce a loss, or reduction, of stabilising ability and whether it was due to specific charge effects or conformational changes due to the increase in net negative charge on the protein.

Hoagland (23) found that β-caseins which were modified to give an increase in net negative charge in the protein were still stabilised by κ-casein against calcium precipitation, and secondly that the modified β-casein itself had become more soluble in the presence of calcium ion.

Hill (18) suggested that there is a peptide region of high positive charge, containing lysine, histidine and arginine, and that this region was related to the stabilising ability of κ-casein for αs1-casein. Kason et al (25) found that αs1-casein reacted with PEI, a polyethylenimine polymer with high primary amine content. These workers suggested that some positive region on the κ-casein molecule could react similarly.
Kenkare and Hansen (27) suggested that the interaction between $\alpha_s$- and $\kappa$-casein is hydrophilic in nature, based on the temperature dependence of association and dissociation of these proteins. Waugh (74) suggested that this temperature effect could be due to a different type of calcium binding among the proteins.

The problems of the type of bonding within the protein become even more complex when it is realised that $\beta$-casein can leave the micelle structure and not re-enter it, and also by the fact that although the ratio of $\alpha_s$: $\beta$: $\kappa$-casein in milk is 3:2:1, Zittle (82) demonstrated that ratios of $\alpha_s$: $\kappa$ of 10:1 can be stabilised in the presence of calcium ion. Many authors have attempted to ascertain the interaction ratio of $\alpha_s$ to $\kappa$-casein in model systems.

Waugh and von Hippel (77) suggested that the interaction ratio of $\alpha_s$: $\kappa$-casein was 4:1. However, Noble and Waugh (47) later suggested that the interaction ratio was close to unity. Garnier (13) suggested that the interaction ratio is unity, but later this author (12) suggested that the $\kappa$-casein species involved in the interaction was a trimer, and that this combined with a monomer of $\alpha_s$-casein, hence the interaction ratio of $\alpha_s$: $\kappa$-
casein was 1:3 on a monomer basis. Parry et al (51) using gel filtration techniques, showed a single interaction product with a weight ratio of unity, whereas Kenkare and Hansen (27) suggested a variable interaction ratio but predominantly $\alpha_s \lambda: \kappa$-casein of 4:1.

It is obvious from the literature that the type of interactions involving the caseins and the interaction ratios among the different species are not clearly established.
INTRODUCTION

Recent studies by Herskovits (17) and Noelken and Reibstein (49), using optical rotatory dispersion have demonstrated that caseins are unordered with little helical structure. According to the classical definition of protein structure caseins exist as random coils. However, such a structure is rather difficult to reconcile with the physical and chemical properties of caseins, particularly of \( \kappa \)-casein: Talbot and Waugh (69) suggested a S-S-bonded open chain polymer as a possible form for \( \kappa \)-casein.

Swaisgood and Brunner (66), McKenzie and Wake (36), McKenzie (35) and Mackinlay and Wake (37) demonstrated the existence of disulfide bridges in \( \kappa \)-casein. Payens (52) postulated the possibility of hydrophobic interactions as a principal cause of aggregation and considered the variation in the sedimentation coefficient with concentration to be due to entanglement of the random coil fibres of \( \kappa \)-casein. Recently, Parry and Carroll (50), and Chesseman and Knight (6) reported that
the \( \kappa \) -casein in milk is structured like an aggregated globular protein.

It has been demonstrated by Weber and Laurence (80) and Stryer (62, 63) that 8-anilinonaphthalene-1-sulfonate (ANS)\(^1\) can be used as a probe for hydrophobic regions and the quantum yield of fluorescence and the wavelength of maximum emission of the probe bound to the protein varies depending upon the polarity of the region of attachment.

Results of spectrofluorometric studies on \( \kappa \) -casein are presented to demonstrate the existence of hydrophobic regions in the molecule and changes in molecular conformation, under varying environmental conditions, measured by the variations in energy transfer (10, 63, 64) and the polarisation of fluorescence (78, 79).

\(^1\)The following abbreviation was used:

ANS, 8-anilinonaphthalene-1-sulfonate
MATERIALS AND METHODS

Kappa-casein was prepared by the method of Zittle and Custer (85) and was electrophoretically pure on polyacrylamide gel.

The magnesium salt of ANS was prepared from the technical grade sodium salt (Eastman Kodak Company, Rochester, N.Y.), by precipitation with saturated magnesium acetate. The crude product was recrystallised from hot water and filtered through a bed of activated charcoal and Hyflo Super Cel. This was repeated until a constant molar absorbance, 5700, at 350 nm in 0.1M phosphate buffer pH 6.5, was obtained.

Fluorescence spectra were recorded on a Moseley, Model 7004A X-Y Recorder, manufactured by Hewlett-Packard, connected through a Photomultiplier Microphotometer to an Aminco-Bowman Spectrophotofluorometer, No. 4-8202. Temperatures above and below ambient were maintained by a Sargent Thermoniter and by a Blue M Control Flow Cooling Unit, respectively.

The number of hydrophobic regions were calculated using the Klotz (29) equation,

$$\frac{P}{Dx} = \frac{1}{n} \left( 1 + \frac{K_a}{D(1-x)} \right)$$

(1)
where \( P \) = the protein concentration
\( D \) = the ANS concentration
\( x \) = the fraction of the dye bound to the protein
\( K \) = the dissociation constant of the dye-protein complex
\( n \) = the number of binding sites.

A plot of \( \frac{P}{D}x \) against \( (1-x)D \) gives a straight line with intercept \( \frac{1}{n} \). For this calculation, a constant concentration of \( 4.0 \times 10^{-5} \text{M} \) was used for \( \kappa \)-casein throughout the studies except for the results appearing in Figures 1 and 2. The concentration of ANS was varied between \( 2.6 \) and \( 6.6 \times 10^{-5} \text{M} \).

Fluorescence data is reported as relative fluorescent intensity, which is the ratio of the fluorescent intensity of the sample to that of a solution of \( \kappa \)-casein \( 4.0 \times 10^{-5} \text{M} \), when both are conjugated with ANS at a concentration of \( 2 \times 10^{-6} \text{M} \).

The polarisation, at room temperature, was calculated using the equation of Azumi and McGlynn (1),

\[
P = \frac{I_{EE} - I_{EB}}{I_{EE} + I_{EB}} \frac{I_{BB}}{I_{BE}}
\]

where \( P \) = polarisation
\( I \) = fluorescent intensity
The polariser was two disks of polacoat formula 105 UV film set at mutual right angles. The analyser was two disks of polaroid HN38 film set at mutual right angles. An excitation wavelength of 350 nm, and an emission wavelength of 480 nm or the wavelength of maximum emission of samples, where applicable, were used. Energy transfer was calculated by the decrease in the emission at 350 nm, excitation 290 nm, of the $\kappa$-casein, when conjugated with ANS. The molar ratio of protein to ANS was constant at 10:1.

Slit positions on the spectrofluorometer were as follows: for fluorescent yield and emission wavelength determinations - #2, #5, 4mm; #3, #4, #7, 3mm; for energy transfer - #2, #5, none; #3, #4, #7, 1mm; for polarisation - #2, 4mm; #3, #4, 3mm; #5, none; #7, 2mm.

The molecular weight of 20,000 was assumed for $\kappa$-casein monomer. Protein concentrations were determined from the absorbance at 280 nm.

Sodium dodecylsulfate solutions were prepared in 0.05M Tris-HCl buffer pH 8.0. Urea solutions were adjusted to pH 7.0. Phosphate buffers, 0.05M, were used for pH values between 5.0 and 7.0; 0.05 Tris-HCl for pH 8.0 and 9.0; 0.05 bicarbonate for pH 10.0; pH 11.0 and
12.0 were obtained by adding NaOH to the sample. The results presented are averages of four (4) readings; two duplicates at different times.
Effect of \( \kappa \)-Casein Concentration

Figure 1 shows the relationship between relative fluorescent intensity, hydrophobic regions and concentration of \( \kappa \)-casein. The relationship between relative fluorescent intensity and hydrophobic regions is inverse. The number of hydrophobic regions tended to approach 0.5 per molecule as the concentration of \( \kappa \)-casein increased, and approximately four regions per molecule were obtained by diluting to \( 5.0 \times 10^{-6} \) M. At low concentrations of \( \kappa \)-casein, the number of hydrophobic regions was large and the relative fluorescent intensity low. However, the relative fluorescent intensity increased and the number of hydrophobic regions decreased with increasing concentrations. Concentrating dilute solutions or diluting concentrated solutions yielded the same values for the number of hydrophobic regions and relative fluorescent intensity as those in figure 1 at the same concentration of \( \kappa \)-casein. Also as the concentration of \( \kappa \)-casein was increased, the energy transfer increased with a slight decrease in polarisation (Fig. 2).
Figure 1. Effect of κ-casein concentration upon the number of hydrophobic regions (••••) and relative fluorescent intensity (△△△△) of κ-casein-ANS conjugate, pH 6.5, 0.05M phosphate buffer.
Figure 2. Effect of $k$-casein concentration on energy transfer ($\bullet-\bullet$) and polarisation ($\triangle-\triangle$).
Effect of NaCl

At low concentrations of salt from zero to 0.01M, there was a marked increase in relative fluorescent intensity and a decrease in the number of hydrophobic regions (Fig.3). These phenomena were accompanied by a sharp rise in energy transfer, and a decrease in polarisation (Fig.4). At NaCl concentrations higher than 0.01M these changes continued, however, at a reduced rate.

Effect of Urea

As the concentration of urea was increased the relative fluorescent intensity rapidly dropped, then levelled off after 4M; also, there was a significant red shift in the wavelength of maximum emission from 480 to 518 nm (Fig.5). Figure 6 indicates that there was a very low level of energy transfer and the polarisation decreased with increasing urea concentration.

Effect of SDS

As the concentration of SDS\(^2\) was increased up to \(^2\)The following abbreviation was used: SDS, sodium dodecylsulfate.
Figure 3. Effect of salt concentration of relative fluorescent intensity (▲-▲) and number of hydrophobic regions (●-●) for the κ-casein-ANS conjugate. pH adjusted to 7.0.
Figure 4. Effect of salt concentration on energy transfer
(○—○) and polarisation (△—△) of κ-casein.
Figure 5. Effect of urea on relative fluorescent intensity (●—●) and maximum emission wavelength (▲—▲) of κ-casein-ANS conjugate. pH 7.0
Figure 6. Effect of urea on energy transfer (●—●) and polarisation (△—△) of κ-casein.
0.005M there was a rapid decrease in relative fluorescent intensity followed by a slight increase as SDS concentration was further increased to 0.1M. Concomitant with this variation in relative fluorescent intensity with SDS concentration there was a marked red shift in the wavelength of maximum emission. At higher SDS concentrations there was again a slight blue shift (Fig. 7). As SDS concentration increased the energy transfer and the polarisation decreased revealing the minimum at an SDS concentration of 0.01M. At higher concentration of SDS there was again a slight increase in the energy transfer and the polarisation (Fig. 8).

Figure 9 shows the effect of SDS concentration on ANS alone. Dodecylsulfate itself increased the relative fluorescent intensity but to a lesser extent than the $\kappa$-casein-SDS mixture.

**Effect of pH**

As the pH was increased from 5.0 the relative fluorescent intensity passed through a minimum at pH 5.5 and a maximum at pH 6.0. As the pH was further increased there was a continuous decrease in relative fluorescent intensity up to pH 12.0. There was no apparent change in wavelength of maximum emission with pH until it exceeded 11.0 (Fig. 10).
Figure 7. Effect of sodium dodecylsulfate on relative fluorescent intensity (θ-θ) and maximum emission wavelength (Δ-Δ) of κ-casein-ANS conjugate. pH 8.0.
Figure 8. Effect of sodium dodecylsulfate on energy transfer (θ-θ) and polarisation (Δ-Δ) of κ-casein-ANS conjugate.
Figure 9. Effect of sodium dodecylsulfate on relative fluorescent intensity of ANS. pH 8.0.
Figure 10. Effect of pH on relative fluorescent intensity ($\Delta - \Delta$) and maximum emission wavelength ($\bullet - \bullet$) of $\kappa$-casein-ANS conjugate.
Effect of Temperature

As the temperature increased from 4 to 60°C there was a decrease in relative fluorescent intensity, but no change in the number of hydrophobic regions (Fig. 11). This decrease was accompanied by a decrease in the energy transfer (Fig. 12).
Figure 11. Effect of temperature on relative fluorescent intensity (■—■) and number of hydrophobic regions (▲—▲) of κ-casein.
Figure 12. Effect of temperature on energy transfer of κ-casein.
DISCUSSION

The relationship between the polarity of the region of bound ANS, fluorescent yield and emission wavelength, has been demonstrated by Stryer (63). The more non-polar the environment of the probe, the greater is the fluorescent yield and the shorter is the wavelength of maximum emission. Stryer (62) and Stryer and Haugland (64) further demonstrated the relationship between energy transfer and distance of separation of donor and acceptor based on the Forster's equation (10). The variations in fluorescent yield, emission wavelength and energy transfer when considered along with polarisation (78, 79) should give a good indication of conformational changes, aggregation or disaggregation, size and rigidity in a macromolecule.

From figure 1 it is apparent that there was some conformational change as there were changes in the polarity of the environment of the ANS, judging from variation in relative fluorescent intensity, and also a change in the number of hydrophobic regions with concentration. These changes in polarity and number of hydrophobic regions with concentration are suggestive of concentration dependence of aggregation through hydrophobic interactions as postulated by Payens (52) and of a similar concentration dependence of
aggregation for $\alpha$ s i- (67), and $\beta$ -caseins (60). However, the data on energy transfer and polarisation (Fig.2) did not support this concept. Although it is possible to have increased energy transfer due to aggregation, it should result in a concomitant increase in polarisation because of a larger molecular volume and an increased relaxation time, for a rigid molecule. If the molecule is not rigid it is unlikely to lead to increased energy transfer. In fact there was a very slight change in polarisation which is contrary to the expectation. This decrease in polarisation may be due to a greater degree of internal rotation which decreases molecular rigidity, a closer approach to a sphere or a decreased molecular volume (2, 78, 79).

A decreased molecular volume could easily account for increased energy transfer due to decreased donor-acceptor distance of separation, decreased number of binding regions due to steric limitations resulting from the decreased molecular volume, and increased relative fluorescent intensity due to more closely packed non-polar side chains. This phenomenon is similar to the concentration dependence of sedimentation coefficient (58). If $\kappa$ -casein at a neutral pH forms elongated macromolecules as Payens (52) suggested it is possible that the steric
exclusion which contributes to the geometrical lower viscosity of surrounding regions (3, 58) will decrease as the concentration of \( \kappa \)-casein increases because of entanglement of molecules (52). This increases the actual viscosity in the surrounding region substantially and decreased molecular volume and Stoke's radius of molecules.

Increasing the ionic strength by the addition of NaCl leads to a more compact structure as judged from a decrease in polarisation, an increase in energy transfer and relative fluorescent intensity, and a decrease in the number of hydrophobic regions.

Due to the high content of apolar side chains in \( \kappa \)-casein (21), it is expected that increasing the concentration of NaCl in the solvent would result in a more intense hydrophobic interaction (71) as was observed for \( \alpha_s \)l- and \( \beta \)-caseins by Waugh, et al. (75). Furthermore intensification of hydrogen bonds may not be neglected (9). Such interactions might lead to a more compact structure.

Increasing the ionic strength would have an effect of reducing electrostatic interactions, leading to increased disorganisation if the molecule existed as a monomer, or to dissociation if a polymer. In the former
case this change would lead to decreased polarisation, relative fluorescent intensity and energy transfer; in the latter case it would also lead to decreased polarisation and probably decreased relative fluorescent intensity and energy transfer. The experimental results did not support this, thus it appears that electrostatic interactions do not play a major role in the structure.

Treatment with urea and SDS, which ruptures hydrogen bonds and hydrophobic interactions (26, 39, 40), amply demonstrate that there is some order associated with the \( \kappa \)-casein molecule. Considerable reduction in relative fluorescent intensity, energy transfer, red spectral shift and decreased polarisation (Fig. 5, 6, 7 and 8) are all suggestive of loss of organisation in molecules. A significant decrease in polarisation is indicative of a high degree of intramolecular free rotation due to a decrease in molecular rigidity. This is in agreement with the work of Leslie, et al. (32) who demonstrated the rigidity of the \( \kappa \)-casein aggregate. From NMR studies the aggregate possessed some level of rigidity as compared with the other caseins. The aromatic region was not resolved with \( \kappa \)-casein treated with 7M urea which then leads to some flexibility of the amino acid residues. Cheeseman and Jeffcoat (5) and Cheeseman and Knight (6) also demon-
strated the disruptive effect of SDS on κ-casein by a marked decrease in the sedimentation coefficient and variations in the spectral characteristics.

At higher concentrations of SDS there was an apparent order generated as judged by a blue spectral shift and increases in relative fluorescent intensity, energy transfer and polarisation. This could be due partly to decreased solvent polarity as is suggested from figure 9 where the relative fluorescent intensity of ANS alone was increased with increasing SDS concentrations and simultaneously due to the development of some helix-like structure as suggested by Jirgensons and Capetillo (24).

The effect of pH upon the κ-casein structure was reflected by the relative fluorescent intensity and the number of hydrophobic regions (Fig. 10). Apart from a minimum at pH 5.5 the relative fluorescent intensity gradually decreased up to pH 12.0. This decrease is readily explained by an increase in molecular volume due to electrostatic repulsion. The large spectral shift above pH 11.0 is indicative of a highly significant structural change.

Since hydrophobic interactions are endothermic
in character (45), increasing temperatures should introduce a higher level of aggregation through hydrophobic interactions as suggested by Payens (52) or at least to a more compact structure due to increased intra-molecular hydrophobic interactions. Conversely decreasing temperatures should invite dissociation. The results presented in figures 11 and 12 did not support this assumption. Low temperatures increased the relative fluorescent intensity and the energy transfer compared with elevated temperatures indicating a role for hydrogen bonding in maintaining the molecular structure of κ-casein. However, even at 60°C there was still considerable structure remaining as relative fluorescent intensity and energy transfer (Fig. 11 and 12) were higher than those for κ-caseins dissociated with urea and SDS (Fig. 5, 6, 7 and 8). At those elevated temperatures the contribution of hydrogen bonds to the maintenance of structure is not expected to be very significant and the structural integrity would be due predominantly to hydrophobic interactions.

These results are in accord with the findings of Zittle (84), Parry and Carroll (50), Leslie, et al. (32) and Talbot and Waugh (69) that the κ-casein exists in a polymeric form with a structure but not necessarily α-helix configuration. Kappa-casein, 4.0 X 10^{-5} M in
0.05M phosphate buffer pH 7.0, had a relaxation time of 480 nanoseconds. This would compare with a sphere of molecular weight 600,000 or an ellipsoid of axial ratio 3:1 if the molecular weight of 200,000 (69) was used.

As polarisation in urea and SDS without disulfide reducing agents was remarkably low, it is possible that disulfide bridges are not important for the conformation of the \( \kappa \)-casein molecule separated by the method of Zittle and Custer (85). This structure seems to depend mainly upon hydrogen bonds and hydrophobic interactions.

Finally, from these data, it is possible that there is no concentration dependent aggregation but a concentration dependent conformational or shape change in \( \kappa \)-casein molecules.
CHAPTER II. INTERACTION OF $\kappa$ - WITH $\alpha_s$-$\lambda$-CASEIN BY FLUORESCENCE POLARISATION.

INTRODUCTION

Since the fractionation of $\alpha$-casein into fractions $\alpha_s$ and $\kappa$, calcium sensitive and insensitive respectively (77), there has been a great interest in the $\kappa$-casein fraction because of its unique stabilising ability towards the other caseins.

Studies have been done on the interaction between $\kappa$- and $\alpha_s$-$\lambda$-caseins, which stabilises $\alpha_s$-$\lambda$-casein against calcium precipitation, and could act as precursor to stable casein micelles (47, 65). The techniques used and the results obtained were varied regarding the interacting ratio of $\alpha_s$-$\lambda$- to $\kappa$-casein and the type of interaction, i.e. hydrophilic, hydrophobic or electrostatic. Waugh and von Hippel (77) studied the interaction between $\alpha_s$-$\lambda$- and $\kappa$-casein and found a variable interaction ratio, but found that the $\alpha_s$-$\lambda$: $\kappa$ ratio was predominantly 4.

Noble and Waugh (47) found that the $\alpha_s$-$\lambda$: $\kappa$ ratio was low and close to unity. Garnier (12) found an
interaction ratio of 1 mole $\alpha_s$ to 3 mole $\kappa$-casein. However, in an earlier paper, Garnier et al. (13) reported an interaction ratio of unity. Parry et al. (51) found the interaction ratio to be unity, using gel filtration.

Kenkare and Hansen (27) found that the interaction product was of variable composition, and suggested that the interaction between the $\alpha_s$ and $\kappa$-casein was hydrophilic in nature. This is in contrast with the work of Garnier et al. (14) who found a positive entropy for the reaction, suggestive of hydrophobic interaction. This postulation of hydrophobic interaction seems to be feasible, in the light of the inhibition of the interaction at temperatures 2 to 6°C and spontaneity of the same reaction at 37°C (47).

Payens (52) suggested that the main interacting force between the caseins, in maintaining the micelle structure, is hydrophobic in nature, directed within the micelle. This concept was based on the amino-acid composition of the caseins, the temperature dependence of aggregation of the caseins and the established hydrophobic nature of aggregation of $\alpha_s$ and $\beta$-caseins.
Hill and Wake (21) discussed the amphiphile nature of \( \kappa \)-casein and suggested that the \( \alpha_S 1^\kappa \)-casein interaction is significantly hydrophobic in nature.

However, in addition to the suggestion of hydrophilic and hydrophobic interactions, there have recently been suggestions of non-specific electrostatic interactions. Woychik (81), Pepper et al. (53), Talbot and Waugh (69) have modified lysine residues in \( \kappa \)-casein using various techniques and found that the modification of increasing numbers of lysine residues reduced the stabilising ability towards \( \alpha_S 1 \)-casein until it was lost completely after 5 or more residues were modified.

Hill and Laing (20) found that the modification by photo-oxidation of histidine residues in \( \kappa \)-casein reduced its stabilising ability for \( \alpha_S 1 \)-casein. Nakai, et al. (43) noted that the modification of histidine in \( \kappa \)-casein caused decreased stabilising ability when there was aggregation.

It is obvious that the interactions between \( \alpha \) - and \( \kappa \)-casein are far from clear. Varying interaction ratios have been reported, and mechanisms involving hydrophilic, hydrophobic and non-specific electrostatic
interactions have been postulated.

In this chapter are reported the results of a study on the interaction of $\alpha_s$- and $\kappa$-caseins, using fluorescence polarisation.
MATERIALS AND METHODS

Kappa and $\alpha_s^I$-caseins were prepared according to the method of Zittle and Custer (85).

Dansylated $\alpha_s^I$-casein was prepared by reacting $\alpha_s^I$-casein, 280 mg/40 ml, in carbonate buffer ($\text{NaHCO}_3$, 0.4M - $\text{Na}_2\text{CO}_3$, 0.1M, pH 9.2) with a suspension of 6 mg dimethylamino-naphthalene sulfonycholride, in 1 ml of the same buffer. The reaction was carried out in an ice bath for 20 minutes with constant stirring. The reaction mixture was then centrifuged at 4°C, the supernatant dialysed against distilled water at 4°C for 48 hours and lyophilised. One dansyl residue was introduced per mole of $\alpha_s^I$-casein (mol.wt. 27,000).

Imidazole buffer, 0.08I, pH 6.8, was prepared by mixing 0.01M imidazole and 0.01M HCl in a ratio of 6:4, with 0.07M NaCl added thereafter. Samples of $\kappa$ - and dansylated $\alpha_s^I$-caseins were prepared in 0.08M imidazole buffer, pH 6.8

Temperatures above and below ambient were maintained by a Sargent Thermonitor and a Blue M constant flow cooling unit, respectively. Polarisation was measured
using an Aminco-Bowman Spectrophotofluorometer #4-8202 with Zenon lamp and Blank-Subtract Photomultiplier. Slits were as follows: #2, 5; 4mm; #3, 4; 2mm; #7, 1mm. Polacoat UV 105 and Polaroid HN38 were used for the polariser and analyser, respectively. Excitation and emission wavelengths were 350 nm and 508 nm, respectively.

Polyethyleneimine PEI 1000 from Dow Chemicals in 0.08M imidazole buffer, pH 6.8 was added to the αs1-casein solution, 4.0 × 10^{-5} M, to give a final concentration of PEI of 0.04 mg/ml.
Fluorescence polarisation of a macromolecule is mainly dependent on the geometry and rigidity of the molecular structure. Changes in molecular conformation due to aggregation and interaction will, therefore, result in changes in the polarisation, because these changes modify molecular geometry and volume in the macromolecule (16, 28, 61, 78, 79). Thus the polarisation technique will be a useful tool for the study of the interaction between \( \alpha_s \) and \( \kappa \)-casein. By labelling one of the caseins with dansyl chloride, in our case \( \alpha_s \)-casein, it is possible to follow the interaction between the two caseins by measuring changes in polarisation.

Weber (78) demonstrated that if more than one oscillator, corresponding to more than one macromolecule is solution, are simultaneously excited, the observed polarisation, \( \bar{P} \), and the fluorescent intensity, \( F \), emitted by the components, are represented by the equation:

\[
\bar{P} = \frac{\sum_i F_i P_i}{\sum_i F_i}
\]

(3)

The observed polarisation of the interacting system \( \alpha_s \)- and \( \kappa \)-casein, would therefore be:
\[
P = \frac{F_1 P_1 + F_2 P_2 + F_3 P_3}{F_1 + F_2 + F_3}
\] (4)

where the subscripts 1, 2, 3 refer to \(\alpha_s\) \(\lambda\)-casein and their interaction product.

However, since only \(\alpha_s\) \(\lambda\)-casein was fluorescent by dansylation, the concentration of the dansylated \(\alpha_s\) \(\lambda\)-casein was kept constant and there was no change in fluorescent intensity with interaction, equation (4) can be simplified to:

\[
\bar{P} = \frac{(F_0 - F_3) P_1 + F_3 P_3}{F_0}
\] (5)

where \(F_0\) is the fluorescent intensity of original dansylated \(\alpha_s\) \(\lambda\)-casein, as the unreacted \(\kappa\) -casein would not contribute to the fluorescence and polarisation of the mixture.

Rearranging equation (5) we obtain:

\[
\bar{P} - P_1 = \frac{F_3}{P_3 - P_1} \frac{1}{F_0}
\] (6)

This equation is eventually the same as the equation used by Dandliker et al. (7).

Since the interaction between \(\alpha_s\) \(\lambda\) - and \(\kappa\) -casein is known to be negligible at 2 to 6°C and to be
spontaneous above 37°C (47) it is possible, using equation (6), to predict the polarisation changes of dansylated \( \alpha_s 1 \)-casein during titration with non-fluorescent \( \kappa \)-casein at these and intermediate temperatures.

Since there is no reaction, i.e. the equilibrium constant is indeterminate at 2 to 6°C, \( F_3 \) and \( P_3 \) are equal to 0, equation (6) simplifies to:

\[
\bar{F} - P_1 = 0, \text{ therefore } \bar{F} = P_1
\]  

(7)

Thus there should be no change in the polarisation of \( \alpha_s 1 \)-casein titrated with \( \kappa \)-casein in this temperature range. At 37°C, the association constant is infinite and there is spontaneous conversion of \( \alpha_s 1 \)-casein to \( \alpha_s 1-\kappa \)-casein complex. If enough \( \kappa \)-casein is added to the \( \alpha_s 1 \)-casein, such that all the \( \alpha_s 1 \)-casein is reacted, the only fluorescent species present in the mixture would be \( \alpha_s 1-\kappa \)-casein complex. In equation (6) \( F_3 = F_0 \) and the equation simplifies to \( \bar{F} = P_3 \), i.e. the observed polarisation is due only to the \( \alpha_s 1-\kappa \)-casein complex. Further addition of \( \kappa \)-casein to the reaction mixture, after all the fluorescent labelled \( \alpha_s 1 \)-casein has reacted, should not affect the observed fluorescent yield as the unreacted \( \kappa \)-casein is non-fluorescent.
Equation (6) can be used to calculate $F_3$ from the observed polarisation $\bar{P}$ since $P_1$ and $P_3$ are constant for a given temperature and viscosity, and $F_0$ is constant if the concentration of $\alpha_s\lambda$-casein is kept constant during the experiment, and there is no change in fluorescent intensity with interaction. The observed polarisation should, therefore, vary directly with the amount of $\kappa$-casein added to the reaction mixture.

For conditions of complete interaction, i.e. $37^\circ C$ or above, $\bar{P}$ should increase linearly with added $\kappa$-casein as all would be converted to the $\alpha_s\lambda$- $\kappa$-casein complex. As stated previously, after all of the $\alpha_s\lambda$-casein has reacted, there is no further increase in observed polarisation.

For conditions of incomplete interaction, between 6 and $37^\circ C$, because of the equilibrium conditions existing between reactants and products, all the $\kappa$-casein added is not converted to $\alpha_s\lambda$- $\kappa$-casein complex; therefore the increase in polarisation with added $\kappa$-casein would be non-linear and less than that for the complete interaction. As the equilibrium constant decreases at lower temperatures (47) a fixed amount of $\kappa$-casein added to $\alpha_s\lambda$-casein would result in less interaction at reduced temperatures, and, as a result, a lower level of increase in the observed polarisation.
From equation (6) it is possible to calculate the equilibrium constant at any temperature and concentration of added $\kappa$-casein, since $\frac{F_3}{F_0}$ is equivalent to the fraction of $\alpha_s 1$-casein reacted, and the reacting mole ratio is 0.93:1 (Fig. 13 and 15).
RESULTS

The changes in polarisation of a 4.0 \times 10^{-5}\text{M} solution of dansylated \(\alpha_s\) \text{-casein} due to titration with \(\kappa\) -casein at 4, 17, 24, 30, 35 and 40\(^\circ\text{C}\) are presented in figure 13.

At 4\(^\circ\text{C}\) there is no change in polarisation of \(\alpha_s\) \text{-casein} when titrated with \(\kappa\) -casein. At 40\(^\circ\text{C}\) there is a linear increase in the polarisation of \(\alpha_s\) \text{-casein} when titrated with \(\kappa\) -casein up to a concentration of 4.3 \times 10^{-5}\text{M}. Further addition of \(\kappa\) -casein does not effect the polarisation of the \(\alpha_s\) \text{-casein}.

At the intermediate temperatures, 17, 24, 30 and 35\(^\circ\text{C}\) the polarisation of the \(\alpha_s\) \text{-casein} increases curvilinearly when titrated with \(\kappa\) -casein. The slopes of these lines decreases with decreasing temperature. The interaction ratio of \(\alpha_s\) \text{-}: \(\kappa\) -casein is 0.93:1 based on the studies carried out at 40\(^\circ\text{C}\).

The effect of PEI and temperature on the polarisation of dansylated \(\alpha_s\) \text{-casein} when titrated with \(\kappa\) -casein are presented in figure 14. The PEI results in a higher value for the polarisation of dansylated \(\alpha_s\) \text{-}
Figure 13. Polarisation of dansylated $\alpha_{\text{s}1}$-casein, 4.0 x $10^{-5}$M titrated with $\kappa$-casein at various temperatures.

- $\bigcirc-\bigcirc$ 40°C
- $\bullet-\bullet$ 35°C
- $\triangle-\triangle$ 30°C
- $\bigtriangleup-\bigtriangleup$ 24°C
- $\blacktriangle-\blacktriangle$ 17°C
- $\square-\square$ 4°C
Figure 14. Polarisation of dansylated $\alpha_{s_1}$-casein, $4.0 \times 10^{-5}$M with and without added PEI titrated with $\kappa$ -casein at various temperatures.

-●-● 50°C No PEI
-○-○ 40°C No PEI
-□-□ 40°C PEI added;
-△-△ 4°C No PEI
-▲-▲ 4°C PEI added.
casein at 4 and 40°C, although the slope of the curves at the respective temperatures are the same when titrated with \( \kappa \)-casein.

The changes in polarisation of a 6.0 \( \times 10^{-5} \) M solution of dansylated \( \alpha\) \( s \) 1-casein due to titration with \( \kappa \)-casein at 4 and 40°C are presented in figure 15 and show the same characteristics as the lower concentration (Fig. 13).

Figure 16 shows a plot of log \( K \) against \( \frac{1}{T} \) from which \( \Delta H \) was calculated.

Figure 17 shows the Perrin plot of the \( \alpha\) \( s \) 1-\( \kappa \)-casein complex. Temperature ranges 37°C to 50°C. From this figure it is possible to calculate the polarisation of the \( \alpha\) \( s \) 1-\( \kappa \)-casein complex at lower temperatures.

Table I shows the variation in polarisation of dansylated \( \alpha\) \( s \) 1-casein and polarisation of dansylated \( \alpha\) \( s \) 1-\( \kappa \)-casein complex with temperature.

Table II shows the variation in association constant as calculated with the aid of equation (6) and data presented in Table I and figure 13, with temperature. Concentration of dansylated \( \alpha\) \( s \) 1-casein was 4.0 \( \times 10^{-5} \) M.
Figure 15. Polarisation of dansylated $\alpha_s\beta$-casein, $6.0 \times 10^{-5}$M titrated with $\kappa$-casein at 40$^\circ$C, $\bullet$-$\bullet$ and 4$^\circ$C $\triangle$-$\triangle$. 
Figure 16. Log $K$ for the association equilibrium plotted against the equilibrium temperatures.
Figure 17. Perrin plot for $\alpha_{sl^{-}} - \kappa$-casein complex. Temperature range, 37 to 50°C.
TABLE I

VARIATION IN POLARISATION OF $\alpha_s\ 1$- AND $\alpha_s\ 1-\kappa$- CASEIN COMPLEX WITH TEMPERATURE.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>$\alpha_s\ 1$</th>
<th>$\alpha_s\ 1-\kappa$-complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.283</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>0.238</td>
<td>0.322</td>
</tr>
<tr>
<td>24</td>
<td>0.232</td>
<td>0.305</td>
</tr>
<tr>
<td>30</td>
<td>0.216</td>
<td>0.292</td>
</tr>
<tr>
<td>35</td>
<td>0.200</td>
<td>0.288</td>
</tr>
<tr>
<td>40</td>
<td>0.185</td>
<td>0.268</td>
</tr>
<tr>
<td>50</td>
<td>0.170</td>
<td>0.246</td>
</tr>
</tbody>
</table>
TABLE II

VARIATION IN ASSOCIATION CONSTANT OF THE $\alpha_{s 1^-}$ -CASEIN INTERACTION WITH TEMPERATURE.

<table>
<thead>
<tr>
<th>Concentration of $\kappa$-casein X 10$^5$ M</th>
<th>Association Constant X 10$^{-4}$</th>
<th>$17^\circ$C</th>
<th>$24^\circ$C</th>
<th>$30^\circ$C</th>
<th>$35^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td></td>
<td>0.99</td>
<td>1.71</td>
<td>2.81</td>
<td>6.80</td>
</tr>
<tr>
<td>2.0</td>
<td></td>
<td>0.96</td>
<td>1.63</td>
<td>2.88</td>
<td>6.81</td>
</tr>
<tr>
<td>3.0</td>
<td></td>
<td>1.02</td>
<td>1.68</td>
<td>2.93</td>
<td>7.61</td>
</tr>
<tr>
<td>4.0</td>
<td></td>
<td>1.00</td>
<td>1.61</td>
<td>3.00</td>
<td>7.51</td>
</tr>
<tr>
<td>6.0</td>
<td></td>
<td>1.02</td>
<td>1.60</td>
<td>3.05</td>
<td>7.40</td>
</tr>
<tr>
<td>8.0</td>
<td></td>
<td>1.06</td>
<td>1.74</td>
<td>3.05</td>
<td>7.27</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>0.97</td>
<td>1.63</td>
<td>3.09</td>
<td>6.82</td>
</tr>
<tr>
<td>12.0</td>
<td></td>
<td>0.97</td>
<td>1.70</td>
<td>3.00</td>
<td>6.87</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.00</td>
<td>1.66</td>
<td>2.97</td>
<td>7.16</td>
</tr>
</tbody>
</table>
ΔF and ΔS were calculated from the following equations and are listed in Table III.

\[ \Delta F = -RT \ln K \]  \hspace{1cm} (8)

\[ \Delta S = \frac{\Delta H - \Delta F}{T} \]  \hspace{1cm} (9)
<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Equilibrium Constant X 10^{-4}</th>
<th>$\Delta F$ kcal/mole</th>
<th>$\Delta H$ kcal/mole</th>
<th>$\Delta S$ EU</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>1.00</td>
<td>-5.29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>1.66</td>
<td>-5.71</td>
<td>3.33</td>
<td>30.4</td>
</tr>
<tr>
<td>30</td>
<td>2.97</td>
<td>-6.19</td>
<td>4.67</td>
<td>35.8</td>
</tr>
<tr>
<td>35</td>
<td>7.16</td>
<td>-6.82</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
DISCUSSION

Dandliker et al. (7) Kierzenbaum et al. (28) reported a method for estimation of the association constant between antigen and antibody by fluorescence polarisation. The basic equation in those papers and ours are the same if there is no change in the fluorescent intensity after interaction.

\[
\frac{F_b Q_b}{F_f Q_f} = \frac{P - P_f}{P_b - P}
\]

Dandliker's (10)

\[
\frac{F_b Q_b + E_f Q_f}{F_b Q_b} = 1 + \frac{P_b - P}{P - P_f} = \frac{P_b - P_f}{P - P_f}
\]

\[
= \frac{F_0}{F_3}
\]

(11)

The molecules in a solution fluoresce independently and the observed intensity is simply the sum of the intensities from all the individual molecules. There was no change in fluorescent intensity upon interaction which was indicated by constant intensity for dansylated \( \alpha \_s 1 \)-casein with increasing \( \kappa \)-casein concentration at 40°C. In this case Dandliker's \( Q_f, Q_b \) and \( Q \) become equal and their equation (10) can be simplified.

In the case of antigen-antibody interaction the
heterogeneity of binding sites for antibody was reported (46). However, in our case the binding sites were assumed to be homogeneous (a = 1) within samples used of α\(s_1\)- and κ-caseins, and this is supported by the interacting mole ratio of approximately 1 and by the fact that there is one hydrophobic site per monomer unit.

The equilibrium constants described in this paper are average association constants for the poly-disperse system. These constants are for the association between dansylated α\(s_1\)-casein and unlabelled κ-casein which should not be significantly different from those between unlabelled α\(s_1\) - and κ-casein, as demonstrated for bovine serum albumin and its antibody by Kierzenbaum et al. (28), since the level of dansylation was one molecule per monomer unit of α\(s_1\)-casein. Such a low level of modification would preferably be at the terminal amino-group, and as such should not significantly interfere with the interaction.

In the calculation of the association constant, concentrations of α\(s_1\)- and κ-casein were determined as monomer units. If the interacting species is a polymer, for example N-mer, with the interaction ratio being 1:1, the equilibrium constant would be decreased by the factor
N, as the molar concentrations of all the species would be decreased by this factor. Values of N for \( \alpha_s \) -casein range from 3 to 5 (52). Such a value would have a very slight effect upon the values of the thermodynamic parameters and would not change the direction of variation of the parameters with temperature.

The stability of casein micelles is probably a reflection of many parameters, one of which is undoubtedly the interaction between the caseins. The endothermic nature of interaction of the caseins has been known for sometime. This endothermic nature of associations of the caseins has been suggested as being due to conformational change in the protein and subsequent changes in the calcium ion binding (74). However, Nemethy and Scheraga (45) have postulated that the endothermic nature of interactions could be due to hydrophobic interactions in the proteins, with a subsequent increase in both the enthalpy and entropy of the system.

The results presented in Table III clearly demonstrated the endothermic nature of the interaction between \( \alpha_s \) - and \( \kappa \) -casein. The association constant increased with temperature, and at 40°C the interaction was complete. The increasing ease of reaction was also reflected in the lower level of free energy with temperature.
In agreement with the theory of hydrophobic interactions (45) there was a positive enthalpy and entropy. These parameters both increased with temperature, indicative of the hydrophobic nature of the interaction. These results confirm the suggestions of Garnier et al. (14) who found a positive entropy for the $\alpha_s$ $\kappa$-casein interaction, which indicated the hydrophobic nature of the interaction, and also Payens (52) who suggested that the interaction would be hydrophobic in nature.

From the 40 or 50°C curves in figures 13, 14 and 15 the interaction ratio $\alpha_s$ $\kappa$ was 0.93:1 on a molar basis, as evidenced by the linear increase in polarisation of $\alpha_s$ $\kappa$-casein with added $\kappa$-casein, until the above ratio was reached; then there was no further increase in polarisation, that is no further interaction.

The linear relationship between polarisation and added $\kappa$-casein, is indicative of only one reaction product. If there were an interaction product or products with a higher ratio, such a linear relationship would not exist. The first addition of $\kappa$-casein would lead to a maximum increase in polarisation, corresponding to the highest possible ratio of $\alpha_s$ $\kappa$ under the experimental conditions; as this would represent the largest possible molecule.
Further addition of $\kappa$-casein would decrease the $\alpha_{s1}:\kappa$ ratio; therefore the molecular weight of the interaction product, and hence the polarisation would also decrease. Since a curvilinear relationship was not obtained the possibility of multiple interaction products is precluded. This reaction ratio was independent of concentration as shown in figures 13, 14 and 15. These results are in agreement with those of Noble and Waugh (47), who found that $\alpha_{s1}$- and $\kappa$-casein reacted in a low weight ratio and also of Parry et al. (51) who found that the interacting weight ratio of $\alpha_{s1}$: $\kappa$-casein is unity.

Kason et al. (25) have shown that there is an interaction between $\alpha_{s1}$-casein and polyethylenimine which is electrostatic in nature. That such an interaction does occur is evident from figure 14, where the interaction between polyethylenimine-treated $\alpha_{s1}$-casein and $\kappa$-casein was compared with the interaction between $\alpha_{s1}$- and $\kappa$-casein at 4 and 40°C. In both cases the slopes at the two temperatures were identical, however, the polarisation values for the polyethylenimine-treated $\alpha_{s1}$-casein was higher than for the untreated $\alpha_{s1}$-casein. Two conclusions can be drawn from these results. Firstly, the polyethylenimine-treated $\alpha_{s1}$-casein is a larger molecule, indicative of an interaction product as demonstrated by
Kason et al. (25). Secondly, the non-specific electrostatic nature of the interaction product of polyethylenimine and αs1-casein neither interfere with the αs1-κ-casein interaction, nor affect the interaction ratio. This indicates that the interaction is quite specific and not electrostatic in nature.

In the previous chapter κ-casein was shown to have one hydrophobic region. This is in total agreement with the results presented above, namely that the interaction ratio of αs1-κ is unity and also that the reaction is hydrophobic in nature.
CHAPTER III. EFFECT OF MODIFICATION OF CHARGED GROUPS OF \( \kappa \)-CASEIN ON ITS STRUCTURE AND STABILISING ABILITY.

INTRODUCTION

The calcium insensitive fraction of whole casein, \( \kappa \)-casein, is unique among the caseins in its ability to inhibit precipitation of other caseins in the presence of calcium ions (77). However, interaction of the caseins in the presence of calcium ions lead to the very stable casein micelles which exist in milk.

It is generally accepted, that the unusual stability of the casein micelle is due to structure maintained by hydrophobic interactions, non-specific electrostatic attractions and calcium phosphate salt bridges.

Payens (52) and Hill and Wake (21) suggested that the main interacting force is hydrophobic in nature, directed within the micelle. Talbot and Waugh (69) Pepper et al. (53), Woychik (81) and Hill and Laing (20), suggested that non-specific electrostatic attraction plays a role in the \( \kappa \)-casein stabilising ability, probably involving the \( \varepsilon \)-amino groups of lysine in \( \kappa \)-casein. However, it was not clear
whether the lysine residues in the $\kappa$-casein played a direct role in interaction, or whether this was due to structural changes induced in the protein as a result of charge differences. Talbot and Waugh (69) found a decrease in stabilising ability after one lysine residue was modified, whereas Pepper et al. (53) found it necessary to modify five residues in $\kappa$-casein before there was any loss in stabilising ability.

Rose and Colvin (57) on the other hand, suggested the possibility of a calcium phosphate bridge involving the $\epsilon$-amino group of $\alpha_s$- and $\beta$-caseins. Hoagland (23) however, found that the $\epsilon$-amino group of $\beta$-casein was not necessary for stabilisation with $\kappa$-casein.

Hill (18) suggested the presence of a positive region in $\kappa$-casein involving a peptide sequence with lysine, arginine and histidine, which was necessary for interaction. Hill and Laing (20) had previously demonstrated that modification of histidine by photo-oxidation leads to loss of stabilising ability. Nakai et al. (43) modified the histidine of $\kappa$-casein residues with diisopropyl fluorophosphate and found no change in its stabilising ability.
Kolar and Brunner (30) were able to partially disrupt the micellar structure by cooling, and Rose (55) suggested that the association of $\alpha_s$- and $\beta$-caseins is through hydrophobic bonds. In chapters I and II it has been demonstrated that $\kappa$-casein possesses some level of structure which can be disrupted by the normal dissociating agents, and also that the interaction between $\alpha_s$- and $\kappa$-casein was accompanied by thermodynamic changes suggestive of hydrophobic interaction.

It was decided to test whether these two positions were reconcilable. The lysine residues of $\kappa$-casein were modified step-wise by carbamylation (53), to give a series of $\kappa$-caseins with increased net negative charge. The free carboxylic acid functions of glutamic and aspartic acid were esterified in a step-wise manner to give a series of $\kappa$-casein samples with increased positive character. The effects of these modifications on structure and stabilising ability of $\kappa$-casein were then investigated.
MATERIALS AND METHOD

Kappa and \( \kappa \) caseins were prepared by the method of Zittle and Custer (85) and were electrophoretically pure on polyacrylamide gel. \( \beta \)-casein was prepared by the method of Nakai et al. (44). Carbamylated \( \kappa \)-caseins were prepared according to the procedure of Pepper et al. (53), using reaction times of 0, 15, 30, 45, 60, 90, 120 minutes, and 20 hours.

The free carboxylic acid groups were converted to glycine methyl esters, according to the method outlined by Lin and Koshland (31). Reaction times used were 5, 10, 15, 27, 40 and 60 minutes. Glycine methyl ester concentration used was 0.25M. Extent of lysine modification was calculated from the percentage decrease in the lysine peak after amino acid analysis, and assuming the lysine content to be nine residues per molecular weight of 20,000. Carboxylic acid groups modified were determined from the increase in the glycine peak, calculated as above, assuming three glycine residues per molecular weight of 20,000.

Changes in electrophoretic mobility of the modified samples were determined using a 10\% polyacrylamide gel, Tris-glycine buffer pH 8.7 with 4.5 M urea and 2- mer-
captoethanol. The stabilising ability was determined according to the method of Pepper et al. (53) with the following modifications: (a) the ratio $a_s l^{-1} \kappa$-casein was 5, and (b) the final concentration of calcium chloride was 0.02M.

Relative fluorescent intensity is the ratio of the fluorescent intensities of the sample to that of $\kappa$-casein at a protein concentration of $4.0 \times 10^{-5}$M and an ANS concentration of $2.0 \times 10^{-6}$M. These concentrations of protein and ANS were used throughout the experiment. Polarisation and energy transfer were determined using the technique and instrumentation previously described. ANS from Eastman Chemicals was purified as previously described. Solutions were prepared in 0.01M Imidazole buffer.
RESULTS

The changes in polarisation, relative fluorescent intensity, percentage energy transfer, wavelength of maximum emission and stabilising ability with different levels of esterification of carboxylic functions of \( \kappa \)-casein are presented in figures 18, 19 and 20. As the level of modification increases, there is a decrease in relative fluorescent intensity and an increase in wavelength in maximum emission (Fig. 18). Both of these changes are indicative of a decrease in hydrophobicity. Increasing the level of modification decreases the energy transfer and polarisation of \( \kappa \)-casein (Fig. 19), indicative of structural change. Concomitant with this structural change is a loss in stabilising ability and calcium insensitivity (Fig. 20).

The changes in polarisation, relative fluorescent intensity, percentage energy transfer, wavelength of maximum emission and stabilising ability, with different levels of carbamylation of \( \varepsilon \)-amino group of the lysine residues in \( \kappa \)-casein are presented in figures 21, 22 and 23. As the level of lysine modification increases, there is initially an increase in relative fluorescent intensity. After one residue is modified, the relative fluorescent intensity decreases progressively with further modification. Concom-
Figure 18. Effect of modification of carboxylic acid functions of $\kappa$-casein on relative fluorescent intensity $\bullet-\bullet$, and wavelength of maximum emission of ANS conjugate $\Delta-\Delta$. 
Figure 19. Effect of modification of carboxylic acid functions of $\kappa$-casein on % energy transfer $\bullet-\bullet$, and polarisation $\triangle-\triangle$. 
Figure 20. Effect of modification of carboxylic acid functions of $\kappa$-casein on its ability to stabilise $\alpha_s$-casein $\bigcirc-\bigcirc$ and on its own solubility in the presence of 0.02M calcium ion $\Delta-\Delta$. 
Effect 21. Effect of modification of ε-amino groups of lysine of κ-casein on relative fluorescent intensity ▽▽, and wavelength of maximum emission of ANS conjugate ○○.
Figure 22. Effect of modification of $\varepsilon$-amino group of lysine of $\kappa$-casein on % energy transfer $\bullet\circ$ and polarisation $\nabla\nabla$. 
Figure 23. Effect of modification of $\varepsilon$-amino group of lysine of $\kappa$-casein on its ability to stabilise $\alpha_s$-casein $\circ\circ$ and on its own solubility in the presence of 0.02M calcium ion $\downarrow\downarrow$. 
tant with these changes in relative fluorescent intensity, there is initially a decrease in the wavelength of maximum emission followed by a rapid increase (Fig. 21). There are very small changes in the energy transfer and polarisation of $\kappa$-casein with modification of up to five lysine residues. Further modification leads to very rapid decreases in energy transfer, polarisation and stabilising ability for $\alpha_s$-casein (Figs. 22, 23).

Table IV shows the polarisation, percentage energy transfer, relative fluorescent intensity and wavelength of maximum emission of $\alpha_s$, $\beta$, and $\kappa$-caseins.

The electrophoretic patterns of the modified samples were as expected for proteins with changes in the charge characteristics. With increasing levels of lysine modification the anionic character, and hence the electrophoretic mobility, increased. With the modification of carboxylic functions, however, the anionic character decreased and mobility decreased, and eventually the migration was towards the cathode at high levels of modification.
**TABLE IV**

**COMPARISON OF THE FLUORESCENT PARAMETERS OF \( \alpha_s l^- \), \( \beta \), AND \( \kappa \) -CASEINS.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Relative Fluorescent Intensity</th>
<th>Polarisation %</th>
<th>Energy Transfer</th>
<th>( \lambda_{\text{max}} ) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \kappa ) -casein</td>
<td>1.00</td>
<td>0.246</td>
<td>12.0</td>
<td>477.5</td>
</tr>
<tr>
<td>( \alpha_s l^- ) -casein</td>
<td>0.37</td>
<td>0.198</td>
<td>7.0</td>
<td>482.4</td>
</tr>
<tr>
<td>( \beta ) -casein</td>
<td>0.31</td>
<td>0.173</td>
<td>12.0</td>
<td>482.4</td>
</tr>
</tbody>
</table>
The modification of charged groups in κ-caseins has been shown to lead to a loss in stabilising ability (18, 19, 20, 53, 69, 81, 84), and suggests the possibility of electrostatic interactions between the caseins, particularly involving the ε-amino group of lysine. The results presented here support the finding that modification of charged groups induces a loss of stabilising ability (Figs. 20, 23). However, this loss of ability to stabilise αs1-casein against precipitation was achieved by modifying either the ε-amino group of lysine residues as the above authors have done, or by modification of carboxylic groups. Furthermore, at high levels of modification of carboxylic groups, the κ-casein reacted like αs1- and β-caseins and it was precipitated by calcium ions.

Finally, although low levels of modification of lysine residues changed the stabilising ability only slightly modification of carboxylic functions even at low levels had a more pronounced effect upon the stabilising ability.

Changes in fluorescent parameters of a macromolecule are a good indication of structural changes (63). Figures 18, 19, 21, 22 show very significant changes in the fluorescent parameters measured, indicative of structural
changes in the macromolecule and further that these changes increase with increasing levels of modification.

According to Stryer (63), if the 8-anilinonaphthalene-1-sulfonate is bound to a region of a macromolecule, structural changes which result in an increase in polarity of the region of the molecule, lead to a decrease in fluorescent intensity, and increase in wavelength of maximum emission. These spectral changes are due to solvent reorientation around the excited state during its lifetime.

The shifts in wavelength of maximum emission and the decrease in fluorescent intensity, as measured relative to unmodified κ-casein, (Figs. 18, 21) are indications that there is increased exposure of the ANS binding region to the solvent water molecules, or at least the molecule acquires a new conformation, whereby charged groups have caused an increase in polarity of the region. This situation could be brought about by a swelling of the macromolecule, or by disruption of bonds maintaining the molecular rigidity thus allowing freer rotation and a reduced clustering of the apolar groupings. The fact that there is a decrease in percentage energy transfer (10) from the tryptophan to ANS, (Figs. 19, 22) could support either of the concepts, as both would lead to an increased distance of separation of
the absorbing typtophan group and the emitting ANS mole-
cule. However, the variation in the polarisation of the
molecule cannot be accounted for by swelling in the mole-
cule. Any swelling would lead to an increased molecular
volume with concomitant increased relaxation time and
polarisation, assuming that there was no change in molecular
rigidity (16, 61, 78, 79).

The observed decrease in polarisation can only be
the result of two effects, a decreased molecular volume,
and/or a decrease in the molecular rigidity. If there were
a decrease in molecular volume, while maintaining rigidigy,
the percentage energy transfer would increase as this
would probably lead to a shorter donor-acceptor distance
of separation. Secondly, a decreased molecular volume could
lead to higher fluorescent yield and shorter wavelength of
maximum emission, as the apolar groups would be forced into
closer contact with each other, away from the solvent
environment. Hence a smaller loss of energy would occur
during the lifetime of the excited state, due to solvent
reorientation. Thus the decrease in polarisation cannot be
due to a contraction in molecular volume. Such a contraction
would be difficult to envisage when the modifications lead to
conditions of increased repulsive forces.
The only explanation for the decrease in polarization, which is compatible with the data presented in figures 18, 19, 21, 22 is that there is some disruption in the molecule which leads to a decrease in rigidity. Such a decrease in rigidity would allow a certain degree of free rotation, and could quite easily decrease the non-polarity of the hydrophobic region, either by disrupting the structure which maintained the conformation of the hydrophobic group, or by increasing the aqueous nature of the environment of the hydrophobic region. This would result in decreased fluorescence and increased wavelength of maximum emission. The free rotation also would effect the percentage energy transfer by allowing variations in the donor-acceptor orientation factor (10). It would appear, therefore, that by modification of \( \kappa \)-casein, some of the rigidity inherent in the molecule is lost.

In the previous chapters, evidence has been presented in support of a hydrophobic interaction between \( \kappa \) -and \( \alpha_s \)-casein, which prevented the latter from being precipitated by calcium. The fact that increasing levels of modification of \( \kappa \)-casein (Figs. 20, 23), leads to decreasing stabilising ability, concomitantly with decreasing hydrophobicity (as measured by decreasing relative fluorescence, and increased wavelength of maximum emission),
lends support to the concept of hydrophobic interaction between $\alpha$- and $\kappa$-casein.

If the data for $\alpha$- and $\beta$-casein are compared with that for $\kappa$-casein, (Table IV), the low values of relative fluorescent intensity and polarisation, and the high wavelength of maximum emission of the two former caseins are quite readily apparent. It would seem that $\alpha$- and $\beta$-caseins are highly disorganised, random coils and have a high level of free rotation compared to $\kappa$-casein. However, when 12 carboxylic acids, functions of $\kappa$-casein were modified, this protein more closely resembled $\alpha$- and $\beta$-casein, in its hydrophobic characteristics and susceptibility to calcium ion precipitation (Figs. 18 - 20; Table IV). This lends further support to the concept of hydrophobic interaction among the caseins.

Although the electrostatic forces are not directly related to the ability of $\kappa$-casein to stabilise $\alpha$- and $\beta$-casein against calcium ion precipitation, these forces are important in maintaining the structural integrity of the molecule. Conversely, the molecular changes could be due solely to an increase in repulsive forces in the molecule with increasing levels of modification. Finally,
as can be seen from figures 21, 22, 23, low levels of modification of lysine residues in $\kappa$-casein do not have as pronounced an effect on the $\kappa$-casein as low levels of carboxylic residue modification. Whereas the carboxylic acid modification leads to an immediate effect upon structure and stabilising ability, (Figs. 18, 19, 20) the fluorescent parameters for similar levels of lysine modification suggest increased hydrophobicity (Fig. 21). These data might suggest that some lysine residues are on the surface, and not directly related to maintenance of the structure. Modification would therefore only have the effect of reducing the polarity with a concomitant apparent increase in hydrophobicity. The carboxylic acid functions seem to be more related to structural integrity, probably internally. If this situation exists in $\kappa$-casein, it offers an explanation for its solubility in the presence of calcium ions due to preferential intramolecular calcium ion binding. This is due to the location of the carboxylic functions in the interior of the molecule. Secondly, the lysine residues on the surface would repel the approaching calcium ions.

This concept of structural changes in $\kappa$-casein with increasing levels of modification is supported by the work of Pepper et al. (53) and Woychik (81) who found a marked decrease in the sedimentation constants of $\kappa$-casein
with high levels of modification of the lysine ε-amino groups.

The calcium binding capacity of αs- and κ-caseins is similar (41). The reason for higher solubility of κ-casein than αs1- and β-caseins in the presence of calcium ion, could be related to the fact that there is structure in κ-casein which is predominantly lacking in αs-, β-casein, and high charge-modified κ-casein. Because of the more open structure in αs, β, and highly charge-modified κ-casein, intermolecular calcium ion binding might occur, leading to polymerisation whereas, in the more rigid κ-casein structure, intramolecular calcium ion binding may predominate. The possibility of hydrophobic interaction leading to aggregation because of a decrease in electrostatic repulsion due to calcium binding cannot be overlooked. However, the weak hydrophobic character of αs1-casein as judged from Table IV would seem to negate this, unless there were a conformational change after calcium ion binding, resulting in increased hydrophobicity.
GENERAL DISCUSSION AND CONCLUSIONS

The long held belief that \( \kappa \)-casein was structureless seems to be unfounded. Kappa-casein although highly aggregated, assumes a structure similar to that of a globular protein. This structure is related to its ability to stabilise \( \alpha_\text{s} \)-casein against calcium precipitation.

Kappa-casein has a well defined hydrophobic region, changes in which along with other fluorescent characteristics, have been used as an index of structural change in the molecule. The interaction with \( \alpha_\text{s} \)-casein involves this hydrophobic region and the thermodynamic parameters \( \Delta S \) and \( \Delta H \) are indicative of an interaction which is highly hydrophobic in character.

Chemical modification of \( \kappa \)-casein leading to changes in positive or negative charges on the protein affect the hydrophobic character of \( \kappa \)-casein, and subsequently to a decrease in the stabilising ability of \( \kappa \)-casein for \( \alpha_\text{s} \)-casein against calcium ion precipitation.

It is therefore concluded that the charged groups of \( \kappa \)-casein are important in maintaining the structural integrity of the molecule, but are not directly related
to the interaction with the other caseins. It is also suggested that interaction with calcium ion might be predominantly intramolecular in \( \kappa \) -casein and intermolecular in \( \alpha_s \) - and \( \beta \) -casein, hence the possibility of high levels of aggregation of the latter proteins in the presence of calcium ions.

Questions naturally arise as to the relationship of these data to micelle structure. Since the interaction between \( \alpha_s \) - and \( \kappa \) -casein is on a 1:1 mole basis, all the \( \alpha_s \) - and \( \beta \) -casein cannot be directly interacted with \( \kappa \) -casein. Also \( \beta \) -casein readily separates from the micelle on cooling, thus the interaction involving \( \beta \) -casein is hydrophobic and not electrostatic in nature. Since the hydrophobicity of \( \kappa \) -casein is much higher than \( \alpha_s \) - or \( \beta \) -casein, this would suggest that the weakest hydrophobic bond would involve \( \alpha_s \) - and \( \beta \) -casein. Hence it is suggested that the \( \beta \) -casein is interacted with \( \alpha_s \) -casein through hydrophobic bonding. The polymer of \( \alpha_s \) - \( \beta \) -casein is then bound to the \( \kappa \) -casein through hydrophobic interaction, to form a small calcium free micelle. Such an interaction must have the effect of either limiting the level of aggregation, in the presence of calcium ion, or of obscuring partially the negative charges on the \( \alpha_s \) - and \( \beta \) -caseins. It is concluded that the latter possibility is more likely, as the more
random coil $\alpha_S$- and $\beta$-caseins would have to assume some geometric rigidity after the interaction with the more structured $\kappa$-casein.

The role of calcium ion in the micelle would therefore be involved in increasing the micelle size by bridging smaller casein micelles together. Such bridges could probably involve carboxylic and phosphoric acid functions. The increased rigidity produced by such bridging would further limit the random character of the proteins, and lend further stability to the micellar structure.
LITERATURE CITED


33. Linderstrom-Lang (1929)