K-casein and its Interaction in Human Milk Micelles

Barbara C. Dev

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Abstract

**κ-CASEIN AND ITS INTERACTION IN HUMAN MILK MICELLES**

by

Barbara C. Dev

Human milk κ-casein was isolated from the acid-precipitated casein fraction on Sephadex G-200 and Mono Q HPLC. Samples of κ-casein purified from different donors were found similar in both amino acid and carbohydrate composition. A molar extinction coefficient of 11.2 was determined on the basis of amino acid analysis with a norleucine internal standard. Tracer κ-casein for human micelle studies was \(^3\)H-labeled in the sialic acid moiety of its carbohydrate. The human micelle system was investigated in its native form at 37°C. Micelle reformation by re-equilibration in skimmed milk at 4°C (overnight) followed by 3 h at 37°C was studied with and without the labeled κ-casein tracer. After the micelles were fractionated by ultracentrifugation into sized pellets, the mole ratio of κ/β-casein was determined by reverse phase HPLC and the six forms of phosphorylated β-casein were quantitated by anion exchange HPLC. In all systems, the relative amount of κ-casein increased inversely with the micelle size, suggesting a surface location for κ-casein in the micelles.
Support for the surface location also came from the re-equilibrated $^3$H-$\kappa$-casein micelle study, where the labeled $\kappa$-casein increased linearly with the micelle surface area/volume ratio. Of the $\beta$-caseins, 0-P and 1-P showed greatest variability with micelle size. The proportion of 0-P within the $\beta$-casein fraction decreased with decreasing micelle size but to a greater extent in the re-equilibrated system compared with the native. This indicates that the lack of ability to form Ca$^{++}$ ion bridges permitted 0-P $\beta$-casein to dissociate at low temperatures but hampered re-association at 37°C. These observations suggest the biosynthetic process is not as simple as component aggregation from whey solution. $\beta$-Casein with 1-P appeared to increase as the micelle surface area increased, supporting a surface position for 1-P and its potential to stabilize micelles against precipitation by Ca$^{++}$.

Collectively these findings support a surface position for $\kappa$-casein in agreement with the Slattery-Evard model for bovine milk micelles. However, the presence of $\beta$-caseins with different properties, due to levels of phosphorylation, makes the human micelle more complex.
k-CASEIN AND ITS INTERACTION IN HUMAN MILK MICELLES

by

Barbara C. Dev

A Dissertation in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in Biochemistry

August 1990
Each person whose signature appears below certifies that this dissertation in his opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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E. Clifford Herrmann, Associate Professor of Biochemistry

George T. Javor, Associate Professor of Biochemistry and Microbiology

Subburaman Mohan, Associate Research Professor of Biochemistry, Medicine and Physiology

R. Bruce Wilcox, Professor of Biochemistry
ACKNOWLEDGMENTS

I wish to express my appreciation to the Biochemistry Department at Loma Linda for allowing me this opportunity to pursue graduate studies. It was a fortunate opportunity to work in a group with Dr. C. W. Slattery, author of the Slattery-Evard bovine micelle model, who was my research director, Dr. S. M. Sood, an established researcher in the milk field and Sally DeWind, whose helpful technical assistance was invaluable. I am grateful to Dr. T. DeWind for his computer assistance. I am also indebted to the members of my committee; Dr. E. C. Herrmann, Dr. G. Javor, Dr. S. Mohan and Dr. R. B. Wilcox for their technical advice and support and Dr. B. Taylor (who was a committee member during the project's formative stages).

However, progress would not have been possible without the consideration, support and assistance given to me by my family and friends.

This project was supported in part by Dr. C. W. Slattery's NIH Grant #HD21123.
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Objectives for this Study

Isolation and Characterization of $\kappa$-Casein

Human Milk Micelle Studies

$\kappa$-Casein in Size-fractionated Micelles

Detection of $\kappa$-Casein Using Tritium Labeled Protein

$\beta$-Casein in Size-fractionated Micelles

Possible Effects on Micelle Structure and Models

Materials and Methods

Milk Donors

Casein Separation

Sephadex G-200 Column

Extracti-Gel D Column

HPLC With Mono Q Column

Polyacrylamide Gel Electrophoresis

HPLC Zorbax Protein-Plus Separation

$\kappa$-Casein Characterization

Amino Acid Analysis

Amino Sugar Analysis

Extinction Coefficient Determination

Gas Chromatographic Sugar Analysis

Calcium Stabilization
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<td>ANS</td>
<td>8-anilino-1-naphthalene sulfonic acid</td>
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<td>CB</td>
<td>Coomassie Blue</td>
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<td>DISC-PAGE</td>
<td>discontinuous polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>DPM</td>
<td>disintegrations per minute</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ESA</td>
<td>ethyl-stains-all</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>gMP</td>
<td>glycomacropeptide</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>n</td>
<td>number of samples</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PAGGE</td>
<td>polyacrylamide gradient gel electrophoresis</td>
</tr>
<tr>
<td>PAS</td>
<td>periodic acid Schiff's base</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Time</td>
<td>Supernatant Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------------------</td>
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- SDS: sodium dodecyl sulfate
- SMUF: simulated milk ultrafiltrate
- TEMED: \(N,N,N',N'\)-tetramethylethylenediamine
- TFA: trifluoroacetic acid
1. INTRODUCTION

This study was initiated to elucidate some of the structural and functional features of \( \kappa \)-casein in human milk. Only two caseins have been verified in human milk: \( \beta \)-casein, the major component, which is precipitated in the presence of physiological concentrations of calcium ion, and \( \kappa \)-casein, which stabilizes \( \beta \)-casein in milk, allowing the formation of large suspended aggregates of casein molecules. These physiological protein aggregates (micelles) also contain \( \text{Ca}^{++} \), \( \text{Mg}^{++} \), phosphate, and citrate, and are the efficiently-packed carriers of nutrients for the infant. Human \( \kappa \)-casein has been investigated using the established properties of bovine \( \kappa \)-casein as a guide, but this has often been misleading. For this reason it is important to be aware of the properties of the bovine milk system. But equally important are the observed differences between bovine milk and human milk.

The isolation of human \( \kappa \)-casein proved to be more difficult than would be expected considering that there are established procedures to obtain bovine \( \kappa \)-casein. Human \( \kappa \)-casein, though similar in amino acid sequence, has proven more complex in its post-translational modifications.
1.1. **Milk Composition**

The human and the bovine milk compositions in weight percent are given in Table 1. There are some important differences to be noted. Though the total solids and fat are similar, the amount and the distribution of protein between casein and whey is important. The casein is the acid-precipitated protein of milk, which naturally forms large aggregates and gives even skim milk the opaque appearance characteristic of milk. The whey proteins are soluble components in milk solution even at pH 4.6, the isoelectric precipitation point of bovine casein. There is 3.5 times more protein in bovine milk, most of it casein. The casein to whey ratio for bovine milk is 2.9/0.6 (4.8), but in human milk the casein/whey ratio is only 0.4/0.6 (0.67). Kunz and Lonnerdal (1990) and Harzer and Bindels (1988) cite evidence that the casein/whey ratio in human milk increases as the period of lactation extends. However, the casein/whey protein distribution accounts for a large difference in the amount of protein and the type of protein present in an equal volume of human milk and cow's milk, and is a prime consideration when infant formula is made from bovine milk. The amino acid distribution is different for casein and whey, which directly effects the nutrient balance the infant receives and its ability to utilize the nutrients. Because the concentration of lactose is higher in human milk (7.0%) than in bovine (4.9%), proportionately more of the calories
### TABLE 1

Bovine and Human Milk Composition (Weight Percent)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Bovine</th>
<th>Human</th>
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<tr>
<td>Total Solids</td>
<td>13.5</td>
<td>11.9</td>
</tr>
<tr>
<td>Fat</td>
<td>4.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Casein Protein</td>
<td>2.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Whey Protein</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Lactose</td>
<td>4.9</td>
<td>7.0</td>
</tr>
<tr>
<td>Ash</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\)Farrell and Thompson (1988)
in human milk are supplied by the carbohydrates. The fact that the ash content of bovine milk is 4 times higher indicates that there are more minerals present in bovine milk; however, this does not represent the amount available to the infant since the transport of minerals by the proteins in human milk appears more effective. From this brief list of components it is evident that these two milks are not the same, in that the human milk contains less protein, which is predominately whey protein, and a greater amount of lactose. Human milk is thus a source of infant nourishment that promotes slower growth than bovine milk, because the predominance of calories are supplied from carbohydrates rather than protein.

1.1.1. **Protein Distribution**

The fact that the whey proteins predominate among the human milk proteins suggests that they may have important functions. There is some change in the distribution of whey and casein in human milk over the extended lactation period. This general distribution is summarized in Table 2. A comparison of the distribution of proteins in the whey of the two milks shows them to be remarkably different. The majority of human whey proteins contribute to the nutrient value, the anti-bacterial protection, or the metal ion carrying capacity of human milk. Casein, α-lactalbumin, and milk serum albumin are considered to be nutritional proteins in that they undergo digestion. Lactoferrin binds with iron
Table 2
Casein and Whey Protein Distribution in Human and Bovine Milk\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>Human mg/ml</th>
<th>% of total protein</th>
<th>Bovine mg/ml</th>
<th>% of total protein</th>
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<tr>
<td>Total Protein</td>
<td>9.0</td>
<td>100.0</td>
<td>33.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Casein</td>
<td>2.7</td>
<td>30.0</td>
<td>26.0</td>
<td>79.7</td>
</tr>
<tr>
<td>Whey Protein</td>
<td>6.3</td>
<td>70.0</td>
<td>6.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1.5</td>
<td>16.7</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>1.9</td>
<td>21.1</td>
<td>1.2</td>
<td>3.7</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>not present</td>
<td></td>
<td>3.2</td>
<td>9.8</td>
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<tr>
<td>Serum Albumin</td>
<td>0.4</td>
<td>4.4</td>
<td>0.4</td>
<td>1.2</td>
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<tr>
<td>Immunoglobulins</td>
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<td>14.4</td>
<td>0.7</td>
<td>2.1</td>
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<td>Lysozyme</td>
<td>0.1</td>
<td>1.1</td>
<td>not present</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>1.1</td>
<td>12.3</td>
<td>0.8</td>
<td>2.4</td>
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\textsuperscript{a}Kunz and Lonnerdal (1990)
and other metals and helps fight bacterial infections due to competition with bacteria for iron in the infant's gut. Lysozyme also protects the infant by degrading the bacterial cell wall. The immunoglobulins present, even in mature human milk, are predominantly secretory IgA and have been shown to pass through the gastro-intestinal tract of the infant intact.

1.1.2. Caseins
1.1.2.1. Bovine Caseins

Originally the bovine acid-precipitated casein or curd was thought to be a single protein. However, casein bands were separated on starch gel electrophoresis and named by their position on the gel with the slowest moving band called α-casein, the next β-casein and the fastest moving band κ-casein. The first casein protein isolated precipitated in the presence of physiological Ca\textsuperscript{++} ion concentration, which prompted Linderstrom-Lang (1929) to search for a casein component that stabilized the calcium-sensitive casein and formed micelles. The casein fraction: α-, β-, and κ- were found to be phosphoproteins, but only α- and β-caseins were Ca\textsuperscript{++} ion sensitive (precipitated by physiological Ca\textsuperscript{++} ion concentration). The fastest moving band in electrophoresis (κ-casein) is a glycoprotein with 5% carbohydrate and is Ca\textsuperscript{++} ion insensitive (not precipitated by physiological Ca\textsuperscript{++} ion concentration).
1.1.2.2. Human Caseins

1.1.2.2.1. \( \beta \)-Casein

Groves and Gordon (1970) were the first to demonstrate that the multiple bands seen in urea polyacrylamide gel electrophoresis (PAGE) of human casein belonged to a single protein that differed in charge and migration because of the number of phosphate groups covalently linked to the polypeptide. They were able to isolate the six forms of the protein on a diethylaminoethyl (DEAE) cellulose column with step gradients of phosphate buffer. The protein was found in a non-phosphorylated form as well as with one to five covalently linked phosphates. This was the first demonstration of the intricacy of post-translational modification present in human casein but not seen in its bovine counterpart. The position and order of phosphate addition is shown in Figure 1 as established by Greenberg et al. (1984). The importance of a fixed order of phosphorylation is that all of a single type of \( \beta \)-casein (i.e. 0-P, 1-P, 2-P, etc.) will have the same properties, such as binding to \( \text{Ca}^{++} \) ion and the ability to interact with other molecules including anion exchange chromatographic supports. By experimental identification of the amino acid residues bearing phosphoryls, Greenberg et al. (1984) determined that in \( \beta \)-casein containing a given number of phosphates, the phosphoryl locations were identical with the exception that the one phosphate form (1-P) can be phosphorylated at either of two positions.
Figure 1. Sites of Phosphorylation Near the N-Terminus of Human β-Casein
NH₂-ARG-GLU-THR-ILE-GLU-SER-LEU-SER-SER-SER-GLU-GLU-SER-ILE-PRO

\[ \text{or} \]

\[ \text{NH₂-ARG-GLU-THR-ILE-GLU-SER-LEU-SER-SER-SER-GLU-GLU-SER-ILE-PRO} \]

1-P

2-P

3-P

4-P

5-P

\[ \text{NH₂-ARG-GLU-THR-ILE-GLU-SER-LEU-SER-SER-SER-GLU-GLU-SER-ILE-PRO} \]
The positioning of the phosphorylation results from the casein kinase specificity for a tripeptide amino acid sequence of Ser/Thr-X-A identified in seven species by Mercier and Chobert (1976). The location of phosphorylation is a Ser or Thr which is followed by any amino acid (X) and in turn by an acidic amino acid (A). Serine residues are phosphorylated before threonines and the phosphorylation of a serine converts it to a new acidic amino acid (A).

1.1.2.2.2. \( \kappa \)-Casein

Many investigators looked for \( \alpha \)-casein and \( \kappa \)-casein in human casein, but electrophoretic separation did not give definitive evidence. Occasionally a broad diffuse band was observed on gels but it was slower moving than \( \beta \)-casein and it did not stain clearly with Coomassie Blue or Amido Black. Many attempts were made to isolate human \( \kappa \)-casein in fractions which contained sugar or sialic acid residues and showed some of the characteristics of bovine \( \kappa \)-casein, such as stabilizing \( \beta \)-casein from Ca\(^{++} \) ion precipitation, calcium ion insensitivity, or cleavage by chymosin (rennin) to give a faster moving band (para \( \kappa \)-casein). \( \kappa \)-Casein enriched samples were partially purified by several groups (Malpress and Seid-Akhavan, 1966; Alais and Jolles, 1969; Nagasawa et al., 1967, 1970; Groves and Gordon, 1970; Toyoda and Yamauchi, 1973). However, it was Chobert et al. (1976) who first isolated a part of \( \kappa \)-casein by an indirect approach. Anticipating that \( \beta \)-casein in human milk must also be
stabilized by κ-casein in the presence of Ca\(^{++}\) ion, they used the milk-splitting enzyme rennin, which was known to cleave bovine κ-casein at a specific peptide bond between Phe and Met residues to yield the glycomacropeptide (gMP) and para-κ-casein, to cleave the human κ-casein. They were able to isolate the glycomacropeptide, which was soluble in trichloroacetic acid, while the para κ-casein precipitated with the other casein protein. Chobert et al. (1976) sequenced the majority of the gMP and in 1980, Fiat et al. completed the sequencing and analyzed the gMP for secondary structure. They found many prolines in probable β-turns which also contained ten threonine and serine residues as sites of O-linked glycosylation (Figure 2). The sugars in O-glycosylation differ in content and method of building the branched-chains of sugar from those with N-glycosylation where the sugars are bound to the nitrogen of asparagine. Mannose and glucose are not present in O-glycosylated sugars. The many possible β-turns and chains of hydrophilic sugars suggest a more extended secondary structure for the human polypeptide than the bovine gMP.

The entire human κ-casein molecule was not isolated until 1981 by Yamauchi et al. They used a molecular sizing gel in the presence of anionic detergent, Sodium Dodecyl Sulfate (SDS), to eliminate the natural aggregation of the caseins. This human κ-casein had an amino acid composition
Figure 2. Human $\kappa$-Casein Glycomacropeptide Secondary Structure According to Fiat et al. (1980).
rennin cleavage end

- amino acid
- helical
- β sheet
- coil
- β turn
- non-glycosylated Threonine
- O-glycosylated Threonine
- proline
+ or – charged amino acid

C-terminus
and polypeptide length similar to bovine $\kappa$-casein. However, particularly noteworthy was that the human molecule contained 40% carbohydrate by weight compared to 5% for bovine $\kappa$-casein. They found fucose and N-acetylglucosamine in addition to galactose, N-acetylgalactosamine and sialic acid present in bovine $\kappa$-casein. The large amount of carbohydrate gave human $\kappa$-casein a larger molecular weight than $\beta$-casein and characteristics peculiar to a highly glycosylated protein, i.e. not binding Coomassie Blue well and separating as a broad diffuse band in electrophoresis. Brignon et al. (1985) also isolated the para-$\kappa$-casein fragment and were able to complete the sequencing of the entire $\kappa$-casein molecule. Their analysis found the sugar content to be even larger than previously reported (closer to 50% of the weight of the molecule). The O-linked sugar chains which have been identified in bovine and human $\kappa$-casein are shown in Figure 3. The second and third sugar chains were found in bovine $\kappa$-casein and are the sialylated form of the first sugar chain (Figure 3). Since, the sialic acids were removed before the analysis of the human sugars to simplify the procedure, their location has not been determined.

The summary of casein components in bovine and human milk (Table 3) shows the differences that exist between human and bovine caseins. Bovine milk has more calcium sensitive components ($\alpha_{s1}$, $\alpha_{s2}$, and $\beta$) but each has a fixed number of phosphoryls. These represent 85% of the bovine
Figure 3. Oligosaccharide Chains in Bovine and Human \(k\)-Casein. Numbers 2 and 3 are found in bovine \(k\)-casein.
1. \( \text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

2. NeuAC\(\alpha(2 \rightarrow 3)\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

3. neuAC\(\alpha(2 \rightarrow 6)\)
   \(\text{NeuAC}\alpha(2 \rightarrow 3)\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

4. \( \text{GlcNAc}\beta(1 \rightarrow 6)\text{GalNAc-ol} \)

5. \( \text{Gal}\beta(1 \rightarrow 4)\text{GlcNAc}\beta(1 \rightarrow 6)\text{GalNAc-ol} \)

6. \( \text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

7. \( \text{GlcNAc}\beta(1 \rightarrow 6)\text{Gal}\beta(1 \rightarrow 4)\text{GlcNAc}\beta(1 \rightarrow 6)\text{GalNAc-ol} \)

8. \( \text{Gal}\beta(1 \rightarrow 4)\text{GlcNAc}\beta(1 \rightarrow 6)\text{GalNAc}\beta(1 \rightarrow 3)\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

9. \( \text{Fuc}\alpha(1 \rightarrow 4)\text{GlcNAc}\beta(1 \rightarrow 6)\text{Gal}\beta(1 \rightarrow 3)\text{GalNAc-ol} \)

1,4,5,6,7. Van Halbeek et al. (1985)
2,3. Jollès and Fiat (1979)
8,9. Saito et al. (1988)
<table>
<thead>
<tr>
<th></th>
<th>% Total Casein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Molecular Weight</th>
<th>Amino Acid Residues</th>
<th>Cysteine</th>
<th>Phosphate</th>
<th>Carbohydrate Residues</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>α&lt;sub&gt;s1&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>38</td>
<td>23,600</td>
<td>199</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>Human</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>α&lt;sub&gt;s2&lt;/sub&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>11</td>
<td>25,200</td>
<td>207</td>
<td>2</td>
<td>10</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>Human</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>35</td>
<td>24,000</td>
<td>209</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>b</td>
</tr>
<tr>
<td>Human</td>
<td>70</td>
<td>24,000</td>
<td>212</td>
<td>0</td>
<td>0-5</td>
<td></td>
<td>c</td>
</tr>
<tr>
<td><strong>K</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>12</td>
<td>19,000&lt;sup&gt;*&lt;/sup&gt;</td>
<td>169</td>
<td>2</td>
<td>N.D.</td>
<td>5</td>
<td>b</td>
</tr>
<tr>
<td>Human</td>
<td>N.D.</td>
<td>(17,707)&lt;sup&gt;**&lt;/sup&gt;</td>
<td>158</td>
<td>1</td>
<td>N.D.</td>
<td>52</td>
<td>d</td>
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<tr>
<td></td>
<td></td>
<td>(39,000)&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>e</td>
</tr>
</tbody>
</table>

N.D. Not Determined
*Peptide
**Peptide and Carbohydrate
<sup>a</sup>Farrell and Thompson (1988)
<sup>b</sup>Ruettimann and Ladisch (1987)
<sup>c</sup>Greenberg et al. (1984)
<sup>d</sup>Brignon et al. (1985)
<sup>e</sup>Sood et al. (1990b)
casein. The cysteine residues present in $\alpha_{S2}$ and $\kappa$-casein form disulfide bonds and polymers, which are cleaved by reducing agents. Human $\kappa$-casein with a single cysteine does not exhibit any chemical change when treated with a reducing agent. The different levels of phosphorylation in human $\beta$-casein in a single milk sample have been shown to have a characteristic distribution (Sood et al., 1985). See Table 4. The $\beta$-caseins with two and four phosphates are the dominant species. However, the samples do exhibit a wide range of variability. Sood et al. (1985) did not include two samples in the table in which unusually elevated levels of zero and one phosphate $\beta$-casein were observed.

Human milk samples have shown less consistency in acid precipitation of the caseins than bovine milk (Alais and Jolles, 1962; Toyoda and Yamauchi, 1972; and Azuma et al., 1981). The more highly phosphorylated caseins are the easiest to precipitate, therefore any appreciable change in $\beta$-casein distribution toward the less phosphorylated forms would effect the results of acid precipitation.

The high levels of glycosylation found in human $\kappa$-casein give it a higher molecular weight with a large hydrophilic and steric effect concentrated toward the C-terminus of the protein. The fact that human $\kappa$-casein does not form disulfide crosslinks must also cause differences in the interactions of human casein when compared to bovine caseins, where polymers of $\kappa$-casein and $\alpha_{S2}$-casein
TABLE 4
Percentage of Each Form of \( \beta \)-Casein in Samples of Human Milk\(^a\)

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>1</td>
<td>4.1</td>
<td>10.3</td>
<td>29.6</td>
<td>16.7</td>
<td>28.5</td>
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<td>2</td>
<td>8.1</td>
<td>11.1</td>
<td>26.2</td>
<td>11.6</td>
<td>30.4</td>
<td>12.6</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>9.0</td>
<td>31.6</td>
<td>16.8</td>
<td>31.5</td>
<td>7.9</td>
</tr>
<tr>
<td>4</td>
<td>6.3</td>
<td>12.6</td>
<td>34.8</td>
<td>18.6</td>
<td>22.1</td>
<td>5.6</td>
</tr>
<tr>
<td>5</td>
<td>5.6</td>
<td>6.5</td>
<td>24.8</td>
<td>14.9</td>
<td>37.4</td>
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<td>6</td>
<td>5.4</td>
<td>8.0</td>
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<td>15.8</td>
<td>30.9</td>
<td>15.4</td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>5.0</td>
<td>22.2</td>
<td>15.5</td>
<td>41.1</td>
<td>11.7</td>
</tr>
<tr>
<td>8</td>
<td>2.6</td>
<td>8.0</td>
<td>24.7</td>
<td>16.1</td>
<td>32.9</td>
<td>15.7</td>
</tr>
<tr>
<td>9</td>
<td>1.4</td>
<td>3.8</td>
<td>17.3</td>
<td>10.3</td>
<td>42.6</td>
<td>24.6</td>
</tr>
</tbody>
</table>

Mean and SD  \( 4.6 \pm 2.1 \)  \( 8.3 \pm 2.9 \)  \( 26.2 \pm 5.1 \)  \( 15.1 \pm 3.9 \)  \( 33.0 \pm 5.7 \)  \( 12.8 \pm 3.6 \)

\(^a\)Sood et al. (1985)
are observed. In their chapter on caseins as calcium binding proteins, Farrell and Thompson (1988) listed the percentage of human \( \kappa \)-casein as 27%. However, Kunz and Lonnerdal (1989a) list the percentage of \( \beta \)-casein and \( \kappa \)-casein with question marks, which is more realistic since the amounts of \( \kappa \)-casein present have not been accurately determined.

The SDS-PAGE separation of \( \kappa \)-casein, as isolated by Yamauchi et al. (1981), produced a positive glycoprotein stain (PAS) in a broad red diffuse band that migrated more slowly than \( \beta \)-casein. It was not stained strongly with Coomassie Blue. Green (1986) reported a \( \kappa \)-casein with a faster electrophoretic mobility than \( \beta \)-casein which stained positively with Ethyl-Stains-All (ESA), a cationic carbocyanic dye that stains certain phosphoproteins blue and certain sialic-acid containing proteins blue-green, while other proteins are dyed red or not at all. Green (1986) obtained a fraction of "kappa-like" casein from Groves and Greenberg who had purified the sample by the method of Groves and Gordon (1970). The sample was Ca\(^{++}\) insensitive, stabilized caseins against Ca\(^{++}\) precipitation, was cleaved by chymosin, and was different in amino acid composition than \( \beta \)-casein. Green demonstrated that this "kappa-like" casein, which stained blue-green on ESA-PAGE, was cleaved by chymosin to yield a broad, faster-moving blue-green band and a red band at the dye front. However, it is important to note that
under the same conditions, they did not observe that human skimmed milk treated with chymosin gave gMP and para-κ-casein. A 2-dimensional electrophoretic gel study, which showed the non-reactivity of human skimmed milk to the same concentration of chymosin that cleaves bovine milk, prompted Anderson et al. (1982) to report that there was no κ-casein in the casein complex of human milk.

In 1988 and 1989(b), Kunz and Lonnerdal described a method to improve the separation of human casein and whey by precipitation at pH 4.3 and 60 mM Ca\(^{++}\) with further fractionation of the whey proteins by fast protein liquid chromatography (FPLC) on an anion-exchange column (Mono Q). They have now published the results of this separation (Kunz and Lonnerdal, 1990). They designated the first peaks as whey proteins, the next as glycosylated caseins, and finally the phosphorylated proteins. The gradient SDS-PAGE of the glycoprotein fractions showed the same band in color and location as reported by Green (1986). The phosphorylated β-caseins were more firmly bound and eluted last. Sugar analysis was performed for the neutral sugars and sialic acid to identify the glycosylated protein peaks. Reducing agents had no effect on the separation results. The last 4 peaks were identified as phosphorylated β-casein and the multiple peaks before those were identified as glycosylated proteins. Neither the non-phosphorylated β-casein nor the β-casein with one phosphoryl group were designated. Kunz
and Lonnerdal noted that α-lactalbumin elutes between the glycosylated and phosphorylated peaks. They also mentioned that the increase in casein during lactation was mainly due to glycosylated casein. There was no evidence of the higher molecular weight PAS-positive κ-casein in this separation.

1.2. The Casein Micelle

Interest in the milk casein micelle has been the driving force for many investigations in regards to casein protein-protein interactions, calcium ion-protein interactions, and the coagulation process, which occurs when κ-casein is enzymatically cleaved by rennin (chymosin) and is of great importance in cheese production in the dairy industry. The results of these studies have been reviewed by Slattery (1976), McMahon and Brown (1984), Ruettimann and Ladisch (1987), and Farrell and Thompson (1988).

The protein aggregates of casein are commonly called micelles because of the large number of molecules in a cluster and the amphipolar nature of α-, β-, and κ-caseins (Bloomfield and Mead, 1973), and the fact that they are colloidal suspensions. Their massive structure causes the characteristic opaqueness or turbidity of milk by scattering light. Carroll et al. (1968) analyzed electron micrographs of bovine micelles. In bovine milk the average micelle has been estimated to be 150 nm in diameter with a molecular weight of $5 \times 10^8$ containing 25,000 monomers. They were found to have a wide distribution of size from 50 nm to over
300 nm in diameter. Later studies indicated that the majority of aggregates may be in a smaller diameter size range (Holt et al., 1978). The fact that protein aggregates have a size-distribution is one of several important properties of the micelle system that must be taken into account in a viable micelle model. Biological systems typically exhibit a set number of subunits in an organized unit, e.g., in large enzyme complexes, so that the size distribution is noteworthy and may have a functional role.

The α- and β-caseins exhibit regions of very high hydrophobicity in their amino acid sequences and also regions rich in acidic amino acids and phosphoryl groups such as the N-terminus of β-casein, giving them amphipathic characteristics (Payens, 1982). In addition to the caseins (αS1, αS2, β, and κ), calcium and phosphate are an integral part of micelles and are involved in micelle interactions (Slattery and Evard, 1973).

1.2.1. Bovine Milk Micelle Models

The proposed models for bovine milk micelles have been summarized in the dissertation of Carlson (1982). The type of model with its components and the experimental evidence supporting the model are detailed in his summary and shown in Table 5. There are three major different models: the coat-core, the internal structure and the subunit. An example of each is represented in Figure 4. The coat-core
### TABLE 5
Summary of Bovine Micelle Models, Carlson (1982)

<table>
<thead>
<tr>
<th>RESEARCHERS</th>
<th>MODEL TYPE</th>
<th>STRUCTURE</th>
<th>EXPERIMENTAL EVIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waugh &amp; Noble</td>
<td>Coat-core</td>
<td><em>K</em>-Casein coat, <em>αs1</em>-<em>,β</em>-casein core. No specific role for calcium apatite.</td>
<td>Spontaneous formation of centrifuge stable &quot;micelles&quot; from mixture of <em>αs1</em>, <em>K</em>-casein.</td>
</tr>
<tr>
<td>(1965)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waugh &amp; Talbot</td>
<td>Coat-core</td>
<td></td>
<td>Increasing <em>K</em>-content causes average micelle size to decrease in accordance with new surface area created by <em>K</em>-casein.</td>
</tr>
<tr>
<td>(1970)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Payens (1966)</td>
<td>Coat-core</td>
<td>Similar to Waugh model except proposed a surface + binding role for calcium salts and surface position for <em>β</em>-casein</td>
<td>Same as above plus explains importance of calcium apatite and solubilization of <em>β</em>-casein on cooling. Easy access of rennet to <em>K</em>-casein.</td>
</tr>
<tr>
<td>Parry &amp; Carrol</td>
<td>Coat-core</td>
<td><em>K</em>-Casein core, <em>αs1</em>- and <em>β</em>-casein coat. Some <em>K</em>-casein free in solution (serum)</td>
<td>Ferritin labeled antibodies against <em>K</em>-casein did not adhere to &quot;coat&quot;. Reaction of serum with rennet and recombination with micelles caused more rapid clotting, indicating serum <em>K</em>-casein hydrolysis was sufficient to cause clotting (refuted by other authors).</td>
</tr>
<tr>
<td>(1969)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RESEARCHERS</td>
<td>MODEL TYPE</td>
<td>STRUCTURE</td>
<td>EXPERIMENTAL EVIDENCE</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Rose</td>
<td>Internal structure</td>
<td>$\beta$-Casein acts as nucleation point for attachment. $K$-Casein binds to other end of a &quot;strands of $\beta$&quot;</td>
<td>The properties of $\beta$- and $\alpha$-casein suggest hydrophobic bonding. Highly porous micelle structure is evident.</td>
</tr>
<tr>
<td>Garnier &amp; Ribadeau-Dumas</td>
<td>Internal structure</td>
<td>Trivalent trimer of $K$-casein acts as a &quot;node&quot; to link chains of casein</td>
<td>Porous structure, oligomers of $K$-casein (trimers) and fact that carboxy peptidase attacks all the caseins with equal ease cited as evidence.</td>
</tr>
<tr>
<td>Morr</td>
<td>Subunit</td>
<td>Submicelles with core of $\alpha_{\gamma_1}$, $\beta$-casein and coat of $K$-casein linked together with bridges of calcium apatite</td>
<td>Electron microscopic studies seem to show submicellar structure. When calcium apatite is removed milk micelles form submicelles. Porous nature allows rennet attack.</td>
</tr>
<tr>
<td>Slattery &amp; Evard</td>
<td>Subunit</td>
<td>Submicelles formed of $\alpha-, \beta-, K$-casein. $K$ is localized on one side of submicelle. Submicelles aggregate to form micelles</td>
<td>Electron microscopy studies. Hydrophobic nature of $\alpha, \beta$-caseins contribute to stability of micelles. Size is restricted by $K$-casein charge. Porous nature allows access to $K$-casein.</td>
</tr>
</tbody>
</table>
Figure 4. Coat-Core, Internal Structure and Subunit Models of Bovine Micelles.
COAT-CORE MODEL
Adapted from Waugh and Talbot, (1970)

INTERNAL STRUCTURE MODEL
Adapted from Garnier and Ribadeau-Dumas, (1970)

SUBMICELLE MODEL (SURFACE VIEW)
Adapted from Slattery and Evard, (1973)
model features a central core of $\alpha$- and $\beta$-casein, protected by a coat or layer of $\kappa$-casein. The core, as described by Talbot and Waugh (1970), is made of aggregates of spherical shape, composed of $\alpha$- and $\beta$-caseins in soap micelle-like orientation with their hydrophobic C-terminus in the center and the hydrophilic acidic amino acids of the N-terminus at the outer circumference. The attack by rennin on the outer layer of $\kappa$-casein releases the hydrophilic gMP, which allows further aggregation of the micelles and their coagulation. The internal structure model is based on a uniform distribution of all the caseins with a definite pattern of orientation. In this model, the cleavage of $\kappa$-casein in an inside location is explained by the porous nature of the micelle, which was demonstrated by the easy access of enzymes to the interior caseins in the micelle structure (Ribadeau-Dumas and Garnier, 1970). The third model considered is the subunit type of Morr (1967) and of Slattery and Evard (1973) which is an extension of the Waugh coat-core model. The subunits are called submicelles, and are visible in electron micrographs of both human and bovine milk as multi-particle aggregates inside the micelles. They are spherical in shape and postulated to contain $\alpha$-, $\beta$-, and $\kappa$-casein. In Morr's proposal, the $\kappa$-casein is uniformly distributed on the outside of a 30 nm sphere, while the Slattery-Evard model postulates that $\kappa$-casein replaces some of the $\alpha$- and $\beta$-casein molecules and is associated in a portion of the
submicelle represented by the darker areas shown in the Slattery-Evard model in Figure 4. The presence of the hydrophilic portion of the C-terminus of κ-casein limits hydrophobic aggregation and also any Ca²⁺ bridging between submicelles. The observation that κ-casein and its sugars are more prevalent in the smaller micelles, which are prevented from growing by the hydrophilic effect of the κ-casein (Sullivan et al., 1959; Bloomfield and Morr, 1973; Slattery, 1978), is consistent with this model. In 1977, Slattery demonstrated that such an uneven distribution of κ-casein could explain the wide size distribution observed in micelles.

In addition to the models described above, Schmidt (1982) proposed an extension of the Slattery model which included some submicelles that had no κ-casein and further emphasized the role of colloidal calcium phosphate in linking the subunits together. The importance of calcium phosphate is demonstrated by the fact that its removal by dialysis or by EDTA causes micelles to dissociate into submicelles (Schmidt and Buchheim, 1970; Carroll et al., 1971).

If it appears from this discussion that over the years a unified model of micelle structure is emerging, then it is important to include the contrary views of the milk scientists at the Hannah Research Center, who support a model without subunit structure, i.e., a gel-like internal structure with hydrophilic arm extensions, the C-terminus of κ-
casein (described as hairy micelles) (Holt and Dalgleisch, 1986; Horne, 1986). They ascribe the submicelle structure evidence to artifacts brought on by experimental manipulation, since micelles exist in equilibrium with milk whey solution and any isolation procedure perturbs this critical balance. This is particularly possible in electron microscopy because the micelles must be fixed for observation and this involves chemical cross-linking with harsh chemicals such as glutaraldehyde. Such treatment would be expected to affect these very hydrated protein aggregates and would explain the difficulties experienced in obtaining good E-M pictures of human milk (Ruegg and Blanc, 1982; Carroll et al., 1985).

1.2.2. Casein Biosynthesis

Any acceptable micelle model must be consistent with the biosynthetic pathway for milk. The biosynthesis of the caseins is a very involved process since they are secretory proteins, which are processed in a very ordered series of events including extensive covalent modification. Mepham et al. (1982) and Neville and Neifert (1983) reviewed the results from experiments with many different systems. The secretory proteins have a signal peptide that directs their translation into the lumen of the rough endoplasmic reticulum (ER). N-Glycosylation has been shown to take place in the ER but O-glycosylation enzymes are present in the golgi. The proteins are transported in transfer
vesicles to the cis side of the golgi for phosphorylation and O-glycosylation. Though many factors in the selective processing of secretory proteins from the rough ER to the golgi have been elucidated, Lodish (1988) points out that the process has not been simplified by these discoveries and appears to be very complex and specific for each system. The site of Ca\textsuperscript{++} ion addition is important because micelle formation is dependent on the presence of Ca\textsuperscript{++} ions, the concentration of which has been found to be ATP-dependent in mouse and rat golgi-derived vesicles (Neville et al., 1981; West, 1981). After post-translational modification, secretory vesicles containing the caseins bud from the trans golgi and are transported to the cell membrane, where their contents undergo exocytosis. A simplified diagram of this process is shown in Figure 5.

In E-M pictures of bovine mammary gland cells, micelles can be seen in the secretory vesicles (Carroll et al., 1970). A possible order of events for micelle formation begins with casein synthesis, followed by phosphorylation, aggregation to form submicelles, aggregation of the submicelles to form micelles, caused by an increase in calcium and phosphate concentration, glycosylation of exterior k-casein and then the formation of secretory vesicles. This sequence includes glycosylation after micelle formation, which was suggested by Slattery (1978) to explain the increased amount of carbohydrate in the small micelles and
Figure 5. Secretory Pathway of Casein Biosynthesis and Micelle Formation.
1. Signal peptide directed insertion of nascent chain into rough endoplasmic reticulum (rer) lumen. Proteins processed through smooth endoplasmic reticulum (ser).

2. Proteins are transferred to cis face of golgi by transfer vesicle (tv).

3. The caseins undergo post-translational modification in the golgi involving phosphorylation and O-glycosylation.

4. Casein micelles observed in secretory vesicles (sv) budded off from trans golgi. This step involves a concentration of casein proteins.

5. Secretory vesicles are transported to cell membrane and undergo exocytosis.
the presence of more non-glycosylated \(\kappa\)-casein in large bovine micelles. Takeuchi et al. (1984) investigated the post-translational modification enzymes in rat and cow mammary glands and have also suggested that phosphorylation of \(\kappa\)-casein probably precedes glycosylation since the rate of phosphorylation is reduced by prior glycosylation.

1.2.3. The Human Micelle System

Measurements from E-M photographs of human milk micelles indicate a smaller average diameter (43 nm) than in bovine milk (140 nm). These photographs were more difficult to obtain than with bovine milk and could only be achieved with micelles pelleted by centrifugation (Carroll et al., 1985). This may be because of the smaller size of human micelles and their lower calcium and phosphate content, and also because the caseins are a smaller percentage of the total protein content of human milk.

To understand the interactions in the formation of the human milk micelle, one must begin with a foundation of the more recently established properties of the individual constituents of this micelle and its properties in self-association. The unique characteristics of human \(\beta\)-casein (six different phosphorylated forms) and \(\kappa\)-casein (ten-fold increase in glycosylation over bovine \(\kappa\)-casein) potentially have important functions in human micelle formation.
1.2.3.1. Individual Forms of β-Casein

Sood et al. (1985), (1988), and (1990a) and Slattery et al. (1989) have made extensive studies of the physical properties of the individual forms of β-casein, which make up the majority of the human micelle protein components. First, the authors investigated β-casein with five phosphoryl groups (5-P β-casein), which has the same degree of phosphorylation as bovine β-casein. Using sedimentation and viscosity studies similar to those applied to bovine β-casein by Waugh et al. (1971), Sood et al. (1985) described a molecule with a molecular weight of 24,400, hydrated with 2.6 g H2O/g protein with an axial ratio of 5 suggesting a prolate ellipsoid of 10 nm length and 2 nm width. Both 0-P and 3-P studies also gave these results (Sood et al., 1988, 1990a). This shape correlates with the amphipathic nature of the molecule, having a phosphorylated charged N-terminus and a majority of the amino acid chain dominated by hydrophobic residues (Sood et al., 1990a). The hydration is twice the amount for bovine β-casein (Sood et al., 1979).

From calcium binding studies by equilibrium dialysis, Sood et al. (1985, 1988 and 1990a) determined that one Ca++ ion was strongly bound for each phosphate covalently-linked to the β-casein i.e. 5-P bound 5 Ca++ ions/molecule and 3-P bound 3 Ca++/molecule. The 0-P form showed no strong calcium binding at physiological Ca++ ion concentration but at
high concentration (300 mM) appeared to bind calcium. In 1989, Azuma et al. investigated the individual forms of the human β-caseins in regard to their Ca$^{++}$ ion binding properties and calcium ion-dependent precipitation. The calcium ion binding exhibited a linear relationship, increasing with the number of covalently-linked phosphates; however, they found more than 1 mole of calcium ion/mole of phosphate (1-P bound 1 to 2 moles of Ca$^{++}$ ions and 5-P with 7 to 8 moles of Ca$^{++}$ ions). But, 0-P was found to bind 6.2 moles of calcium ions/mole, which is inconsistent with their other results. Azuma et al. (1989) stated that a good method for this determination is difficult to find because of the precipitation of the more phosphorylated β-caseins.

Azuma et al. (1989) studied the effect on the hydrophobicity of β-casein of the number of covalently-bound phosphates, determined by the elution pattern on a C-4 reverse phase column in 5 mM phosphate buffer at pH 7.0 with a linear gradient of 34% to 40% acetonitrile. The phosphorylated forms of β-casein were eluted in order of their phosphorylation with 5-P β-casein eluting first and 0-P last which was evidence that under these conditions (pH 7.0) the level of phosphorylation inversely effected the hydrophobicity.

1.2.3.2. β-Casein Self-Association

In studies of the self-association of non-phosphorylated human β-casein by Sood et al. (1988), 0-P was more hydrophobic than the phosphorylated β-caseins since
it dissolves the least readily at 37°C in low salt buffer at pH 7.0. In the three forms of β-casein studied (0-P, 3-P, and 5-P), self-association was favored by factors enhancing hydrophobic interactions, i.e. increase in temperature and increase in ionic strength. A sharp transition was seen between 25 and 30°C, from monomer to aggregate, indicating a conformational change enhancing hydrophobic interactions. The aggregates were considered to be spherical and ranged from 9-12 nm in radius, with 48 to 105 monomers, and were highly hydrated, containing 0.86-3.0 g H₂O/g protein. The phosphoryl groups give the N-terminus a net negative charge while the rest of the molecule favors hydrophobic interaction. To form aggregates of limiting size and cause salting out of protein required increasing ionic strength with increasing number of negatively charged phosphates; 0-P was the least soluble and 3-P was less soluble than 5-P. The salt ions shield the repulsive charges of the phosphate esters which favors aggregation. The limiting size for 0-P was reached in 0.10 M NaCl, in 0.20 M NaCl for 3-P, and 5-P did not reach maximum size until 0.25 M NaCl. Sood et al. (1988) explained that the stability given by electrostatic repulsion of the larger number of phosphates needed increasing amounts of salt to be overcome as the net charge on the molecule increased. Hydrophobic interaction alone is a very important force in self-association as was seen in the
dephosphorylation of bovine \( \beta \)-casein which caused an increase in size of aggregation (Yoshikawa et al., 1974).

Similar results were found by Azuma et al. (1989) who reported that in the Ca\(^{++} \)-dependent precipitation at 37\(^\circ\)C, 3-P, 4-P, and 5-P acted very similarly, while 0-P, 1-P, and 2-P were slower to aggregate. This study was done by observing the increase in turbidity at \( A_{320} \) nm when the protein and Ca\(^{++} \) ion solution was transferred from ice-cold water to a 37\(^\circ\)C spectrophotometer cell. When the individual \( \beta \)-caseins were incubated at 37\(^\circ\)C for 60 min, 0-P and 1-P were turbid while the other forms of \( \beta \)-casein were not, indicating the increased self-association of the 0-P and 1-P.

The association of the monomer was described by Sood et al. (1985) as a spherical aggregation of amphipathic molecules in the form of prolate ellipsoids, similar to the monomers in the core of the Waugh model (Waugh, 1971). See Figure 4. The distribution of charge toward one end of the molecule also could explain the concentration-dependent change in the plot of reduced viscosity versus concentration which was seen with the phosphorylated \( \beta \)-caseins but not seen with 0-P. The molecules at low protein concentration could align with the flow in the viscometer but as the concentration of the protein increased, the asymmetric charge distribution would prevent alignment and increase the reduced viscosity (Sood et al., 1989).
Further information concerning structure and possible important sites involved in the interactions of the individual caseins may be obtained by analyzing the homologous amino acid and mRNA sequences in bovine and human caseins. Holt and Sawyer (1988) investigated the predicted secondary structure and the homologous amino acids in the sequences of caseins in four species. They applied four different methods to determine secondary structure and only when three methods matched did they accept the structure as a positive prediction. For the $\beta$-caseins, the signal peptide, the 3'and 5' non-coding regions and the major phosphorylation site are similar for all four species. The region of phosphorylation in calcium-sensitive proteins ($\beta$-caseins) has an $\alpha$-Helix-loop-$\alpha$-Helix structure, which gives it a prominent position in the molecule for calcium binding. When the authors put the indicated $\alpha$-helix sequences near the N-terminus in a helical net diagram, which aligns the amino acids according to their secondary structure, two polar side chain clusters and a hydrophobic patch are similar in the four species. One cluster is mainly acidic (Glu, Gln and Asp), the other has Lys in place of Glu (Lys, Gln and Asp) and with the hydrophobic patch define this amphipathic region. Though the remainder of the amino acids in the $\beta$-caseins are different from one species to the next, the overall high hydrophobicity of the amino acids is maintained.
Slattery et al. (1989) studied hydrophobic interactions in self-associating systems for the extremes of human β-casein phosphorylation (0-P and 5-P) by fluorescence monitoring of the intrinsic tryptophan and the binding of 8-anilino-1-naphthalene sulfonic acid (ANS), (a non-polar binding site probe). The changes in fluorescence intensity and polarization were followed from 5°C to 45°C with and without 10 mM CaCl₂. All the fluorescence studies indicated that the proteins underwent a temperature-dependent conformational change to increase hydrophobic interactions. These were also enhanced by calcium ions. The complete comparison cannot be drawn because 5-P in the presence of 10 mM CaCl₂ precipitates at the transition temperature. The fluorescence intensity change was less apparent for the 5-P and Slattery et al. (1989) suggested that the phosphoryl groups cause a less flexible, constrained environment, which was already more non-polar than the 0-P at temperatures below the conformational transition.

There was some indication that self-aggregation may be similar to the first step in the formation of micelles. Slattery et al. (1989) reported laser light-scattering results for 0-P and 5-P which indicated that a submicelle-sized aggregate (20-30 monomers) was formed initially. With 0-P, when calcium was then added at 37°C, a minimum sized micelle aggregate was formed which contained 12-15 submicelles. These results reinforce the importance
of both hydrophobic and calcium phosphate interactions in
micelle formation (Slattery et al., 1989).

1.2.3.3. Human κ-Casein

With a further purified sample of the κ-casein that
they had reported initially in 1981, Azuma et al. (1984a,b)
performed more characterization studies. This sample did
not precipitate with the addition of Ca\(^{++}\) (12.5-37.5 mM) and
in sedimentation experiments showed no significant effect of
temperature (6\(^{\circ}\)C and 37\(^{\circ}\)C), verifying that human κ-casein
was Ca\(^{++}\) ion insensitive and did not self-associate. In
sedimentation studies, bovine κ-casein was reported to be
polymerized due to disulfide cross-linking (McKenzie and
Wake, 1961). This difference between the human and bovine
protein is caused by the higher cysteine content of the
bovine κ-casein. They applied the equations of Simha and
Perrin to relative viscosity and sedimentation data in the
method that Waugh et al. (1970) used for bovine β-casein,
and found the best fit was a prolate or oblate ellipsoid
with a high axial ratio, indicating an elongated or fibrous
structure rather than a spherical or globular molecule for
the κ-casein molecular shape. Circular dichroism experi-
ments suggested that the structure had little α helix (2%),
but 34% β sheet and 26% β turns. These results are consis-
tent with the gMP secondary structure proposed by Fiat et
al. (1980) for the amino acid sequence (Figure 2). They
also established that this κ-casein was cleaved by both
rennin and pepsin (the natural endopeptidase) into a trichloroacetic acid-soluble glycomacropeptide and two time-dependent acid-insoluble para-κ-caseins and that there was not much difference in the enzymatic rate of cleavage of κ-casein by the bovine or human enzyme.

The amino acid sequence of the calcium insensitive proteins (κ-caseins) maybe divided into two major groups (Nakhasi et al., 1984). Human, pig, mouse, and rat are in the more glycosylated group, which has more propyl residues and hydrophobic residues. The other group includes cow, sheep, goat and water buffalo. The area around the chymosin-sensitive bond is highly similar for secondary structure, and indicates the sequence β-turn-β-strand-β-turn for all the species of known sequence, (Holt and Sawyer, 1988). Possible sites of phosphorylation in the κ-caseins do not show the same prominent position as in the calcium-sensitive caseins. They do not have the flexible extended end location but are farther along the polypeptide chain and also they are not in clusters, which probably relates to their being calcium insensitive. It is interesting to see which amino acids are identical in the bovine and human κ-casein gMP. Figure 6 shows the secondary structure proposed by Fiat et al. (1980) with the identical bovine amino acids also indicated (Holt & Sawyer, 1988).
Figure 6. Human κ-Casein Glycomacropeptide Secondary Structures (Fiat et al., 1980) With the Identical Amino Acids of Bovine κ-Casein gMP Indicated by Shading.
rennin cleavage end

- amino acid
- helical

\[ \beta \text{ sheet} \]

- coil

\[ \beta \text{ turn} \]

\[ \text{non-glycosylated Threonine} \]

\[ \text{O-glycosylated Threonine} \]

\[ \text{proline} \]

+ or − charged amino acid

C - terminus
1.2.4. Reconstituted Micelles

Yamauchi et al. (1981) isolated a sample of human $\kappa$-casein and combined it with human $\beta$-casein and 10 mM $\text{CaCl}_2$ to test its ability to form micelles. They showed that a 20/1 $\beta$-casein to $\kappa$-casein ratio formed spherical micelles, which were visible by E-M. With an improved preparation of $\kappa$-casein, involving further removal of SDS from the isolated protein, Azuma et al. (1984a) carried out sedimentation velocity studies to investigate $\kappa$-casein and its interaction with $\beta$-casein. A new faster-moving sedimentation peak was observed when $\kappa$-casein and $\beta$-casein were mixed in a 3/1:$\beta/\kappa$ ratio and the $\kappa$-casein peak was eliminated. $\kappa$-Casein combined with a third of the $\beta$-casein to form a complex that was separable as a higher molecular weight species on DISC-PAGE. There was no interaction evident in sedimentation studies of $\kappa$-casein mixed with lactoferrin even though lactoferrin is difficult to remove as a contaminant in $\kappa$-casein purification.

1.2.4.1. Size-fractionated Reconstituted Micelles

In 1985, the same group (Azuma et al.) reconstituted human micelles from $\kappa$-casein and $\beta$-casein and investigated their properties according to their relative sizes. Artificial micelle formation was observed in a 7/1:$\beta/\kappa$ ratio in 10 mM $\text{CaCl}_2$ although 5 mM $\text{CaCl}_2$ was found to be sufficient to cause micelle formation. The micelles formed were separated into three size groups by differential
centrifugation. The largest micelles had the most β-casein (16/1:β/κ) and the smallest amount of carbohydrate (25%) in the κ-casein whereas, the smallest micelles were 3/1: β/κ with 43% carbohydrate (wt/wt) in the κ-casein. These results agree with Slattery's (1978) bovine micelle studies which found an inverse relationship for the amount of κ-casein and the amount of glycosylation in κ-casein with the size of native micelles. The urea DISC-PAGE of size-fractionated artificial micelles showed the presence of each form of the β-caseins with an increase of 5-P β-casein evident in the largest sized micelles.

1.2.4.2. Mixtures

An interesting complexity of the interaction of the different forms of β-casein was seen in the study of Ca\(^{++}\) ion stabilization of mixtures of 5-P individually with 0-P, 1-P, and 2-P and 4-P with 1-P. The 1-P form protected 4-P and 5-P from Ca\(^{++}\) ion precipitation, 2-P showed slight stabilization but 0-P appeared to cause precipitation. Thus the β-caseins with fewer phosphoryls exhibited Ca\(^{++}\) ion stabilizing properties similar to κ-casein. This may be important in the formation of micelles (Azuma et al., 1989).

1.2.4.3. Reconstituted Micelles of κ-Casein With Individual β-Caseins

In addition to the study of κ-casein with whole human β-casein containing the six different types of
phosphorylated β-caseins, recombination studies of κ-casein were carried out individually with 0-P, 3-P, and 5-P β-caseins (Azuma et al., 1985). The 5-P β-casein formed micelles faster than whole β-casein but to the same extent. β-Casein with three phosphoryls was slower to form micelles and 0-P β-casein only increased slightly in turbidity, indicating that 0-P β-casein differs as a participant in micelle formation from the phosphorylated β-caseins, although 0-P was found to be present in all the micelles separated from whole casein when analyzed by Urea-PAGE. The 0-P form appeared to be disadvantageous to micelle formation. To add to the questions about the function of 0-P in human milk is the report of Monti and Jolles (1982) who isolated 0-P in the whey protein fraction and called it temperature-sensitive protein (galactothermin). The biosynthetic conditions may also influence the incorporation of 0-P in the native micelle.

1.3. Objectives for this Study

It has been noted that when human milk micelles are acid precipitated (pH 4.5), the curd that is formed is softer and less dense than that formed from bovine milk. This property may determine the digestibility of milk in the acid environment of the infant stomach since Tomomasa et al. (1987) has reported that the stomachs of breast-fed babies empty faster than those fed bovine milk formula. The overall purpose of this research is to determine how the
differences in the composition and post-translational modification of the proteins in the human casein system can lead to micelle structural differences which may be related to the differences in curd properties. The individual human \(\beta\)-caseins were purified and investigated for their self-association properties. The increased water of hydration over bovine \(\beta\)-casein suggests the possibility of a more open and sponge-like structure for human casein (Sood et al., 1985, 1988, and 1990a). The different sized micelles for bovine milk have been separated by differential high speed ultracentrifugation (Slattery 1978; Davies & Law 1983; Dalgleisch et al., 1989), size-exclusion gel chromatography (McGann et al., 1980; Donnelley et al., 1984) or by controlled-pore glass chromatography (Ekstrand et al., 1978), but there are no reports of the separation of human milk micelles according to their sizes. The compositional differences in these fractions is a key to understanding the interactions in the human milk micelle. However, before compositional differences can be determined, pure human \(\kappa\)-casein must be available as well as pure human \(\beta\)-casein.

This study describes the isolation of pure \(\kappa\)-casein and its use in determining the composition of size fractionated human micelles and discusses the implication of the results on possible micelle structure. Specific objectives related to each aspect of the study are listed below.
1.3.1. **Isolation and Characterization of \( \kappa \)-Casein**

Many aspects of the human system are not defined, because it has been very difficult to isolate pure human \( \kappa \)-casein. As mentioned in this introduction, there are many reports in the literature (Kunz & Lonnerdal 1988; Anderson 1982; Green 1986; Chtourou et al., 1985) of human \( \kappa \)-casein with properties that conflict with those of the \( \kappa \)-casein isolated by Yamauchi et al. (1981) and Brignon et al. (1985). These inconsistent reports make it necessary first to isolate \( \kappa \)-casein in a pure form and characterize it before any study involving its contribution to the human micelle can be undertaken. Fundamental properties, such as the extinction coefficient for \( \kappa \)-casein, the distribution of the caseins between the whey (soluble proteins) and the micelles, and any variation of sugar content in samples from different donors need to be investigated.

1.3.2. **Human Milk Micelle Studies**

Since the only human caseins reported so far are \( \beta \)- and \( \kappa \)-casein, it would appear that their interaction in the human milk micelles would be a simplified version of the bovine system. However, the existence of six different forms of phosphorylated \( \beta \)-casein and the heterogeneity of the sugars in \( \kappa \)-casein point to the possibility of more complex interactions in the human micelle system.
1.3.2.1. *k*-Casein in Size-fractionated Micelles

In the bovine system the amount of *k*-casein increases with the decrease in size of micelle and increased surface area and this relationship is the basis for the surface position for *k*-casein in the model of Slattery and Evard (1973). Furthermore, the surface location of *k*-casein, which limits submicelle association, has been used to explain the size distribution of bovine micelles (Slattery, 1977). For these reasons the investigation of size-fractionated human micelles is considered to be the foundation of the study of human milk. Human micelles have not been fractionated from milk in its native system because the abundance of whey proteins complicates the separation. Since the micelle system is in equilibrium with the whey solution, isolation from this native system is particularly important.

1.3.2.2. Detection of *k*-Casein Using Tritium Labeled Protein

*k*-Casein has been difficult to detect in human milk because it is present in much smaller amounts than β-casein and is usually identified by electrophoretic separation and stained with PAS, a relatively insensitive glycoprotein stain. As part of the project it was decided to enhance the detection of *k*-casein with a tritium label which could be used for more sensitive identification of this protein.
Additional studies of the distribution of $\kappa$-casein and $\beta$-casein may then be carried out in re-equilibrated human milk that has tritium labeled $\kappa$-casein incorporated in the micelles.

1.3.2.3. $\beta$-Casein in Size-fractionated Micelles

As noted in the introduction concerning human $\beta$-casein, recent reports have established that the individual forms of $\beta$-casein vary in important properties involved in micelle interactions. These include calcium ion binding, calcium ion stabilization and self-association. For this reason it was considered of value to examine the distribution of the individual forms of $\beta$-casein in size-fractionated micelles.

1.3.3. Possible Effects on Micelle Structure and Models

The results of the distribution of $\kappa$-casein and $\beta$-casein in the different size micelles may be interpreted in terms of their implications concerning the interactions of the human micelle and their relation to the bovine micelle models. Valuable additional information is available from the known amino acid sequences of these proteins. The sequences for $\kappa$-casein and $\beta$-casein will be compared for both species, and predicted properties related to association such as secondary structure, hydrophobicity and charge distribution applied to add insight into possible interactions in the micelle.
2. MATERIALS AND METHODS

2.1. K-Casein Isolation

2.1.1. Milk Donors

Milk donors were selected who were not taking any medication, could supply milk in excess of the quantity needed by their babies, and had the potential to contribute samples for the length of the study. The mother completed a consent form which was approved by the University Institutional Review Board. Mature milk was collected after the cessation of colostrum and the intermediate period, that is, after one month from the beginning of nursing. The milk sample was frozen as soon as it was collected and kept at -20°C until processed.

2.1.2. Casein Separation

The method used for human casein isolation (Sood et al., 1985) was modified for the initial acid precipitation of casein. This modification eliminated two steps that diminished the amount of κ-casein obtained. These steps caused the cleavage of carbohydrate from the glycoprotein thereby decreasing the molecular weight of κ-casein. This affected the separation of κ-casein from β-casein based on molecular weight. The first discontinued step involved the addition of 1 N NaOH to the isoelectric precipitate which
caused the cleavage of O-glycosidic bonds between the sugars and the protein and the second was the overnight stirring at 4°C of the precipitated solution in acetic acid at pH 4.0 which affected the acid-labile sialic acids.

Five hundred milliliters of milk was thawed at room temperature and skimmed after centrifugation in a GS-3 rotor on a Sorval RC5B centrifuge (DuPont Instruments, Wilmington, DE). The sample was spun at 6K rpm (6100 x g) for 20 minutes at 0°C to 5°C. Solid lipid was carefully removed with a cold spatula from the side and top of the opaque liquid. The solution was diluted with an equal volume of distilled water and was adjusted to pH 4.5 with 1 N HCl, the isoelectric point of bovine casein. The flocculent precipitate and solution were stirred for one hour at room temperature and spun in the GSA rotor at 11K rpm (20,000 x g) for 20 min at 25°C to 30°C. The pellets from multiple tubes were collected and suspended in 25 ml of distilled water and the pH readjusted to 4.5. This smaller volume was spun in the SS-34 rotor at 10K rpm (12,000 x g) for 20 min at 25 to 30°C. The pellets were collected and suspended in 50 ml of cold ethanol and filtered on a sintered glass filter at room temperature. The precipitate was resuspended in 25 ml of cold ether, filtered and then washed with a second 25-ml portion of ether and filtered. The precipitate was allowed to air-dry overnight on a covered watch glass.
2.1.3. **Sephadex G-200 Column**

The method of Yamauchi et al. (1981) for \(k\)-casein isolation was modified. Sephadex G-200 (Pharmacia, Piscataway, NJ) was prepared by allowing it to swell in distilled water in a 90°C water bath for 5 h. The buffer system for the column was 0.05 M imidazole (pH 7.0), 1% sodium dodecyl sulfate (SDS, Ultra pure grade Schwarz/Mann Biotech, Cleveland, OH), and 0.01% \(\beta\)-mercaptoethanol (research grade, Haake Buchler Instruments, Inc., Saddle Brook, NJ). Special care was taken in pouring the gel into the column because the SDS in the buffer caused foaming. First the column (2.5 x 120 cm) was completely filled with buffer and the swollen gel slurry was poured into a reservoir funnel at the top at room temperature. The swollen gel percolated through the buffer to settle at the bottom. After the column was filled with gel, it was equilibrated with buffer by gravity flow with an operating pressure not exceeding 16 cm of water, and then equilibrated in the upward flow direction for 24 h. The upward buffer flow was maintained by a Microperpex peristaltic pump (LKB, Bromma, Sweden) with a flow rate of 9 ml/h. A 250-mg sample of acid-precipitated casein was dissolved with stirring for 1 h at room temperature in 4 ml of column buffer containing 16 mg SDS and 19 \(\mu\)l of \(\beta\)-mercaptoethanol. The sample was heated for 10 min in a 60°C water bath and then pumped onto the column. The eluate was collected in 150-drop fractions (3 ml/test tube) on a
MultiRac fraction collector (LKB Bromma, Sweden) and the absorbance of each fraction at 280nm ($A_{280nm}$) was determined on a modified DU (Gilford) spectrophotometer (Beckman, Fullerton, CA). The protein peak fractions were pooled to separate $\beta$-casein from the smaller $\kappa$-casein peak. To partially remove the SDS, these fractions were bagged in Spectra/Por dialysis tubing with a molecular weight cut-off of 6,000 to 8,000 (Spectrum Medical Industries Inc., Los Angeles, CA) and dialyzed against 40% methanol for 4 h with a change of solution after 2 h. The bags were then transferred to a distilled water container in the cold room ($4^\circ$C) and the distilled water was periodically changed until the conductivity of the solution approached the conductivity of distilled water. The dialyzed protein was freeze-dried on a lyophilizer (Labconco Corporation, Kansas City, MO). The dried sample was weighed and kept at $-20^\circ$C until used. For longer storage it was kept at $-80^\circ$C.

To obtain sufficient $\kappa$-casein, the column was kept flowing with buffer and consecutive samples were applied until the separation showed signs of deterioration, such as peak broadening.

2.1.4. **Extracti-Gel D Column**

A step to remove the anionic detergent (SDS) from the glycoprotein was necessary before further purification on an anionic exchange HPLC column (Mono Q). The removal of SDS was accomplished by dissolving the lyophilized $\kappa$-casein in
Tris-urea buffer containing 6 M urea, 0.02 M Tris-hydrochloride (pH 8.0), and 0.02 mM dithiothreitol (DTT) and passing the protein solution through a 2.5 x 6.0 cm column of Extracti-Gel D (Pierce Chemical Company, Rockville, IL) with Tris-urea buffer (Elzinga and Phelan, 1984). Ions were removed from the 6 M solution of ultra pure urea (Schwarz/Mann Biotech, Cleveland, OH) by batch treatment with Bio-Rex MSX 501-M (Bio-Rad Laboratories, Richmond, CA) as recommended in BMBiochemica Lab Notes (1989). One-milliliter fractions of the eluted protein were collected and the $A_{280\text{nm}}$ was determined. The protein fractions were pooled and used directly on the Mono Q column.

2.1.5. HPLC With Mono Q Column

The protein solution in Tris-urea buffer from the above procedure was separated on a LKB GTI Liquid Chromatographic System (LKB, Bromma, Sweden) equipped with an anion exchange column HR 5/5 Mono Q (Pharmacia, Piscataway, NJ) and a Superloop (Pharmacia). The Superloop accommodates large sample injections (1 to 10 ml). The LKB GTI System has titanium fittings and a continuous water wash behind the pump piston, which facilitated working with high salt conditions. The protein solution was filtered on an Acro LC13 disposable filter assembly (Gelman Sciences, Ann Arbor, MI) and sonicated in a Branson 220 sonicator (Branson Cleaning Equipment Company, Shelton, CT) to remove dissolved air before an aliquot was injected into the superloop. The
protein was eluted with 0.02 M Tris-hydrochloride (pH 8.0), 6 M urea, 0.02 mM DTT (buffer A) and increasing amounts of buffer B, which was buffer A with 1.0 M NaCl. The buffers were made with deionized water from a NANOpure Water system equipped with an ULTRAfilter (Sybron/Barnstead, Boston, MA), filtered on a cellulosic MSI filter (Micron Separations Inc., Westboro, MA) of pore size 0.22 μm and sonicated to remove dissolved air. The buffers were continuously bubbled with chromatographic grade Helium.

For κ-casein purification, an elongated shallow gradient was used to separate high molecular weight contaminants that eluted after the broad κ-casein peak. The elution started with 10 min of buffer A, then the gradient increased linearly to 10% buffer B over 25 min (fraction 35). This was maintained for 5 min (fraction 40), then increased linearly to 25% buffer B in 20 min (fraction 60) and finally increasing to 100% in 5 min (fraction 65). The flow rate was 1 ml/min and 1-ml fractions were collected on a Helirac (LKB, Bromma, Sweden). The protein peaks established by A_{280nm} readings were pooled, dialyzed against deionized water until the conductivity approached that of background and lyophilized. The dried samples were kept at -20°C or at -80°C for longer periods of time.

2.1.6. Polyacrylamide Gel Electrophoresis

The purity of the protein preparation was determined by SDS-PAGE using a 15% separating gel and a 5% stacking gel as
described by Laemmli (1970). The gels were run on a Mighty Small II electrophoresis apparatus (Hoefer Scientific, San Francisco, CA). They were stained with Coomassie Blue (CB), PAS (periodic acid Schiff's base, a glycoprotein stain) and Ethyl-Stains-All (ESA) which differentially stained sialylated protein, phosphorylated-protein, and unmodified protein.

The PAS method of Segrest and Jackson (1972) was used with modification. The gel was placed in a plastic container with a lid and washed overnight to remove the SDS with 200 ml of fixative solution containing 40% methanol and 7% acetic acid. For all washes, the closed container was agitated on an orbital shaker (Bellco Biotechnology, Vineland, NJ). The mini-gel was covered two times with 250 ml of 0.7% periodic acid in 5% acetic acid for 30 min each. The gel was then carefully drained and the solution changed twice with 250 ml of 0.2% sodium metabisulfite in 5.0% acetic acid for 30 min each. After pouring off this solution, the gel was covered with Schiff's reagent (Sigma Chemical Company, St.Louis, MO). The pink bands that developed at this point were indicative of glycoprotein. A 35mm colored print was taken on Fuji Super HR 100 on a Desk Top light box equipped with a 5000°K color corrected lamp (Logan, Chicago, IL) and then the gel was rinsed with repeated changes of water. The non-glycoprotein bands appeared in addition to the glycoprotein bands after the
excess of magenta dye was washed from the gel. Again a picture was taken to record the position of the bands.

Duplicate gels were run simultaneously with each electrophoretic separation. One gel was stained with PAS and then washed to expose non-glycosylated protein bands. The other gel was stained with Ethyl-Stains-All, destained and stained with CB according to the procedure of Green (1986). Ethyl-Stains-All solution was made fresh for each use and care was taken to minimize exposure to light. Both Stains-All and Ethyl-Stains-All, two different chemicals, are available but this method requires Ethyl-Stains-All (Gallard Schlesinger, Carle Place, NY). The gel for ESA was washed overnight in 25% isopropanol, with agitation, to remove the SDS. In the morning the wash solution was changed 2 to 3 times for 2 h each, which gave a total wash time of 20 h. The gel was drained and covered in the dark with 100 ml of freshly prepared ESA stain. The dye (5 mg ESA) was dissolved in 5 ml of formamide in a flask covered with aluminum foil, to which was added an additional 5 ml formamide, 25 ml isopropanol, 1 ml of 1.5 M Tris-hydrochloride (pH 8.9), and 64 ml of water. The container was covered with aluminum foil and agitated overnight. The gel was destained in water while protected from direct light until the colored bands were visible on a light background. After a photograph was taken of the gel, it was further destained in water with exposure to light until colorless and then stained for 1 h
with a 0.125% CB-R-250 solution in 50% methanol and 10% acetic acid, followed by destaining in a solution of 7% acetic acid and 5% methanol. A photograph was taken when the background had destained. The gels were covered on both sides with moistened cellophane membrane backing (Bio-Rad, Richmond, CA) and dried on a Gel Slab Dryer (Bio-Rad).

2.1.7. **HPLC Zorbax Protein-Plus Separation**

Since κ-casein eluted at the same position as the 1-P and 2-P β-casein peaks when separated by Mono Q HPLC, reverse phase HPLC was used for analytical purposes to detect and quantitate κ- and β-casein individually. κ-Casein was separated from β-casein on a Zorbax Protein-Plus Bio Series HPLC column (DuPont, Wilmington, DE) size 4.6 mm x 25 cm. The column packing was a bonded reverse phase material stable to hydrolysis from pH 1.5 to 7.0. The separation of casein was carried out at pH 2.0 where charges on the sialic acid and phosphate groups were minimized and the difference in hydrophobicity of the caseins was maximized.

A guard column of the same support was installed between the injector and the column on the LKB GTI System. The primary mobile phase was 0.1% trifluoroacetic acid (TFA) in deionized water (pH 2.0) and a gradient suitable for optimum separation was established by the secondary mobile phase, 0.1% TFA in HPLC grade acetonitrile (Baxter, Muskegon, MI). The water-based solution was filtered on a
MSI cellulosic filter (0.22 μm pore size) and the acetonitrile solution was filtered on a 0.2 μm pore size FP-200 Vericel membrane filter (Gelman Sciences Inc., Ann Arbor, MI). The pumps were run at 1 ml/min and 200 μl of protein solution containing less than 300 μg protein in 6 M urea was injected. The eluting proteins were detected at 280 nm and collected in 1-ml fractions.

2.2. κ-Casein Characterization

2.2.1. Amino Acid Analysis

The amino acid composition of acid hydrolyzed κ-casein was determined by Dr. J. W. Blankenship of the Connective Tissue Laboratory, Jerry L. Pettis Memorial Veteran's Hospital, Loma Linda, CA using a Biotronik LC5001 amino acid analyzer (Munich, West Germany). A 0.3 mg sample of κ-casein was hydrolyzed in 1.0 ml of 6N HCl for 22 h at 110°C prior to analysis.

2.2.2. Amino Sugar Analysis

The amino sugars are detected by amino acid analysis, but are more sensitive to acid hydrolysis than the amino acids, which necessitated determining them by a modified procedure. The method of Fanger and Smyth (1970) was applied to 1 mg of glycoprotein that was hydrolyzed in 1.0 ml of 6 M HCl at 100°C for 4 h. The amino sugars were separated from incompletely digested peptides on a column made from a pasteur pipette filled with Dowex 2X4 (Bio-Rad) in a
buffer of 1% N-ethylmorpholine (pH 9.0). The volume of the eluate was reduced on a rotary evaporator and dried in a vacuum desiccator over P₂O₅. The residue was dissolved in sodium citrate sample dilution buffer (Pierce) and the amino sugars were determined with the Biotronik used for the amino acid analysis.

2.2.3. Extinction Coefficient Determination

It is difficult to determine the concentration of a glycoprotein by the common methods because glycoproteins are hygroscopic, which causes problems in weighing the samples. Furthermore, the carbohydrate moiety contains nitrogen in amino sugars and sialic acids which contribute heterogeneity to the sample's non-protein nitrogen and eliminate the validity of the Kjeldahl analysis. For these reasons it was particularly valuable to know the extinction coefficient of κ-casein.

The method of Walsh and Brown (1962), which incorporated norleucine, an "unconventional" amino acid, as an internal standard in amino acid analysis, was applied. The solution of κ-casein (approximately 1 mg/ml) was prepared and the A₂₈₀nm reading taken. Norleucine (Pierce, Rockville, IL) was added to give a final concentration of 0.5 mM. The sample of protein with the internal standard was hydrolyzed and analyzed for amino acid composition on the Biotronik according to the procedure described in 2.2.1. The amount of each amino acid in κ-casein can be corrected
for experimental loss by determining the factor effecting the norleucine concentration.

The amount of proline in $\kappa$-casein was used as the known specific parameter to determine the concentration of $\kappa$-casein, because both Yamauchi et al. (1981) and Brignon et al. (1985) had obtained 28 moles proline per mole of $\kappa$-casein. The moles of proline corrected for experimental loss were converted into moles of $\kappa$-casein and its concentration determined. In this manner the extinction coefficient was calculated from the concentration and absorbance of the initial $\kappa$-casein solution by the use of Beer's Law.

\[ \varepsilon = \frac{A}{b \cdot c} \]

2.2.4. Gas Chromatographic Sugar Analysis

The method of Bhatti et al. (1970) was adapted to determine carbohydrate content of $\kappa$-casein. This analysis was done in the laboratory of Professor Vasu Dev at California State Polytechnic University, Pomona. Initially the sugars of the glycoprotein were hydrolyzed with anhydrous methanolic HCl, forming the methylated derivatives. These were then reacylated with acetic anhydride and finally reacted with trimethyl-silylating reagent to form the trimethylsilylated ethers. Each sugar formed several derivatives. The amount of an individual sugar's derivatives was determined in relation to a known amount of derivatized mannitol as an internal standard.
A milligram sample of each of the two glycoprotein samples was weighed and dissolved in 1.0 ml of water. This solution was filtered through an Acro LC13 with a pore size of 0.2 μm (Gelman Sciences, Ann Arbor, MI) and the $A_{280\text{nm}}$ and $A_{320\text{nm}}$ readings were taken on a Gilford DU modified spectrophotometer. Four hundred microliter aliquots of the glycoprotein solutions and 0.1 μmoles of mannitol standard were placed in 2 ml Wheaton ampules (VWR Scientific, Cerritos, CA) and dried over $P_2O_5$ in a vacuum desiccator. Five hundred microliters of methanolic HCl were added to each ampule, and bubbled with a steady stream of nitrogen for 30 s. The ampules were quickly sealed and placed in an oven at 90°C for 24 hours. After the vials were cooled to room temperature, $Ag_2CO_3$ was added to the vials in 200 mg aliquots, until the samples stopped bubbling, indicating that they were neutralized. Each sample was then acylated with 50 μl of acetic anhydride, covered with parafilm and placed in the dark for 24 hours. The precipitate was crushed with a glass rod, centrifuged and the supernatant collected with a 50 μl syringe. The precipitate was treated three times with 0.25 ml of anhydrous methanol and centrifuged. The supernatants were transferred to a 1 ml Reacti-Vial (Pierce Chemical Co.) and first dried under a stream of $N_2$ and then in a vacuum desiccator over $P_2O_5$ for 24 h. Each vial was closed with a teflon lined septum cap (Pierce) and 50 μl of Tri-Sil silylating reagent (Pierce)
was added. The vials were turned on their sides, rolled and then shaken by gentle tapping, followed by sonication for 10 minutes. The samples were centrifuged and the supernatant analyzed by a HP-5880 gas chromatograph (Hewlett Packard, Avondale, PA). A fused silica capillary column (J&W, Sacramento, CA) 30 m long and 0.25 mm in diameter and coated with DB-Wax was used for the analysis of the derivatized sugars. The carrier gas was helium with a flow-rate of 1.2 ml/min and a split ratio of 70:1, that is, of 70 parts injected, one part passed through the column. The injector temperature was 200°C and the flame ionization detector at 300°C. The initial temperature of the column was 100°C and the temperature was programmed for a final temperature of 220°C at 2°C/min. Sample injection size was 1 μl and all silylated sugar derivatives were eluted within 60 minutes.

The identification of peaks for each sugar was established separately and the total area response relative to mannitol was determined. The molar response factor of each monosaccharide was determined from the slope of the plot of the mole ratio versus the total peak area ratio of each sugar relative to mannitol.

Initially the gas chromatographic analysis of fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid, (the sugars present in O-glycosylated proteins) was performed for each individual sugar. The chromatograms were analyzed to establish the retention times of the peaks
of the silylated derivatives of each sugar. In order to establish the molar response factor (a weighting factor which is different for each sugar), three standard mixtures of the five sugars and mannitol were derivatized and analyzed. The total peak area response of each sugar relative to mannitol peak area was plotted against the mole ratio of that sugar to mannitol. The slope of the line of this graph was the molar response factor for a given sugar and was used to calculate the amount of that sugar present in the derivatized samples of $\kappa$-casein by applying the following equation.

$$\mu\text{moles sugar} = \frac{(\text{total peak area})(\text{molar response factor})}{\mu\text{mole of internal standard}}$$

2.2.5. Calcium Stabilization

The method of Malpress and Seid-Akhavan (1966) was used to study the $\kappa$-casein stabilization of $\beta$-casein precipitation by calcium ion. Human $\kappa$-casein and human $\beta$-casein with four phosphoryls were dissolved in imidazole-KCl buffer, pH 7.1, to give a 6 mg/ml solution. In this study, a varied amount of $\kappa$-casein (0 to 0.4 ml) was combined with a constant amount of $\beta$-casein (0.5 ml). The total volume was brought to 0.9 ml with imidazole buffer and the mixture was equilibrated at 37°C for 15 min. Afterwards 0.1 ml of 0.1 M CaCl$_2$ was added to each of the mixtures and they were agitated in a 37°C water bath for 1 h. Next, the mixtures were centrifuged at 400 x $g$ for 3 min at 37°C and a 0.5 ml
sample of the supernatant was diluted with 2.5 ml of 0.05 M sodium citrate. The absorbance of the non-precipitated casein was read at 280 and 320 nm. The effect of light scattering in milk protein solutions on the absorbance at 280 nm was corrected by subtracting 1.7 times the absorbance at 320 nm from the apparent absorbance at 280 nm (Sood et al., 1985):

\[
\text{corrected } A_{280\text{nm}} = A_{280\text{nm}} - (1.7)(A_{320\text{nm}}).
\]

The data were plotted as the percent stabilization versus the mole ratio of \( \kappa/\beta \) determined by corrected \( A_{280}/\epsilon \) using the following relationship to calculate the % stabilization:

\[
\text{% STABILIZATION} = \frac{A_{280}(\beta+\kappa+\text{Ca}) - A_{280}(\kappa) - A_{280}(\beta+\text{Ca})}{A_{280}(\beta) - A_{280}(\beta+\text{Ca})}.
\]

2.2.6. Phosphorus Analysis

The determination of phosphorus followed the method reported by Long and Yardley (1970) which involved the formation of colored molybdate solutions. Care was taken to treat the glassware with chromic acid and to wear gloves to minimize background contamination for this sensitive test.

2.2.7. Sialic Acid Analysis

The periodate-thiobarbituric acid method of Aminoff (1961) was used to obtain a colorimetric product from the sialic acids hydrolyzed from \( \kappa \)-casein by sulfuric acid. The Aminoff procedure was chosen because it was reported to give
better results in the presence of fucose (Beeley, 1987), one of the sugars in $\kappa$-casein.

2.3. $\kappa$-Casein Tritium Labeling

2.3.1. Method for $\kappa$-Casein Tritium Labeling

The sialic acid residues of $\kappa$-casein were labeled with tritium by the technique of Van Lenten and Ashwell (1971) involving the oxidation and reduction of the three carbon chain attached to the sugar ring (Figure 7). Selective modification was achieved by mild conditions of low concentration (2 to 5 mM NaIO$_4$) and a 5 to 10 fold molar excess of periodate.

Ten milligrams of purified $\kappa$-casein, dissolved in 2 ml of 0.1 M sodium acetate, was cooled in ice in the dark. An equal volume of 10 mM NaIO$_4$ was added. After 10 min, 100 $\mu$l of ethylene glycol was added to stop the reaction. The solution was then dialyzed overnight in the cold room against one liter of phosphate buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.5) with two changes of buffer. The oxidized $\kappa$-casein was transferred to a small test tube at room temperature and reacted with 30 $\mu$l of 100 mM NaB$_3$H$_4$ for 30 min. The labeled reducing agent solution was made from sodium borohydride [$^3$H] (NEN, Wilmington, DE) (600mCi/mmol) dissolved in 417 $\mu$l of 0.01 N NaOH. Then 60 $\mu$l of non-labeled 100 mM NaBH$_4$ was added. The reduced labeled protein solution was dialyzed overnight in the cold room with two
Figure 7. Synthetic Scheme for Labeling Sialic Acid in k-Casein with NaB\textsuperscript{3}H\textsubscript{4}. 
changes of phosphate buffer, one change of imidazole buffer (0.05 M imidazole, 0.15 M NaCl, pH 7.5) and finally with several changes of deionized water. The sample was then lyophilized and stored at -20°C.

2.3.2. Scintillation Counting

A 10 to 20 μl aliquot of the solution to be counted was pipetted into a 7 ml disposable solvent saver scintillation vial (Kimble, Vineland, NJ). The vial was filled with 5 ml of Universol Cocktail (ICN Radiochemical, Irvine, CA). The vials were capped and vigorously shaken. They were counted in a LS-7500 Scintillation Counter (Beckman Instruments Inc., Fullerton, CA) for 10 min after a 2 h storage in the dark to minimize chemiluminescence. The counts per minute were converted to DPM by correcting for quenching by the H-number method which involves an external standardization by $^{137}$Cs (Beckman Instructions, 1980).

2.3.3. Purification of $[^3$H] $\kappa$-Casein

The products of the tritium labeling of $\kappa$-casein were introduced into a Mono Q column for HPLC to remove products that did not have the binding properties of $\kappa$-casein. The gradient eluants were the same Tris-urea and Tris-urea-NaCl buffers used in section 2.1.5. The column was conditioned with an initial separation of non-radioactive $\kappa$-casein (1.5 mg in 1 ml) to reduce the amount of loss due to non-specific binding of $[^3$H] $\kappa$-casein. The lyophilized sample of $[^3$H]}
$\kappa$-casein (7.05 mg) was dissolved in 7 $\mu$l of HPLC Tris-urea buffer, stirred 10 min with a magnetic stir bar and filtered through an Acro LC13 filter, pore size 0.2 lm. The programmed gradient was designed to yield maximum $\kappa$-casein, while cutting contaminants which eluted initially and after $\kappa$-casein. The sample was divided in two 3.5-ml injections and the protein elution was monitored at A280nm. Fractions were collected every 2 min and 10 $\mu$l aliquots from each fraction were counted in duplicate in the scintillation counter. The fractions were pooled so as to limit the contaminants. The purity of the samples was evaluated by SDS-PAGE and X-ray autoradiograph of the CB stained gel. The gel was treated with autoradiograph En$^3$Hance (NEN Products Dupont, Boston, MA) for 30 min and washed in water for 10 min. The gel was transferred to cellophane and vacuum-dried. The autoradiograph was done on a pre-flashed X-ray film (Kodax-QMAT$^\text{thAR}$). In the dark, the vacuum-dried gel was placed on a X-ray film cassette and covered with the X-ray film. The cassette was closed, covered with aluminum foil and stored at -80°C. It was found that a 4 h exposure of the gel was sufficient to get detail of the location of the radioactivity. An aliquot of this purified sample was used in each of the labeled re-equilibrated micelle experiments. It is referred to as $^3$H labeled $\kappa$-casein.
2.4. **Micelle Preparation**

2.4.1. **Milk Donor**

For the micelle study it was necessary to obtain milk immediately after it was expressed by the donor so that the milk could be maintained at 37°C and the micelle separation begun as soon as possible. A sample of 125 to 200 ml of milk was collected from a donor on the Loma Linda University campus. The milk was from an individual donor and expressed at least two hours after the baby had been nursed. Two donors were used to supply fresh milk for the micelle study.

2.4.2. **Skimmed Milk Preparation**

Before micelles can be separated from human milk, the fat globules, which contain unwanted enzymes, must be removed. A method was developed for this project to maintain the milk sample at 37°C, gently centrifuge the milk and remove the fat. The less dense fat globules rise to the top of the tube forming a discrete fat layer and the skimmed milk can be removed from beneath the fat layer by making a hole in the bottom of the tube.

The milk was slowly agitated in a 37°C water bath until centrifuged. The Beckman SW27 rotor, swinging buckets and centrifuge tubes (polyallomer, 1 x 3.5 in) (Beckman, Palo Alto, CA) were kept in an oven at 40°C. The Beckman ultracentrifuge was prewarmed with the temperature set from 37° to 40°C. The milk sample was divided among the minimum number of centrifuge tubes, weighed in their swinging buckets,
and paired to balance the rotor. They were spun for 10 min at 3.5K (2,200 x g). After the centrifugation, the tubes were carefully removed from the buckets and the bottom of the tube was punctured with a 21 gauge, 1 inch disposable beveled syringe needle attached to a disposable syringe by a luer lock. The needle was inserted perpendicular to the bottom of the tube, which gave the best results for continuous flow. The punctured tube was set on top of a 40 ml Kimax test tube, set in a test-tube rack, to collect the drops of skimmed milk. Collection was stopped when it appeared that the fat layer contents would mix with the milk. The skimmed milk was kept in the 37°C water bath. A protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added to the milk from a stock solution (100 mM PMSF in 100% propanol) to obtain 0.2 mM PMSF in the skimmed milk. The A_{280nm} and A_{320nm} readings of skimmed milk, solubilized in Mono Q-HPLC buffer A, were recorded.

2.4.3. **Micelle Separation by Ultracentrifugation**

The micelles were separated as rapidly as possible, maintaining the milk at 37°C throughout the procedure. The rotor (SW 50.1), the swinging buckets and the centrifuge tubes (polyallomer, 0.5 x 2 in., Beckman) were kept in a 40°C oven in preparation for all the centrifugations at 37°C. Six centrifuge tubes were filled by pipette with 5 ml of 37°C skimmed milk, weighed and paired for their position in the rotor. The first run was centrifuged at 30,000 rpm
(110,000 x g) at 37°C for 2 min after the rotor attained the desired speed. Subsequent centrifugations were for 12, 30, and 60 min. After each, the rotor and the buckets were returned to the oven. The supernatants were pooled, with each tube being drained very carefully so as not to disturb the pellets. If there was a lipid film at the top, it was removed with a Kimwipe (Kimberly-Clark, Rosewell, GA) before draining the supernatant. To remove as much supernatant as possible so as to minimize the amount of whey proteins mixed with the micelles in the pellet, the tubes were supported on their sides with the bottom of the tube slightly elevated, and the inside and the lip of each the tube was dried with a Kimwipe. The supernatant was kept in a 37°C water bath until the next run was prepared. The tubes with the pellets were labeled and covered with parafilm. One less tube than used in the first centrifugation was filled with the 2S (2 min supernatant) for the 12 min run and the excess 2S was covered with parafilm and retained to determine later its corrected A₂₈₀ reading. Centrifuge runs for 12, 30, and 60 min were completed following the above procedure with the decrease of one tube with each run. A flow chart for this procedure is given in Figure 8.

Two sets of the sample were run in parallel by dividing the native milk into two portions, one set of 30 ml (6 tubes of 5 ml) and the other one of 25 ml (5 tubes of 5 ml), which was established to effectively use the limited milk sample.
Figure 8. Flowchart for Separation of Human Micelles by Ultracentrifugation.
Human Milk

Skim
Centrifuge 10 min
2,200 X g. 37 °C

Fat

Skimmed Milk
PMSF

Native 37 °C
Centrifuge 2 min.
110,000 X g. 37 °C

Re-Equilibrated
Overnight 4 °C, 3 h 37 °C

Control
Centrifuge 2 min

Labeled
Centrifuge 2 min

2S
Centrifuge 12 min.
110,000 X g. 37 °C

2P

12S
Centrifuge 30 min.
110,000 X g. 37 °C

12P

30S
Centrifuge 60 min.
110,000 X g. 37 °C

30P

60S

2Sr
Centrifuge 12 min

2Pr

12Sr
Centrifuge 30 min

12Pr

30Sr
Centrifuge 60 min

30Pr

60Sr

2S *

2P *

12S *

12P *

30S *

30P *

60S *

60P *
The number of tubes in a set was determined by three factors: first, the 6 tube capacity of the rotor (the maximum); second, the total amount of milk available for a single experiment, which included the native micelle study, re-equilibrated micelle study and the labeled $\kappa$-casein micelle study; and third, the need to have two pellets, that is, two tubes from each centrifugation (the minimum). It was necessary to start with 5-tubes per study to finish with 2-60 min pellets which were produced from 3-30 min, 4-12 min, and 5-2 min tubes. One pellet was used to determine the contribution of this fraction to the original protein concentration and the amount of the differentially-phosphorylated forms of $\beta$-casein (HPLC-Mono Q separation). The second pellet was frozen and later dissolved to establish its $\kappa$-casein/$\beta$-casein ratio by HPLC Protein-Plus separation.

The micelles were separated in consecutive centrifugations, so that the 2 min run was done for the first set of tubes and then for the second part. Next the two 12 min centrifugations were carried out, followed by 2 spins for 30 min and finally one 60 min run, because there were only a total of 5 tubes remaining in set 1 and set 2.

The contribution of each micelle fraction to the protein in the original skimmed milk solution was determined by reconstituting the pellet to its original volume of 5 ml and by taking the $A_{280\text{nm}}$ and $A_{320\text{nm}}$ readings. The pellet was dissolved by stirring with a glass rod in 2 ml of Tris-urea
HPLC buffer A. The solution and subsequent washes with buffer A were transferred by a pasteur pipette to a 5 ml volumetric flask and filled to volume with the Tris-urea buffer A. The solution in the volumetric flask was stirred with a small magnetic stir bar for at least 15 minutes before a 0.1 ml aliquot was taken and diluted to 5.0 ml with buffer A. The $A_{280\text{nm}}$ reading was taken and corrected for dilution.

2.4.4. Re-equilibration of Micelle Structure After Cooling (Labeled and Control)

In the experiments that involved the addition of labeled $\kappa$-casein, the [$^3$H] $\kappa$-casein (< 1 mg) was dissolved in 200 $\mu$l simulated milk ultrafiltrate (SMUF) obtained from Dr. S.M. Sood. One liter of human SMUF was prepared by combining 0.333 g of MgCl$_2$·6 H$_2$O, 0.334 g citric acid, 0.588 g CaCl$_2$·2H$_2$O, 0.263 g KH$_2$PO$_4$, 0.254 g K$_2$SO$_4$, and 0.3175 g Na$_3$ citrate·2 H$_2$O in less than one liter of distilled water. The solution was stirred until dissolved, adjusted to pH 7.0 with 1 M KOH and water added to make 1 l. For the re-equilibration studies, skimmed milk was stirred overnight in the cold room ($4^\circ C$) to decrease the hydrophobic interaction in the milk micelle. Where appropriate, the labeled $\kappa$-casein was added to the skimmed milk when it was placed in the cold room. In the morning, the milk was transferred from the cold room to an agitating $37^\circ C$ water
bath for a 3 h incubation before beginning the micelle centrifugation procedure as described in 2.4.3.

2.5. Methods of Analysis

2.5.1. Ratio of Individual β-Casein/Total Casein

For each micelle study, the protein composition of the pellets from the fractionated micelles and the final supernatant (60S) was determined by anion exchange HPLC. This column separated β-casein according to the number of phosphates covalently linked to the polypeptide, so that 6 peaks of β-casein were observed. κ-Casein cannot be accurately determined on the Mono Q because it is a minor component and overlaps between β-casein peaks. An aliquot of 4 ml of the buffer A-reconstituted pellet was separated on the HPLC Mono Q and the 1 ml fractions obtained were pooled on the basis of protein peaks, dialyzed against changes of deionized water at 4°C and lyophilized. SDS-PAGE gels were run with the lyophilized proteins and stained with CB, PAS and ESA.

Each HPLC chromatogram was analyzed by the Nelson 3000 Series Chromatography Data System (Nelson Analytical, Cupertino, CA) to obtain retention time, peak area, and area percent. The HPLC data were converted from analog to digital data by the Nelson 760 Series Intelligent Interface (Nelson Analytical). The system was integrated with an IBM PC/XT, an IBM color monitor with a color graphics card and a Canon Color Ink jet printer.
2.5.2. Ratio of $\kappa$-Casein/$\beta$-Casein

The ratio of $\kappa$-casein to $\beta$-casein in the fractionated micelle pellets was determined by reverse phase HPLC separation on a Zorbax Protein Plus column (Material and Methods 2.1.7.). The pellets were dissolved in 1 to 4 ml of freshly prepared solution of deionized 6 M urea with 0.1% TFA. The 6 M urea was deionized with a mixed-bed resin and stored at -20°C in 1.5 ml volumes (BMBiochemica 1989). The amount of urea-TFA solution to be added to the pellet was determined by the observed $A_{280\text{nm}}$ of the reconstituted pellet to give a dilution, that would not overload the column. The Protein Plus column has a maximum load of 200 to 300 $\mu$g and a maximum injection size of 200 $\mu$l, which was the size of the injection loop. Triplicate samples of the pellets were subjected to the HPLC and the data were collected and analyzed by the Nelson Analytical Chromatographic Data System. The $\kappa$-casein and $\beta$-casein peaks were identified by injection of standards and by SDS-PAGE of the isolated pellet peak fractions, which had been freeze-dried by a Speed Vac Concentrator (Savant Instruments Co., Farmingdale, NY).

2.5.3. Protein Analysis

Protein analysis was performed using a micro method involving the Coomassie Blue dye-binding assay (Bio-Rad Laboratories, Richmond, CA). The samples were dissolved in 3 M urea which was also included in the blank. In this micro-assay the lower limit of detection was 1 $\mu$g. This
assay was used because it was able to determine small amounts of protein but the reproducibility with milk caseins was a problem, as reported by Keller and Neville (1986).

2.5.4. **Amino Acid Sequence; PC/Gene**

Structural implications of the amino acid sequences for human κ-casein, bovine κ-casein, human β-casein and bovine β-casein were investigated using the computer analysis program of PC/Gene (IntelliGenetics Inc., Geneva, Switzerland).
3. RESULTS

3.1. K-Casein Isolation and Purification

The separation of k-casein from β-casein was difficult because of the natural aggregation affinity of the caseins, the similarity in their molecular weights and also the much greater abundance of β-casein compared to k-casein as seen in Figure 9. In the separation on the molecular sizing gel column (Sephadex G-200), any procedure that caused the loss of sugar residues from k-casein resulted in a noticeable decrease in the ability to obtain k-casein without a β-casein contamination. Also, any factor that decreased the efficiency of the column, such as a decreased column length, an uneven gel bed, or too rapid elution, dramatically lessened the yield of k-casein free from β-casein. This was evident by the change of the discrete, small k-casein peak to only a shoulder position on the large β-casein peak. A decrease in intact k-casein may also result from the cleavage of the polypeptide into the gMP and para k-casein, which was reported by Beeby and Nitschmann (1963) in bovine k-casein when it was repeatedly acid-precipitated or treated with 6 M urea at pH 4.7 for several hours.

The Sephadex G-200 separation was improved by washing the acid-precipitated casein with cold ethanol and cold
Figure 9. Sephadex G-200 Casein Elution Profile. The tubes of each peak were pooled. Peak 3 $\kappa$-casein and the large peak (6) is $\beta$-casein.
ether which removed much of the lipid material that appeared in the first elution peak and increased the purity of the \(\kappa\)-casein obtained from the Sephadex column.

The composition of proteins in the eluted peaks was checked by SDS-PAGE. \(\kappa\)-Casein stained positive with PAS; Gel A, lane 3, of Figure 10. Several different stains of the SDS-PAGE gels were necessary to give a complete picture of the proteins in the casein separations because the standard protein dye, Coomassie Blue, did not stain the highly glycosylated \(\kappa\)-casein clearly. The PAS stain for glycoprotein was used for a positive \(\kappa\)-casein identification as reported by Yamauchi et al. (1981), but with the established protocol, only the glycoprotein that runs slower than \(\beta\)-casein was stained. The washed gel showed the location of \(\beta\)-casein relative to \(\kappa\)-casein. The glycoprotein bands were often enhanced by the water washes, which was also an advantage because the PAS stain is less sensitive than CB. To obtain a positive PAS result, the gels require more protein applied per well which leads to overloading of the CB bands.

In 1986, Green reported the identification of human \(\kappa\)-casein with ESA on 5 to 15 % gradient SDS-PAGE (SDS-PAGGE) gels. This appeared to be an ideal indicator to differentiate the caseins because phosphoproteins (\(\beta\)-casein) stained blue, sialic acid modified proteins (\(\kappa\)-casein) stained blue-green, and non-modified proteins stained red. However, it should be noted that \(\beta\)-casein also occurs in a
Figure 10. Photograph of SDS-PAGE Gels of Selected Fractions from Figure 9. The numbers above the gel lanes correspond to the designated peaks in Figure 9. (A) Stained with PAS; (B) Gel A washed with water; (C) Duplicate gel stained with ESA; (D) Gel C destained and stained with CB.
non-phosphorylated form and may not be properly identified. The bands indicated by ESA to be glycoproteins (blue-green) moved faster than β-casein (blue) and the PAS-positive glycoprotein behind β-casein was not seen. This stain was also applied to acid-precipitated casein on a 10-20 % SDS-PAGE gel by Kunz and Lonnerdal (1988, 1990) and the PAS-positive band was not observed. This indicates that the staining properties of the PAS-positive κ-casein and the ESA-identified κ-casein are based on different structures or modifications which are not present in both systems, and therefore are probably different proteins. This same result was also observed (Dev et al., 1988) when two identical SDS-PAGE gels containing human acid-precipitated casein were examined. For this reason, the SDS-PAGE gels in this study were stained by all three methods: CB, PAS, and ESA.

A further purification step was necessary to remove the high molecular weight contaminants evident in lane 3 in the SDS-PAGE of the κ-casein separated on the Sephadex G-200 column (Figure 10). An anion exchange HPLC separation in 6 M urea was developed for human casein for this project (Slattery et al., 1989) based on the successful separation of human β-casein on DEAE-cellulose, an anion exchange resin (Groves and Gordon, 1970), and the Mono Q HPLC separation of bovine caseins (Andrews et al., 1985). However, the SDS used in the initial G-200 buffer needed to be removed since it would bind to the HPLC anion exchange column. It was
removed by an Extracti-Gel D detergent exchange column in the presence of 6 M urea. The eluted protein was then applied to the anion exchange column. Due to the heterogeneity of its sugars, \( \kappa \)-casein was eluted as a broad peak shaped by the HPLC gradient. The eluted protein fractions were pooled, dialyzed, lyophilized, and examined for purity on SDS-PAGE (Figure 11). Only the fraction in lane 3 representing Figure 11 was used as purified \( \kappa \)-casein.

3.2. \( \kappa \)-Casein Characterization

The results of the amino acid analysis of human \( \kappa \)-casein are shown in Table 6. The molar amount of each amino acid was based on its relative amount compared to proline which was determined by amino acid sequencing to be 28 moles per mole of \( \kappa \)-casein (Brignon et al., 1985). Tryptophan was not separately established nor was cysteine.

If the results for the individual amino acids of the protein samples of the two donor mothers are rounded to the nearest whole number and compared, they show virtually the same composition. Also included in Table 6 is the amino acid composition from the original sequencing of \( \kappa \)-casein reported by Brignon et al. (1985) and the values from the first isolation of \( \kappa \)-casein (Yamauchi et al., 1981), recalculated by Brignon et al. (1985). The reported values are very similar, with the exception of isoleucine and valine. Isoleucine was lower in the Yamauchi study, as it was in this study. Valine eluted in a region of changing buffer
Figure 11. Photograph of SDS-PAGE Gels from Mono Q-HPLC Separation of K-Casein Partially Purified on Sephadex G-200. The ten gel slots, from left to right, contain the following pooled 1 min fractions: (1) 3-6, (2) 11-14, (3) 18-31, (4) 39-42, (5) 44-50, (6) 51-58, (7) 62, (8) 64, (9) 65-66, (10) empty. (A) PAS stain; (B) ESA stain; (C) Gel A washed with water; (D) Gel B destained and stained with CB.
### TABLE 6
Amino Acid Composition of Human K-Casein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Integer from Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino Acid Analysis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Recalculated first Amino Acid Analysis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sample Mother 1</th>
<th>Sample Mother 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic Acid</td>
<td>12</td>
<td>12.0</td>
<td>12.8</td>
<td>13.0 ± .6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12.6 ± .1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Threonine</td>
<td>18</td>
<td>18.0</td>
<td>17.8</td>
<td>16.6 ± .1</td>
<td>17.5 ± .3</td>
</tr>
<tr>
<td>Serine</td>
<td>7</td>
<td>8.0</td>
<td>7.5</td>
<td>7.2 ± .2</td>
<td>6.8 ± .1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15</td>
<td>15.4</td>
<td>15.2</td>
<td>17.3 ± .3</td>
<td>16.8 ± .2</td>
</tr>
<tr>
<td>Proline</td>
<td>28</td>
<td>28.1</td>
<td>28.7</td>
<td>28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glycine</td>
<td>1</td>
<td>1.9</td>
<td>2.6</td>
<td>1.7 ± .1</td>
<td>1.10 ± .03</td>
</tr>
<tr>
<td>Alanine</td>
<td>14</td>
<td>13.5</td>
<td>13.8</td>
<td>13.9 ± .5</td>
<td>13.9 ± .1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>13</td>
<td>11.1</td>
<td>12.4</td>
<td>5.5 ± .5</td>
<td>5.9 ± .4</td>
</tr>
<tr>
<td>Methionine</td>
<td>1</td>
<td>0.4</td>
<td>1.3</td>
<td>1.20 ± .04</td>
<td>0.80 ± .09</td>
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<tr>
<td>Isoleucine</td>
<td>13</td>
<td>12.0</td>
<td>10.6</td>
<td>8.5 ± .3</td>
<td>9.1 ± .8</td>
</tr>
<tr>
<td>Leucine</td>
<td>3</td>
<td>3.5</td>
<td>4.3</td>
<td>3.9 ± .5</td>
<td>3.8 ± .3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>12</td>
<td>11.3</td>
<td>10.3</td>
<td>8.7 ± 1.4</td>
<td>8.7 ± .8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3</td>
<td>3.1</td>
<td>3.1</td>
<td>3.6 ± .7</td>
<td>3.7 ± .4</td>
</tr>
<tr>
<td>Lysine</td>
<td>4</td>
<td>4.0</td>
<td>3.0</td>
<td>5.5 ± .4</td>
<td>4.6 ± .3</td>
</tr>
<tr>
<td>Histidine</td>
<td>5</td>
<td>5.0</td>
<td>4.9</td>
<td>5.0 ± .1</td>
<td>4.9 ± .1</td>
</tr>
<tr>
<td>Arginine</td>
<td>8</td>
<td>7.7</td>
<td>7.7</td>
<td>7.8 ± .2</td>
<td>7.5 ± .3</td>
</tr>
</tbody>
</table>

**Notes:**

- <sup>a</sup>Brignon et al. (1985)
- <sup>b</sup>Yamauchi et al. (1981) recalculated from 150 residues to 158 by Brignon et al. (1985)
- <sup>c</sup>basis for mole ratio 28 prolines/mole K-casein
- <sup>d</sup>values given are the mean and standard deviation of 3 measurements
and may have been inaccurately determined due to a change in baseline.

Peaks for amino sugars were evident in the amino acid analysis chromatograms but the results were not quantitative unless the hydrolysis was carried out under mild conditions. Therefore in a separate experiment, samples from two different donors were hydrolyzed for 4 h in 4 M HCl and separated from the unhydrolyzed peptides on a Dowex 2 resin prior to their amino acid analysis. The glucosamine to galactosamine ratio for sample 1 was 2.4 and for sample 2 was 2.1 which was later confirmed by the more quantitative gas chromatographic analysis.

The value of the extinction coefficient,
\[ E_{1\text{ mg/100 ml}1 \text{ cm}, 280nm} \], so important for the ability to quantitate \( \kappa \)-casein throughout this project, was determined to be 11.21 using the established polypeptide molecular weight \((17,707)\) reported by Brignon et al. (1985). It was close in value to the number \((E_{1\text{ mg/100 ml}1 \text{ cm}, 280nm} = 11.26)\) determined theoretically from the number of tyrosines and tryptophans (Cantor and Schimmel, 1980).

The sugar content of two different samples, isolated separately from two individual donors, was analyzed by gas chromatography with the results shown in Table 7. Not only do the two samples from different mothers have very similar total carbohydrate content but the individual sugars are present in virtually the same amounts. Although Brignon et
<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>This Study</th>
<th>Yamauchi et al. (1981)(^a)</th>
<th>Brignon et al. (1985)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample I</td>
<td>Sample II</td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
<td>5.26</td>
<td>4.78</td>
<td>4.6</td>
</tr>
<tr>
<td>Galactose</td>
<td>17.15</td>
<td>19.22</td>
<td>21.4</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>17.83</td>
<td>18.51</td>
<td>14.6</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>8.40</td>
<td>9.29</td>
<td>6.6</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>6.89</td>
<td>7.82</td>
<td>6.9</td>
</tr>
<tr>
<td>Total</td>
<td>55.5</td>
<td>59.6</td>
<td>54.1</td>
</tr>
</tbody>
</table>

\(^a\)Recalculated on the basis of 55.0% total carbohydrate.

n.d. - Not determined.
al. (1985) questioned the initial reported values of Yamauchi et al. (1981), these results are also close to the distribution they found. Among the sugars detected, N-acetylgalatosamine is the only sugar that bonds to the peptide chain (Figure 3). It has a unique position in the sugar chain and is indicative of the number of sugar chains attached to the protein. The presence of fucose and N-acetylgalactosamine in human milk is noteworthy since they are absent in bovine milk.

Preliminary determinations of sialic acid content were done by the method of Aminoff (1961). This method was recommended by Beeley (1985) for glycoprotein containing fucose, instead of the procedure of Warren (1959) which is commonly used. By this colorimetric method, only 3.7% sialic acid was detected whereas an average of 7.35% was determined by gas chromatographic analysis.

One of the important characteristics of $\kappa$-casein is its ability to stabilize $\beta$-casein from Ca$^{++}$ ion precipitation. The purified $\kappa$-casein stabilized human 4-P $\beta$-casein in the presence of the physiological concentration of 10 mM Ca$^{++}$ ion (Figure 12). Stabilization increased with the molar ratio of $\kappa/\beta$ and was greater than 95% when the mole ratio exceeded 0.15.

Since the caseins are defined as phosphoproteins it was of interest to determine if phosphorous was present in the $\kappa$-casein. The presence of less than 1 mole phosphate per
Figure 12. $K$-Casein Stabilization of 4-P $\beta$-Casein Against Precipitation by $Ca^{++}$ Ion.
mole of κ-casein was detected on limited amounts of purified material.

3.3. κ-Casein Tritium Labeling

The Van Lenten and Ashwell (1971) method of tritium labeling of sialic acids of glycoproteins worked well for κ-casein. The labeling scheme is given in Figure 7. The labeled product obtained was purified by HPLC on a Mono Q column. The location of the labeled protein fractions was determined by scintillation counting (Figure 13A) and the protein peaks were detected at 280 nm (Figure 13B). These data were compared with the SDS-PAGE gels of this separation and their autoradiograph to determine which protein fractions would be used in ³H-κ-casein experiments. The autoradiograph of the SDS-PAGE gel indicated labeled high molecular weight protein bands in the fractions eluting later than the κ-casein peak (Figure 14: lanes 8 and 9). The peak which eluted before the major κ-casein peak had more radioactive label incorporated than the κ-casein peak and was not included in the tritiated sample to be used later in re-equilibration studies. The pooled protein of fractions 11-29 (Figure 14: lanes 5 and 6) was used.

3.4. Size-Fractionated Micelle Preparations

In this project it was of prime interest to examine size-fractionated micelles in human milk under conditions as close as possible to the native system. In the experiments
Figure 13. Mono Q HPLC Separation of $^3$H-Labeled $\kappa$-Casein. (A) NaCl gradient (—) and protein $A_{280}$ (•) elution patterns; (B) DPM of radioactive $\kappa$-casein superimposed upon the protein $A_{280}$ (•) pattern.
Figure 14. Photograph of Autoradiograph and SDS-PAGE of Non-radioactive and $^3$H Labeled $\kappa$-Casein Separated by Mono Q-HPLC (Figure 13). The ten lane slots of gels A and C, from left to right, contain the following non-labeled fractions: (1) 15-25, (2) 27-32, (3) 33-39, (4) Empty; and the following labeled fractions: (5) 11-15, (6) 17-19, (7) Empty, (8) 31-33, (9) 35-39, (10) Empty. (A) PAS stained; (B) Autoradiograph of Gel C (lanes aligned with gels); (C) CB stained.
with human milk presented here, differential high speed ultracentrifugation was used because it is least disruptive to the equilibrium system of the micelles and their liquid environment (Dalgleish et al., 1989).

Preliminary centrifugation studies on native milk were done in triplicate to establish whether there was reproducibility of micelle size in the pellets separated from a single sample of milk. The pellet distribution was also investigated to evaluate the effect of re-equilibration on the micelles. The results are shown in Table 8. Considering the many variables in the system, these results were very similar for the native system, the re-equilibrated control system and the radioactive re-equilibrated system.

When the native skimmed milk and the tritium re-equilibrated skimmed milk were analyzed for size distribution by laser light scattering, they exhibited similar size distributions (Dr. S. M. Sood, personal communication). Results with the native milk micelles fitted a curve consistent with radii of 22.1 nm (72.6%), 85.9 nm (25.3%), and 336.3 nm (2.0%) with an average radius of 44.50 nm. The tritium labeled re-equilibrated skimmed milk micelles showed a distribution of radii of 19.3 nm (72.5%), 78.8 nm (24.0%), and 440.3 nm (3.6%) giving an average radius of 48.75 nm.

It is difficult to assess the reproducibility of the size distribution among different milk samples because of the natural variations that exist in the different samples.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Donation 6</th>
<th>Donation 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>$4^\circ C - 37^\circ C$ (3 h)</td>
</tr>
<tr>
<td>Pellet (2 min)</td>
<td>$0.563 \pm 0.056^a$</td>
<td>$0.530 \pm 0.018^b$</td>
</tr>
<tr>
<td>Pellet (12 min)</td>
<td>$0.808 \pm 0.062$</td>
<td>$0.772 \pm 0.028$</td>
</tr>
<tr>
<td>Pellet (30 min)</td>
<td>$0.352 \pm 0.031$</td>
<td>$0.358 \pm 0.015$</td>
</tr>
<tr>
<td>Pellet (60 min)</td>
<td>$0.198 \pm 0.017$</td>
<td>$0.180 \pm 0.041$</td>
</tr>
<tr>
<td>Sup. (60 min)</td>
<td>$6.92 \pm 0.38$</td>
<td>$6.66 \pm 0.103$</td>
</tr>
<tr>
<td>Total Pellets</td>
<td>1.92</td>
<td>1.84</td>
</tr>
<tr>
<td>Pellets + Sup.</td>
<td>8.84</td>
<td>8.50</td>
</tr>
<tr>
<td>Skimmed Milk</td>
<td>$8.10 \pm 0.20$</td>
<td>$7.73 \pm 0.27$</td>
</tr>
</tbody>
</table>

$^a$ mean and standard deviation of 3 samples

$^b$ mean and standard deviation of 2 samples
even from the same mother. Human milk composition does change appreciably during the lactation period (Harzer and Bindels, 1989). The milk samples (donations 8 and 9) used in the experiments reported in Tables 9 and 10 were from the same mother but the samples were obtained two weeks apart. However, if the percentages of the pellet protein sedimented at each centrifugation are examined for native, re-equilibrated, and labeled re-equilibrated samples (Table 10), it can be seen that the re-equilibrated samples show a similar size-distribution as the native system. Also it is noteworthy that there is not a noticeable difference between the control re-equilibrated set of pellets and those with added radioactive $\kappa$-casein. This suggests that the labeling of $\kappa$-casein did not adversely effect its interactions in human milk micelles and that the amount of labeled $\kappa$-casein did not effect the equilibrium.

3.5. Analysis of Pellets

The redissolved pellet components were analyzed by the two different HPLC methods. The separation on the anion exchange column (Mono Q) yielded data pertaining to the six individual forms of $\beta$-casein whereas the procedure with the reverse phase column separated the $\kappa$-casein and $\beta$-casein.

3.5.1. $^3$H-$\kappa$-Casein/Total Pellet Protein

The six forms of $\beta$-casein were separated into discrete peaks by the Mono Q-HPLC column. The peak positions were
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Donation 8</th>
<th>Donation 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>4 °C-37 °C</td>
</tr>
<tr>
<td>Skimmed Milk</td>
<td>11.75 a</td>
<td>11.20 a</td>
</tr>
<tr>
<td>Pellet (2 min)</td>
<td>0.82</td>
<td>0.85</td>
</tr>
<tr>
<td>Pellet (12 min)</td>
<td>1.28</td>
<td>1.02</td>
</tr>
<tr>
<td>Pellet (30 min)</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>Pellet (60 min)</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>Total Pellets</td>
<td>2.99</td>
<td>2.72</td>
</tr>
<tr>
<td>Sup. (60 min)</td>
<td>9.75</td>
<td>9.00</td>
</tr>
<tr>
<td>Pellets + Sup.</td>
<td>12.74</td>
<td>11.75</td>
</tr>
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</table>

aValues are the average of two determinations
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Donation 8</th>
<th>Donation 9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>4°C-37°C</td>
</tr>
<tr>
<td>Pellet (2 min)</td>
<td>27.4a</td>
<td>31.3</td>
</tr>
<tr>
<td>Pellet (12 min)</td>
<td>42.8</td>
<td>37.5</td>
</tr>
<tr>
<td>Pellet (30 min)</td>
<td>21.7</td>
<td>22.4</td>
</tr>
<tr>
<td>Pellet (60 min)</td>
<td>8.0</td>
<td>8.8</td>
</tr>
<tr>
<td>Total</td>
<td>99.9</td>
<td>100</td>
</tr>
</tbody>
</table>

*aValues are the average of two determinations*
verified by injecting purified samples of the individual
forms. A Mono Q micelle pellet separation and gradient pro-
file is given in Figure 15. However, \(\kappa\)-casein elutes as a
broad peak as shown in Figure 13. In the normal pellet pro-
tein distribution (Figure 15), the \(\kappa\)-casein peak is small
and overlaps several of the \(\beta\)-casein peaks as can be seen in
SDS-PAGE gels of Mono Q column fractions (Figure 16). How-
ever, the position of \(\kappa\)-casein was clearly established in
the Mono Q separation of the pellet components after re-
equilibration with \(^3\text{H}\)-\(\kappa\)-casein (Figure 17).

Tritium labeled \(\kappa\)-casein was re-equilibrated in three
different samples of skimmed milk. The maximum label sepa-
rated in the same Mono Q HPLC fraction for each of the pel-
lets (Figure 17). There was no difference in the maximum
peak fraction for the reverse phase HPLC elution either.
Since the sialic acids are the site of incorporation for
tritium and the sugar moiety exhibits heterogeneity, there
might be discernible differences in the \(^3\text{H}\)-sugars of size-
fractionated pellets. The fact that some of the peaks of
Figure 17 are skewed differently may be related to that.
The smaller sample size in the reverse phase separation made
any difference less observable.

It would have been useful to determine the specific
activity for the individual \(\kappa\)-casein fractions. However,
the small amounts of \(\kappa\)-casein protein separated by the
reverse phase HPLC were not measurable with accuracy.
Figure 15. Mono Q-HPLC Micelle Pellet Separation Showing the NaCl Gradient and A$_{280}$ Elution Pattern.
Figure 16. Photograph of SDS-Page Gels of Mono Q-HPLC Fractions from a Micelle Pellet and its 60 min Supernatant. Gels A and B are the micelle pellet. The ten gel slots, from left to right, contain the following pooled 2 min fractions. (1) 3-9, (2) 11-12, (3) 13-14, (4) 15, (5) 16-17, (6) 18-19, (7) 20-21, (8) 22-23, (9) 24-26, (10) 27. Gels C and D are the supernatant. The ten gel slots, from left to right, contain the following pooled 2 min fractions. (1) 1-9, (2) 11-12, (3) 13, (4) 14-16, (5) 17-18, (6) 20, (7) 21-24, (8) 25-26, (9) 28-30, (10) 31-34. (A) CB stained, (B) washed PAS stain; (c) CB stained; (D) washed PAS stain.
Figure 17. Separation of $^3$H-K-Casein by Mono Q-HPLC: Elution Pattern of Radioactivity.
The DPM data for each of the labeled micelle separations is tabulated in Table 11. Also, since the spin duration of 2 min, 12 min, 30 min and 60 min sedimented micelles have average radii of 209 nm, 85.3 nm, 54.0 nm, and 38.2 nm respectively (Dr. S. M. Sood, personal communication), the value for the micelle surface area/volume may be determined from the following:

\[
\text{Area/Volume} = \frac{4\pi r^2}{(4/3)\pi r^3} = \frac{3}{r}
\]

In a plot of the normalized DPM against the micelle surface area per volume, the first three data points for the three different micelle separations follow a good linear relationship with the 60-min pellets deviating somewhat from this straight line (Figure 18). It is important to note that these 60-min pellets were the best preparation of those analyzed because they were resuspended and aliquots were counted on the same day as the micelles were separated.

3.5.2. Percentage of Individual \( \beta \)-Casein in the Pellets

A set of the chromatograms from native micelle pellets and supernatant is given in Figure 19. These patterns indicate the relative amount of the different proteins in micelles of decreasing size. The elution position of the individual \( \beta \)-casein peaks is identified in Figure 15 with 2-P and 4-P being the most abundant. The first two peaks eluted include whey proteins, which are loosely bound or not at all retained on the anion exchange column. A comparison of the pellet chromatograms of Figure 19A - 19D with that of
<table>
<thead>
<tr>
<th>Pellet</th>
<th>DPM</th>
<th>A$_{280}$</th>
<th>DPM/A$_{280}$</th>
<th>Normalized</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-2min</td>
<td>15870</td>
<td>0.52</td>
<td>30519</td>
<td>1.00</td>
</tr>
<tr>
<td>4-12min</td>
<td>62020</td>
<td>1.24</td>
<td>50016</td>
<td>1.64</td>
</tr>
<tr>
<td>4-30min</td>
<td>81700</td>
<td>1.16</td>
<td>70431</td>
<td>2.31</td>
</tr>
<tr>
<td>4-60min</td>
<td>52780</td>
<td>0.68</td>
<td>77618</td>
<td>2.54</td>
</tr>
<tr>
<td>8-2min</td>
<td>25640</td>
<td>0.84</td>
<td>30524</td>
<td>1.00</td>
</tr>
<tr>
<td>8-12min</td>
<td>52490</td>
<td>1.22</td>
<td>43025</td>
<td>1.41</td>
</tr>
<tr>
<td>8-30min</td>
<td>46900</td>
<td>0.68</td>
<td>68971</td>
<td>2.26</td>
</tr>
<tr>
<td>8-60min</td>
<td>29100</td>
<td>0.31</td>
<td>93871</td>
<td>3.08</td>
</tr>
<tr>
<td>9-2min</td>
<td>77231</td>
<td>0.79</td>
<td>97761</td>
<td>1.00</td>
</tr>
<tr>
<td>9-12min</td>
<td>151130</td>
<td>1.10</td>
<td>137391</td>
<td>1.41</td>
</tr>
<tr>
<td>9-30min</td>
<td>108730</td>
<td>0.49</td>
<td>221898</td>
<td>2.27</td>
</tr>
<tr>
<td>9-60min</td>
<td>74180</td>
<td>0.18</td>
<td>412111</td>
<td>4.22</td>
</tr>
</tbody>
</table>
Figure 18. Plot of DPM of $^3$H-K-casein in 2 min, 12 min, 30 min, and 60 min micelle pellets versus micelle surface area/volume.
Figure 19. Elution Profiles for a Complete Set of Mono Q-HPLC Separated Micelle Pellets and Supernatant from Donation 9. (A) 2 min, (B) 12 min, (C) 30 min, (D) 60 min pellets and (E) 60 min supernatant.
the supernatant (Figure 19E) shows that one of the whey proteins (α-lactalbumin, identified by SDS-PAGE) overlaps the 2-P β-casein peak. This causes difficulty in quantifying 2-P β-casein. The whey protein that eluted after 40 min is a higher molecular weight glycoprotein than κ-casein, possibly the glyco-phosphoprotein reported by Azuma and Yamauchi (1987).

Data from the Mono Q separation of the last two micelle pellet preparations, which were from the same milk donor, were analyzed for the percentage of the individual forms of β-casein. The data from the three different conditions of separation were averaged since they were very similar (Table 12). Since it was possible to separate the human milk into fractions according to size, the following correlations may be made:

1. In both separations the amount of 0-P in the 2 min pellet was larger in the native sample than in the two re-equilibrated samples. The amount of 0-P was greater in all the pellets from the native milk in the second separation. T-tests on these plot points support the validity of this analytical relationship.

2. The amount of 1-P increased as the length of spin increased or micelle radius decreased for both the milk samples. T-tests on these plot points support the validity of this analytical relationship.
Table 12

Percent Composition of Pellets of Individual β-Caseins from Donations 8 and 9 Separated by Mono Q-HPLC

<table>
<thead>
<tr>
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<th></th>
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<tr>
<td>2-min p</td>
<td>21.1</td>
<td>24.5</td>
<td>12.0</td>
<td>14.6</td>
<td>32.3</td>
<td>30.9</td>
<td>12.2</td>
<td>11.3</td>
<td>18.5</td>
<td>15.2</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>2-min p'</td>
<td>15.6</td>
<td>18.9</td>
<td>12.2</td>
<td>15.6</td>
<td>36.0</td>
<td>30.4</td>
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<td>4.6</td>
</tr>
<tr>
<td>2-min p*</td>
<td>15.8</td>
<td>19.4</td>
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<td>14.3</td>
<td>34.9</td>
<td>33.1</td>
<td>12.6</td>
<td>11.4</td>
<td>20.0</td>
<td>17.9</td>
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<td>4.0</td>
</tr>
<tr>
<td>mean 2-p</td>
<td>17.5</td>
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<td>19.5</td>
<td>17.2</td>
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<td>2.5</td>
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<td>0.6</td>
<td>1.6</td>
<td>1.2</td>
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<td>15.6</td>
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<td>18.6</td>
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<td>13.9</td>
<td>34.2</td>
<td>34.5</td>
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<td>19.4</td>
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<td>12.7</td>
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<td>18.3</td>
<td>6.3</td>
<td>4.4</td>
</tr>
<tr>
<td>mean 12-p</td>
<td>11.7</td>
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<td>33.7</td>
<td>32.4</td>
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<td>0.8</td>
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<td>16.0</td>
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<tr>
<td>30-min p'</td>
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<td>32.3</td>
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<td>11.8</td>
<td>23.1</td>
<td>20.7</td>
<td>5.8</td>
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<tr>
<td>mean 30-p</td>
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<td>15.3</td>
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<td>31.6</td>
<td>30.5</td>
<td>12.2</td>
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<td>21.0</td>
<td>6.1</td>
<td>5.6</td>
</tr>
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<td>0.8</td>
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<td>0.8</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
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<tr>
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<td>20.2</td>
<td>17.6</td>
<td>26.6</td>
<td>28.5</td>
<td>22.3</td>
<td>11.0</td>
<td>9.7</td>
<td>21.2</td>
<td>15.4</td>
<td>7.8</td>
<td>5.8</td>
</tr>
<tr>
<td>60-min p'</td>
<td>13.0</td>
<td>15.6</td>
<td>17.0</td>
<td>18.0</td>
<td>34.0</td>
<td>32.2</td>
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<td>60-min p*</td>
<td>11.7</td>
<td>17.4</td>
<td>16.3</td>
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<td>35.0</td>
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<td>9.1</td>
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<td>16.5</td>
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<tr>
<td>mean 60-p</td>
<td>12.9</td>
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<td>17.0</td>
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<td>32.5</td>
<td>28.5</td>
<td>11.3</td>
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<td>19.8</td>
<td>16.8</td>
<td>7.0</td>
<td>5.4</td>
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<td>4.4</td>
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<td>1.0</td>
<td>1.3</td>
<td>0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>
3. The data for 2-P β-casein was distorted by the presence of α-lactalbumin in the peak. It would be possible to determine the amount of α-lactalbumin per pellet from the amount present in the separation of the pellets by the reverse phase HPLC. Without this correction the amount of 2-P appeared to decrease while the relative amount of whey proteins increased with the longer spins and the increased removal of micelle casein.

4. There is no evident change in 3-P except on the 60 minute pellet which showed a decrease in 3-P content.

5. The amount of 4-P increased during the first three separations but decreased in the final pellet.

6. The 5-P form of β-casein was present in the smallest amounts. It also showed a consistent increase in amount as the time of spin increased.

3.5.3. Ratio of κ-Casein/β-Casein

Applications of purified human κ-casein and human β-caseins samples to the Zorbax Protein Plus reverse phase HPLC column showed κ-casein eluting before β-casein, which separated into several peaks. When solutions of the micelle pellets were analyzed, very complex chromatograms resulted. Many more peaks were evident than anticipated from the number of peaks and their area in the anion exchange HPLC. However, when a solution was analyzed from a pellet that had been frozen rather than refrigerated after the ultracentrifugation, it was apparent that the multiple peaks were
from degradation products. The \( \beta \)-casein peaks were larger in the frozen pellets and many of the other peaks disappeared.

The data included in this section are only for frozen pellets with the exception of the 60-min pellets. The 60p samples were produced in the smallest number in the fractionation procedure i.e. only three 60-min pellets were produced from a complete set of six 2-min pellets and one was needed to determine the protein content of the reconstituted pellet. The 60-min pellets, separated on the reverse phase after refrigeration but without freezing, did not show the decomposition products seen in the other non-frozen pellets which were spun for shorter times. This effect may indicate that the pellets were degraded by bacteria which were sedimented with the larger particles so that there was less contamination in the 60-min pellets.

The pattern of protein elution from the reverse phase column can be seen in the series of chromatograms of the tritium labeled re-equilibrated pellets (Figure 20). Both purified \( \kappa \)-casein and radioactive \( \kappa \)-casein eluted in the first small wide double peak at 20 minutes \( (k_1) \). \( \beta \)-Casein eluted in the two major peaks at the end of the separation: the first at 45 min \( (\beta_1) \) and the second as a split peak at 55 min \( (\beta_2) \). The other major peak, that eluted prior to the \( \beta \)-casein peaks, is \( \alpha \)-lactalbumin. SDS-PAGE gels of the human milk standards (gels A and B) and the pellet fractions
Figure 20. Reverse Phase HPLC Chromatograms of Tritium Labeled Pellets. (A) 2 min labeled pellet including acetonitrile gradient, (B) 12 min, (C) 30 min. Asterisk indicates $^3$H-$\kappa$-casein peak.
from the reverse phase column (gels C and D) are given in Figure 21.

Figures 20, 22 and 23 exhibit a similarity in elution patterns for the three different skimmed milk preparations: native (p), re-equilibrated (p'), and re-equilibrated tritium labeled (p*). Figure 22 contains the chromatograms from the 2-min pellets under the three experimental conditions. The tracings are very similar except for the split peak in the 2p* final β-casein peak. This shoulder peak was detected in samples of purified 5-P β-casein and therefore it has been included in the calculation of total β-casein area.

Figures 20 and 22 also showed that there was a similar change in both of the re-equilibrated pellets (p' and p*) as a function of centrifugation time. It appears that the amount of κ-casein increased with the length of spin and therefore increased as the micelle size decreased.

The SDS-PAGE gels of the pooled reverse phase HPLC peak fractions stained positively for PAS for the k_2 peak in addition to the established κ-casein peak (k_1). In labeled experiments, this second peak did not contain radioactive material, nor did the purified κ-casein elute in this peak. The k_2 peak was present in acid precipitated casein separated by reverse phase HPLC, which indicates that it should be present in the Sephadex G-200 separated proteins.
Figure 21. Photograph of SDS-PAGE of Standards and 12 Min Pellet Fractions Separated by Zorbax Protein Plus-HPLC. Gel A and B contain standards. The slots of gels A and B, from left to right, contain the following: (1) empty, (2) purified K-casein, (3) Lactoferrin, (4) Lysozyme, (5) IgA, (6) IgG, (7) Lactalbumin, (8) Serum Albumin, (9) purified β-casein (4p), (9) molecular weight STDs. The slots of gels C and D, left to right, contain the following 1 min fractions from the 12 min pellet. (1) 22-23, (2) 30, (3) 32-33, (4) 38, (5) 42, (6) 44, (7) 45, (8) 53, (9) 55, (10) Empty. (A) CB stained standards; (B) PAS stained standards; (C) CB stained HPLC pellet fractions; (D) PAS stained HPLC pellet fractions. Slot A contains the following proteins: Cytochrome c (12,400 da), Lactoglobulin (18,400 da), Carbonic Anhydrase (29,000 da), Lactate Dehydrogenase (36,000 da), Ovalbumin (43,000 da), Glutamate Dehydrogenase (55,000 da), Phosphorylase B (95,500 da).
Figure 22. Reverse Phase HPLC Chromatograms of 2 min Pellets of the Three Micelle Preparations. (A) Native, (B) Re-equilibrated and (C) Labeled Re-equilibrated.
132

A

2p

0.02

0.01

0.00

0.00

0.02

0.01

0.00

0.00

0.02

0.01

0.00

0.00

0 10 20 30 40 50 60

Time (Min)

B

2p'

C

2p*

A_{280}
Figure 23. Reverse Phase HPLC Chromatograms of Re-equilibrated Pellets. (A) 2 min, (B) 12 min and (C) 30 min.
To verify the $\kappa$-casein position of $\kappa_2$, the glycoprotein stain was used on a SDS-PAGE gel of the 28-min fraction (Figure 21, Gel D, lane 2). A pink band in the same location as $\kappa$-casein provided the visible verification. Another set of 2p* and 30p* casein fractions with lactoferrin and purified $\kappa$-casein as glycoprotein standards was separated by SDS-PAGE electrophoresis (Figure 21). In this case only faint PAS-positive bands were recorded for $\kappa$-casein fraction $\kappa_1$ and for $\kappa_2$ in the 30p* (Figure 24, Gel B, lanes 6 and 7). The reverse phase HPLC column has a much lower loading capacity (250 $\mu$g in a maximum of 200 $\mu$l) than the Mono Q; therefore, it was possible to inject the entire reconstituted pellet (5 ml) at one time on the Mono Q. The small amounts of protein separated by the reverse phase made it difficult to detect the eluted proteins by SDS-PAGE other than the proportionately larger $\beta$-casein fraction.

These PAS-positive bands have made it necessary to consider including this second peak ($\kappa_2$) with the area assigned to $\kappa$-casein even though it did not show up as such in the $^3$H-$\kappa$-casein separation. It is possible that this fraction did not have sialic acid residues and thus it was not radioactively labeled or another possibility is that the acid-labile sialic acid was cleaved in the separation procedures. The cleavage of sialic acid residues would make the glycoprotein more hydrophobic so that it would be bound
Figure 24. Photograph of Duplicate SDS-PAGE Gels of Fractions from Zorbax Protein Plus-HPLC Separation of 2p and 30p Pellets. The ten slots of the gels, from left to right, contain from the 2 min pellet: (1) $\kappa_1$, (2) $\kappa_2$, (3) $\beta_1$, (4) $\beta_2$, (5) Lactoferrin, (6) purified $\kappa$-Casein, and from the 30 min pellet: (7) $\kappa_1$, (8) $\kappa_2$, (9) $\beta_1$, (10) $\beta_2$. (A) CB stained; (B) PAS stained.
tighter to the reverse phase column. The inclusion of the material in the second peak as \( \kappa \)-casein appears reasonable considering that this study did establish that \( \beta \)-casein also eluted in two peaks.

The primary purpose of reverse phase analysis of the micelles was to quantitate the \( \kappa \)-casein and \( \beta \)-casein components. Ordinarily this would be a straight-forward process of determining the peak areas and converting these to mass units of protein. The conversions are based on determining the areas produced by known quantities of purified proteins. The complication in this case arises from the fact that although the areas under the peaks increased linearly with increased protein concentration, the correlation was different for the 1-P, 5-P, and \( \kappa \)-caseins.

The explanation for the differences in correlation factors is based on the observation in this study that each protein fraction was eluted from the reverse phase column with a different concentration of acetonitrile. When the absorbance of these proteins was scanned with a U.V. spectrophotometer, an appreciable increase in absorbance at 280 nm and a shift in maximum wavelength was observed in acetonitrile solutions. This may be another example of conformational changes of \( \beta \)-casein in a hydrophobic environment, described by Slattery et al. (1989).

The response factor was calculated to be 4.2 ± 0.3 for 1-P, 2.8 ± 0.3 for 5-P and 5.7 for \( \kappa \)-casein. These factors
were used to correct the individual peak areas and to calculate the moles of \( \kappa \)-casein/moles \( \beta \)-casein. The same response factor for \( \kappa \)-casein was used for both the peaks (\( \kappa_1 \) and \( \kappa_2 \)) since no purified protein was available to determine the response of the second peak. However, this peak occurs at a plateau in the gradient concentration and there would be no change in eluant hydrophobicity.

The integrated peak areas obtained for \( \kappa \)-casein and \( \beta \)-casein in the pellet separations were multiplied by the response factors determined above for the \( \kappa \)-casein and \( \beta \)-caseins and the mole ratio for \( \kappa \)-casein/\( \beta \)-casein was determined. The average value for each of the size separations was plotted against micelle area/volume (Figure 25).

The mole ratio values for \( \kappa \)-casein/\( \beta \)-casein increased with increasing surface area of the micelles, which is consistent with a postulated surface position for \( \kappa \)-casein on the micelles. The plot of the mole ratio for \( \kappa \)-casein/\( \beta \)-casein against the surface area/micelle gave a reasonable straight line for the first three points but the data from the 60-min pellet deviated upward (Figure 25). Also, when only the area for \( \kappa_1 \) is used to determine the moles of \( \kappa \)-casein the curve follows a similar pattern (Figure 26).

The above method was used to determine the amount of each casein because it was not possible to measure accurately the \( \kappa \)-casein protein in the eluted fractions. The
Figure 25. Plot of Corrected Mole Ratios of K- and β-Casein Versus Micelle Surface Area/Volume for 2 min, 12 min, 30 min, and 60 min Pellets. The moles of K-casein include $K_1$ and $K_2$ peaks from Figure 20. The moles of β-casein include both $\beta_1$ and $\beta_2$ peaks.
Figure 26. Plot of Corrected Mole Ratios of K- and β-Casein Versus Micelle Surface Area/Volume for 2 min, 12 min, 30 min, and 60 min Pellets. The moles of K-casein only include $K_1$ from Figure 20. The moles of β-casein include both $\beta_1$ and $\beta_2$ peaks.
micro-protein analysis by the Bio-Rad protein was used to assay the pooled concentrated peaks. The peak fractions for \( \beta \)-casein yielded useful data, but the \( \kappa \)-casein fractions were near the lower limits of detection (1 \( \mu \)g) and their triplicate determinations varied widely. This may be due to interference by the sugars of the highly glycosylated \( \kappa \)-casein, as indicated in the assay instructions.

3.5.4. Ratio of Individual \( \beta \)-Caseins to Total Pellet Protein

The data from the Mono Q HPLC (Table 12) and the sum of the \( \kappa \)- and \( \beta \)-casein proteins from the reverse phase HPLC may be combined to give the amount of each individual \( \beta \)-casein and the total protein in each pellet. For donation 9, plots of the ratio of the individual \( \beta \)-caseins to total pellet protein versus the micelle surface area per volume ratio are shown in Figure 27. The data for 2-P \( \beta \)-casein were not used because of the overlap of \( \alpha \)-lactalbumin. Also, the reverse phase determination of the 60 min pellet was not performed on the same instrument at the same time and gave inconsistent data. However, the plots clearly show that 0-P \( \beta \)-casein in the pellets decreased with micelle size while all the others tended to remain constant. The decrease was related to the increase in surface area of the micelles and may have resulted simply from the greater exposure of all of the caseins to the solution as more surface was generated. The 0-P \( \beta \)-casein with no possibility for forming \( \text{Ca}^{++} \) ion
Figure 27. Plot of Mole Ratio of the Individual β-Caseins to Total Pellet Protein Versus the Micelle Surface Area/Volume.

(○) 0-P; (●) 1-P; (△) 3-P; (▲) 4-P; (□) 5-P
bridges, could then dissociate from the micelle while the others could not.

3.6. Amino Acid Sequence Analysis: PC/Gene (TM)

Added valuable information is available from the amino acid sequences of κ-casein and β-casein for both human and bovine with the computer programs available in PC/Gene (TM). The program "Novotny" graphically displays the predicted hydrophobicity and location of charged residues along the amino acid sequence. It also indicates predicted locations of β turns, beta sheet and alpha helix from the Chou-Fasman rules. These rules were also used to give the predicted secondary structure for the glycomacropeptide (Fiat et al., 1980)(Figure 2). The apparent features for human κ-casein include a charge reversal in the amino acid sequence before the enzymatic cleavage site of the gMP and also, the N-terminus is more hydrophobic than the gMP and the hydrophobicity decreases gradually along the sequence from the N-terminus. This would predict a structure with more flexibility in the C-terminus. These major features are also observable for the bovine κ-casein sequence (Figure 28). The refinements of secondary structure predicted by Fiat et al.(1980) are more clearly represented in Figure 2 and they are compatible with these predictions. Although the amino acid sequence analysis does not include the effects of covalent modification, both the caseins are strongly influenced by the presence of the glycosylation so
Figure 28. Novotny Program Predictions of PC/Gene (TM) for Hydrophobicity, Secondary Structure (β-turn, β-sheet, α-helix) and Amino Acid Charge Distribution of Bovine and Human κ-caseins. (A) Human κ-casein, (B) Bovine κ-casein.
Plot of secondary structure curves for sequence CASK$HUMAN.

Plot of secondary structure curves for sequence CASK$BOVIN.
that the 50% more carbohydrate in human $\kappa$-casein will
enhance the hydrophilic nature and negative charge on the
gMP.

The Novotny analysis for human and bovine $\beta$-casein show
similar charge distributions. The charge pattern is much
more complex than in $\kappa$-casein with the charge changing more
often. The entire molecule is hydrophobic for both the
species.
4. DISCUSSION

This research project was divided into two phases. The first part consisted of isolating sufficient quantities of pure \( \kappa \)-casein to characterize and to carry out the labeling study experiments. This involved modifying reported techniques and developing new ones to further purify the sample. It provided an opportunity to develop respect for the complications involved in isolating a glycoprotein that by its very nature forms aggregates with the major component in the separation. In the characterization process the reliability of the method to produce samples that compared with published reports and were reproducible was established. This was particularly important relative to the sugars, which were labile under the initial separation conditions. The observation that two separate samples of \( \kappa \)-casein from two donors had similar sugar composition indicated that the purification method developed was indeed suitable for this glycoprotein.

The problem of detecting \( \kappa \)-casein was resolved by the use of three procedures. First of all, a standard SDS-PAGE gel glycoprotein PAS dye protocol was developed so that it was not only reliable but gave added information concerning the location and the presence of other proteins. When the
labeled sample of $\kappa$-casein was prepared and used as a tracer, the sketchy results of the unlabeled material became well defined. This was evident in the separation procedures on the Mono Q and the reverse phase HPLC. The reverse phase separation of $\kappa$-casein from $\beta$-casein gave the final necessary tool for the project.

The second part of this project involved the investigation of $\kappa$-casein in human milk micelles. The native human micelle system was of prime interest because neither had much been established previously about the micelle itself nor had any comparisons been made with the bovine micelle models. Therefore, any comparisons must be initiated with an investigation to see if the components of the micelles are surface area related as is proposed for $\kappa$-casein in the Slattery-Evard bovine model. Since the surface area is related to size, and the micelles are known to exist in a size-distribution with an average radius of 40 nm, it became important to separate them by centrifugation into different sized fractions. From the distribution of micelle protein in each of the size-differentiated fractions, the number of micelles in each fraction was estimated by using their calculated average radius to establish the average volume of a micelle in each fraction. The total volume of the micelles was approximated by dividing the total weight of the micelles by their density which is the reciprocal of their voluminosity as established by Sood et al. (1976). The
total weight of the micelles was obtained from the weight of the protein present in each fraction after including the correction for the weight of H₂O/g of protein in the micelle. From the total volume of micelles in a fraction and the average volume of a micelle the total number of micelles in a fraction was calculated. The values were 2.3 x 10^{12} micelles (2 min), 4.8 x 10^{13} micelles (12 min), 7.08 x 10^{13} micelles (30 min) and 9.01 x 10^{13} micelles (60 min). This corresponds to a total of 2.1 x 10^{13} micelles/ml of skimmed milk. From this total the percentage abundance of micelles in each fraction was 1.09\% (2 min, r=209 nm), 22.7\% (12 min, r=85.3 nm), 33.6\% (30 min, r=54.0 nm), and 42.7\% (60 min, r=38.2 nm). This distribution is compatible with the reported value of 40 nm radius for the most abundant-sized micelle in human milk.

A second feature that is very important for understanding the design of the micelle is the ability of \( \kappa \)-casein to stabilize \( \beta \)-casein from Ca\(^{++} \) ion precipitation. The \( \kappa \)-casein isolated in this study had the proper stabilization properties. The stabilization ability of \( \kappa \)-casein in bovine milk has been correlated to a surface position for \( \kappa \)-casein that limits aggregation of submicelles. The amount of bovine \( \kappa \)-casein has been reported to increase as the surface area of the micelle increases, (Slattery, 1978). In the present separations of human milk micelles, it was established that the moles of \( \kappa \)-casein relative to the moles of
\( \beta \)-casein increased with the increasing surface area of the micelle. These results support the surface stabilizing role of \( \kappa \)-casein in the human two-casein system. In addition to this \( \text{Ca}^{++} \) ion stabilization which is similar to the bovine system, there were other aspects of \( \text{Ca}^{++} \) ion stabilization in human milk that needed to be examined. Azuma et al. (1989) reported that 1-P \( \beta \)-casein stabilized both 4-P and 5-P \( \beta \)-caseins in the presence of \( \text{Ca}^{++} \) ions and 2-P also had some stabilizing ability. Though 1-P exhibited stabilization properties, it was much less effective than \( \kappa \)-casein in stabilizing 4-P. The results of Azuma et al. (1989) indicated that 1-P reached 90% stabilization with 0.4 mole 1-P/mole 4-P, whereas this study showed that \( \kappa \)-casein stabilized 90% at 0.1 mole \( \kappa \)-casein/mole of 4-P. The composition of these individual forms of human \( \beta \)-casein may be important in micelle formation. Considering that the human micelles are much smaller than the bovine micelles and therefore have more surface area, it is hard to account for the \( \text{Ca}^{++} \) ion stability of human micelles from \( \kappa \)-casein alone, since the protein has not been detected in as large amounts in the human milk as in bovine. For this reason it is important to note that the relative amount of 1-P increased significantly as the size of the micelle decreased (Table 12).

The third aspect of this human micelle study was to evaluate the possible effects in the native system due to the postulated sequence of events in the biosynthetic
pathway of the micelles in the secretory process (Figure 5). Some information can be obtained by comparison of the native system with the re-equilibrated system. In most of the features of these separations, the native and the re-equilibrated systems had the same characteristics and size distribution. As was mentioned in Results, the re-equilibrated human milk in laser light scattering analysis gave a similar size distribution as the native milk. But, it was evident in the results for 0-P β-casein separated by anion exchange (Mono Q) that the 0-P distribution was different for the three micelle systems. Zero-P appeared to be the most sensitive to re-equilibration. The data suggested that 0-P was lost from the micelle during redistribution in the cold followed by reincorporation during re-equilibration, (Table 12). Zero-P β-casein has been reported by Azuma et al.(1989) to be the least micelle-forming of the β-caseins. Zero-P has been detected by Monti and Jolles, (1982) in the whey proteins (non-micelle soluble proteins) and they called it galactothermin.

In both the native and re-equilibrated separations, 0-P showed the same trend i.e. a decrease in the amount of 0-P with smaller micelle size in the first 3 pellets collected. In the 60-min pellets the 0-P increased again but it was still less than in the largest micelles. From this sequence it appeared that 0-P was incorporated in the largest
micelles and was easily lost to solution possibly due to its lack of phosphate to cross-link through Ca\(^{++}\) ion bridges.

In the acid precipitation of casein, the Ca\(^{++}\) ions are displaced by H\(^+\) which may help disrupt the micelle and release the 0-P β-casein. From the results of this study, acid-precipitation of casein appears to incompletely separate micellar casein. The percentage of each β-casein in these studies differed from those noted in the acid precipitated milk (Sood et al., 1985). The most abundant form reported was 4-P and in decreasing order were 2-P, 3-P, 5-P, 1-P and 0-P, (Table 4). In this study the results for 2-P cannot be compared because they have not been corrected for the α-lactalbumin present but the order of abundance of other β-caseins was 4-P> 1-P> 0-P> 3-P> 5-P. This suggests that in acid-precipitated casein there is a loss of 0-P and 1-P, the β-caseins with the least ability to cross-link with Ca\(^{++}\) ion.

The results of the present study indicated that 0-P may be incorporated into the micelle under the conditions of formation but does not re-equilibrate completely. With the decrease in temperature in the re-equilibration studies, the hydrophobic interaction decreases and the β-caseins dissociate from the micelle. The percent of 0-P obtained from acid-precipitated milk has been extremely variable from different donors in our laboratory (Sood et al., 1985) which
could be either the effect of individual milk variation or of isolation procedure.

In the 0-P, 3-P, and the 4-P forms of β-casein in the micelle separation, the 60-min pellet does not continue the trend of the first three fractions. This may mean that the 60-min fraction is most effected by the separation conditions or that some other factor is in operation.

When comparing the results of this study with reconstituted micelle studies of Azuma et al. (1985), it appears that the specificity of the interaction is not retained in the reconstituted micelles. Five-P was the only form of β-casein observed to change in the micelles, reconstituted in 10 mM Imidazole-HCl buffer (pH 7.0) containing 70 mM KCl and 10mM CaCl₂ with a 7/1 mixture of β/κ casein by weight. These were re-equilibrated at 37°C for 1 hour and separated into two size-differentiated fractions and a supernatant by centrifugation. The 1st pellet (large micelle) was 16/1:β/κ by weight, and the 2nd pellet was 6/1 while the supernatant which they called small micelles was 3/1:β/κ. These results do not directly relate to the results of this study and point out the many variables involved. However, a 7/1 ratio of β/κ appeared to be a reasonable starting proportion. The results from initial re-equilibration studies in this project indicated that the one hour equilibrium period probably was not sufficiently long enough for equilibrium to be established so that the large micelles were not well
stabilized with κ-casein and the \( \beta/\kappa \) ratio by weight was actually larger than any determined in this study. The medium micelles were similar in composition to the 4 fractions obtained in this study, while the final solution was probably not micelles but perhaps incompletely aggregated caseins. This is supported by the observation that 35% of the labeled κ-casein remained in the 60s fraction. These differences in the results with the acid-precipitated casein, the reconstituted micelles, and the native micelles indicates the importance of the biosynthetic process and its control to produce native micelles.

Having established the probable surface relationship of κ-casein in the human micelle, it is of interest to describe the interaction on a molecular level. The bovine model of Slattery-Evard proposes a surface position for κ-casein that is concentrated in one area of the submicelle. The ability to compare bovine and human κ-casein by their amino acid sequence gives some suggestion as to how the casein interactions may take place. The computer-assisted analysis of the amino acid sequences of κ-casein and \( \beta \)-casein suggests two possibilities to explain the reversal of charge along the κ-casein polypeptide. The C-terminus is negatively charged and the sugars attached in this region make it very hydrophilic. The N-terminus is more hydrophobic, in addition to having positively charged amino acids. One result of these characteristics could be the interaction of the
N-terminus of one $\kappa$-casein with the C-terminus of another. However, it may also be possible that the interaction of the negatively charged hydrophilic region is with the $\text{Ca}^{++}$ bound to the phosphates at the N-terminus of $\beta$-casein. In the first case, $\kappa$-casein would tend to aggregate together either on the surface layer of a hydrophobic $\beta$-casein core or inserted in the core as an aggregate. In the second case, the hydrophobic parts of $\kappa$- and $\beta$-casein are in the submicelle but the hydrophilic C-terminus of the $\kappa$-casein would be extended outside the hydrophobic core and limit the growth of the micelle. The fact that $\kappa$-casein does not self-associate was reported by Sood et al. (1990b). However, it is possible that the hydrophobic environment of the $\beta$-casein in the micelle would enhance the self-interaction of $\kappa$-casein.

Any model for the human micelle needs to account for the following observations. First of all, $\kappa$-casein is located on the surface and stabilizes the micelle. But, unique to the human micelle, the effects of the individual phosphorylated forms of $\beta$-casein must be given a role in a micelle model. Three-P, 4-P, and 5-P are more hydrophobic at pH 2.0 (which may relate to the conformational change with $\text{Ca}^{++}$ ion binding) than 0-P, 1-P, and 2-P since they elute later from the reverse phase column. These more phosphorylated $\beta$-caseins may make up the central hydrophobic core of the submicelle, oriented in the amphipathic manner.
proposed in the Waugh model. The 0-P, 1-P and possibly the 2-P fit into this core, but are less tightly bound since they do not have as strong Ca\textsuperscript{++} ion binding capability. Calcium ion binding which they lack possibly causes a conformational change that may take place after the \( \beta \)-caseins originally aggregate in the biosynthetic process. Under these conditions 0-P, 1-P and 2-P could extend farther from the center of the micelle, interfering with further Ca\textsuperscript{++} ion cross-linking of the N-terminal region of the more phosphorylated \( \beta \)-caseins in the central core of the micelle. The question remains of how \( \kappa \)-casein interacts on the surface. Perhaps the hydrophobic N-terminus inserts partially into the hydrophobic core leaving the hydrophylic C-terminus with its many carbohydrate chains extended from the surface. From this position \( \kappa \)-casein would shield the \( \beta \)-caseins from Ca\textsuperscript{++} ion precipitation. Though \( \kappa \)-casein's N-terminus is negatively charged, it probably does not have the proper conformation to encourage Ca\textsuperscript{++} ion bridging. The looser binding of the less phosphorylated \( \beta \)-caseins would allow their exchange from the submicelle, aided by the fact that the human micelles are less densely packed than the bovine micelles.
5. FUTURE STUDIES

Questions that arise from this work include the further identification of the second $\kappa$-casein peak ($\kappa_2$). First, $\kappa_2$ must be isolated in sufficient quantity by reverse phase HPLC and sequenced. Its location in the separation by Sephadex G-200 and Mono Q HPLC should also be identified and compared with the material isolated in this project. This protein also needs to be compared with the highly glycosylated phosphoprotein (HGPP) reported by Azuma et al. (1987) which is a glycoprotein of higher molecular weight than $\kappa$-casein that has been found in both the casein and the whey fractions of human milk.

Studies of deglycosylated $\kappa$-casein should be pursued to help clarify the hydrophilic effect of the sugars on the aggregation properties of $\kappa$-casein and the stabilization of the $\beta$-caseins.

It would be of interest to analyze the pellets from a single sample of milk that has been centrifuged differently as well as in the manner described here. One set could be isolated in the manner of the native pellets described in this study and compared with the pellet from all the micelles brought down in one long spin and the pellet formed in acid-precipitated casein, to get an overview of the
effect of the different processes on the micelle sample. An acid-precipitated sample with a final addition of Ca\textsuperscript{++} ion, which was recommended by Kunz and Lonnerdal (1988), would also be of interest and help to unravel the native micelle system and its relation to other human milk preparations.

It would also be of interest to use these HPLC tools to screen donor's milk for any irregular patterns of $\beta$-casein and $\kappa$-casein and see what effects are present in the micelle.

For micelle modeling studies it is necessary to know more concerning the shape of the $\kappa$-casein molecules. When sufficient $\kappa$-casein is available, viscosity studies are planned to determine the shape of the molecule.

From the results of this study it appears that the 0-P and 1-P forms of $\beta$-casein are not as tightly bound and that the more highly phosphorylated forms are more strongly held in the micelle. If bovine milk were modified with $\beta$-casein of lower degree of phosphorylation, a softer curd might be formed and more digestible milk produced. A study involving such modifications could be made of bovine milk to see if it would improve digestibility of infant formula.
6. LITERATURE CITED


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