Abstract

Nonsolvent water measured by cryoscopic forms the lower limit of the solvation water measured by ultracentrifugation. Variations of the solvation of casein in bovine milk under the influence of various factors including concentration, pH, calcium ions, and heat have been determined.

Introduction

The three major constituents of whole casein from cow's milk (α₁-casein, β-casein, and κ-casein) are in almost equal quantities composed of nonpolar side chain and polar side chain amino acids. The latter, by their interaction with the water molecules, assure solvation of casein in its natural surroundings.

All mechanisms that come into play in fixation of water by proteins are not known with precision, nor is it known whether this water constitutes an homogeneous phase. According to Kauzmann (12) the nonpolar groups tend to turn in toward the center of the molecules to avoid the aqueous phase whereas polar groups align themselves on the surface in direct contact with the water molecules.

In milk the problem is more complex due to "micelles" formed by casein and minerals, especially those of calcium and of phosphoric acid. That α₁-casein, β-casein, and κ-casein form stoichiometric associations is known, but only hypotheses following indirect observations can be made as to the structure of these micelles (8, 12, 20, 30). Kappa-casein occupies a key position in this structure because of its stabilizing properties, but its position is still being debated. Phosphocaseinate micelles have a variable molecular weight of from 1 to 300 × 10^6 (18) and are easily sedimented within centrifugal fields in the neighborhood of 50,000 × g.

The micelle has a loose structure of about 70% water. It may be assumed that hydrophobic intramolecular zones retain little water but that, on the contrary, intramicellar spaces in the native state (micelle porosity) limited by the hydrophilic surfaces of the protein molecules, retain a large amount. The water in these zones as well as on the outer surfaces of the micelles may be linked, to a greater or lesser degree, to protein. It is even possible that some of the intramicellar water is retained in a mechanical manner.

The presence of several types of water associated with the protein may be assumed. "Bound water" does not act as a solvent and does not freeze at -18 C, -40 C, or below (7, 14, 19, 28) but forms the nearest layers of water molecules adjacent to the peptide chain, perfectly oriented and linked by hydrogen bonds. Various means exist for determining this bound water, but the results of different authors sometimes disagree.

We assume that water molecules which form an intermediary layer between bound water and the really free water are linked to the preceding layer and among themselves by a lesser number of hydrogen bonds or some other interaction and constitute unfreezable and nonsolvent water. The totality of bound water, unfreezable water, and nonsolvent water, plus the water trapped in the casein pellets during centrifugation, constitute the water of solvation of the protein which is the water retained by the sediment pellets after ultracentrifugation according to Thompson et al. (26).

We have studied variations in solvation of casein under different conditions using the cryoscopic method and ultracentrifugation. The diameter of the micelles may vary considerably with a mean diameter of about 100 nm and a maximum of about 300 nm for cow's milk (11, 18, 24, 30). Certain technological processes modify their size; they increase in size during evaporation which also causes formation of aggregates (10). Does solvation vary when the diameter or density of the
micelles change? We have also tried to determine the influence of total solids, calcium, and colloidal phosphate content of the milk as well as the effects of variations in pH and of diverse heat treatments.

Materials and Methods

Milk. Either bulk skim milk or milk reconstituted from spray skim milk powder was used. The powder was dissolved by stirring for 30 min in demineralized water, and the milk thus obtained was utilized only after having been preserved at 4 C for 24 h for us to work with a stable product in salt equilibria. Both natural milk and reconstituted milk yielded similar results in determining casein solvation. The milk powder was obtained by a low heat process. Normal and concentrated milks are always derived from the same source.

Colloidal phosphate-free milk was prepared by a modification of the Pyne and MacGann technique (20) from the results of Fox and Ernstrom (8):100 ml of milk cooled to 0 C were acidified to pH 4.6 by adding .35 ml of hydrochloric acid, and were dialyzed at 0 C for 96 h against 2000 ml of the same milk non-acidified. Milk for counter dialysis was changed every 24 h.

Heating was by passing the milk through a 2.0 mm inner diameter glass coil plunged into a heated water bath; then the milk was passed through a cooling circuit which brought it rapidly down to a temperature of 20 C. The temperature rise and fall were realized in less than 3 s.

Determination of the solvation water. According to Thompson and others (26, 27), this is the water in the calcium phosphocaseinate sediment pellets separated by the centrifugation of the milk at 68,000 X g for 35 min at 37 C. We also used centrifugal fields of 126,000 and 198,000 X g with the SPINCO L 2 ultracentrifuge, rotor 50. All the data were obtained at 37 C after a holding time at this temperature of 1 h; they were extremely reproducible.

To reveal differences in composition and in solvation among the micelles of different milks, we proceeded according to the following scheme for skim milk at 9.15 and 22.07 total solids: 1st centrifugation, (skim milk) 8,000 X g, — 37 C, 35 min; 2nd centrifugation, (1st supernatant) 17,800 X g, — 37 C, 35 min; 3rd centrifugation, (2nd supernatant) 68,000 X g, — 37 C, 35 min; and 4th centrifugation, (3rd supernatant) 126,000 X g, — 37 C, 35 min. At each step of the experiment the sediment pellets were analyzed as well as the fourth supernatant. The supernatants are easily separated by draining the tubes for 5 min.

Determination of the non solvent water by cryoscopy. The method developed by Newton and Gortner (17) was applied to milk and milk products by Pyenson and Dahle (19). Depression of the freezing point of a sucrose solution in milk is always higher, for an equal quantity of total solvent, than the sum of the corresponding depressions of milk and sucrose solution separately. The sucrose, therefore, is dissolved in only part of the total amount of water in the milk. The difference is made up of the nonsolvent water.

Cryoscopic depressions were measured with a Horvet type cryoscope (25), following the AOAC technique (3), and then with a semi-automatic ADVANCED cryoscope following Abele's technique (1, 2).

We used a sucrose weight equal to a tenth of its molar mass per 100 g of total water, that is, for 89.2 g of solvent as this sugar is hexahydrated in solution. In a given weight of milk containing 100 g of water, the nonsolvent water, e, is calculated by the following equation:

\[ e = 89.2 \times \frac{D_s}{(D_a - D_m)} \]

where:

\[ D_s = \text{augmentation of the cryoscopic depression due to sucrose}; \]
\[ D_a = \text{total cryoscopic depression (milk + sucrose)}; \] and
\[ D_m = \text{cryoscopic depression of milk}. \]

Electrophoretic method. Starch gel electrophoresis in a horizontal cell was according to Wake and Baldwin (29) with a Tris-borate buffer solution, pH 9.3, containing 4.5 M urea and .03 M 2-mercaptoethanol.

Analytical methods. Nitrogen was determined by micro Kjeldahl method (protein = nitrogen X 6.38). We used atomic absorption spectrophotometry for calcium and Fiske and Subarow's method, modified by Bamman et al. (4) for phosphorus.

Results

Validity of the experimental conditions: Ultracentrifugation. We centrifuged a 9.1% reconstituted milk (normal milk) with centrifugal fields of from 8000 to 198,000 X g. The weight of the dry sediment pellets reached its maximum at 68,000 X g and hardly varied afterwards. The conditions given by Thompson et al. (26, 27) are valid; at 37 C there is no need to exceed 100,000 X g to obtain the maximum amount of sedimentary materials from ordinary milk, contrary to the results of Sabarwal and Ganguli (22) at 20 C.

Solvation water diminished slightly above
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68,000 × g: 2.52, 2.43, and 2.41 per g of sedimented proteins for 68,000, 126,000, and 198,000 × g. A further limited compressing of sedimented micelles occurred. However, we may assume the water that accompanies the sediment for this value of the centrifugal field constitutes the solvation water.

The quantities of nitrogen, calcium, and phosphorus sedimented from 1000 g of milk remained stable above 68,000 × g.

Cryoscopy. We used sucrose to determine the nonsolvent water; other sugars lead to results that differ in absolute value (14). We observed that the use of glucose resulted in a proportion of .9 g of nonsolvent water/g of protein in skim milk whereas sucrose gave us a proportion of 1.5 g. On the other hand, the concentration of sugar does not seem to interfere since sucrose concentrations of .166 M, .25 M, .5 M gave us 1.5, 1.6, and 1.6 g of nonsolvent water/g of protein for a single milk.

Influence of milk concentration. Five milks reconstituted from dried skim milk powder, whose contents in total solids (TS) varied from 9.1 to 25.6%, were centrifuged at 68,000, 126,000, and 198,000 × g. Fig. 1 shows that the weight of dry sediment increased linearly with TS. However, above 22% a centrifugal field of 68,000 × g is insufficient.

Solvation of phosphocaseinate varied relatively little with variations in concentration of milk. Fig. 2 shows that solvation water and nonsolvent water, expressed in relation to proteins, develop in inverse order.

Table 1 shows results from analysis of normal milk (9.15% TS) and concentrated milk (22.07% TS) sediment pellets, separated by fractional centrifugation at fields increasing from 8000 to 126,000 × g. The four fractions from each milk did not correspond to the same proportions of the sedimented total. At 17,800

![Fig. 1. Effect of total solids (TS) of milk on the weight variations of sediment pellets.](image)

![Fig. 2. Effect of total solids (TS) of milk on the protein solvation (g of solvation water/g of sedimented protein, or g of non solvent water/g of total milk proteins).](image)

**Table 1. Composition of fractions of phosphocaseinate micelles separated by sequential centrifugation.**

<table>
<thead>
<tr>
<th>Centrifugal field</th>
<th>8,000</th>
<th>17,800</th>
<th>68,000</th>
<th>126,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry sediment</td>
<td>16.6</td>
<td>13.2</td>
<td>4.76</td>
<td>.67</td>
</tr>
<tr>
<td>g/1000 g of milk</td>
<td>18.85</td>
<td>26.5</td>
<td>57.0</td>
<td>5.46</td>
</tr>
<tr>
<td>Nitrogen g/100 g</td>
<td>73.5</td>
<td>79.5</td>
<td>77.0</td>
<td>84.8</td>
</tr>
<tr>
<td>of dry sediment</td>
<td>59.2</td>
<td>62.7</td>
<td>63.3</td>
<td>60.4</td>
</tr>
<tr>
<td>Hydration g/g of</td>
<td>2.82</td>
<td>2.81</td>
<td>2.69</td>
<td>3.55</td>
</tr>
<tr>
<td>proteins sedimented</td>
<td>2.63</td>
<td>2.66</td>
<td>2.50</td>
<td>3.51</td>
</tr>
<tr>
<td>Calcium mg/g of</td>
<td>34.6</td>
<td>27.7</td>
<td>18.3</td>
<td>11.3</td>
</tr>
<tr>
<td>proteins sedimented</td>
<td>41.1</td>
<td>34.8</td>
<td>34.2</td>
<td>28.1</td>
</tr>
<tr>
<td>Phosphorus mg/g of</td>
<td>17.0</td>
<td>16.8</td>
<td>16.2</td>
<td>3.51</td>
</tr>
<tr>
<td>proteins sedimented</td>
<td>19.1</td>
<td>18.8</td>
<td>18.5</td>
<td>14.0</td>
</tr>
</tbody>
</table>

*Normal figures: normal milk (9.15 % TS); Italics: concentrated milk (22.07 % TS).

*In parentheses: % of total sediment.
Farm. 3. Starch gel electrophoresis of the sediment pellets: (1) 68,000 × g, normal milk; (2) 68,000 × g, concentrated milk; (3) 126,000 × g, normal milk; and (4) 126,000 × g, concentrated milk.

85% of the sedimentable substances were separated from the normal milk, as opposed to 42% for the concentrated milk. Moreover, the sediment pellets showed differences in composition according to their origins. Those coming from the concentrated milk were poorer in nitrogen materials and richer in phosphate and calcium (Table 1). We also observed that they contained more lactose. On the other hand, solvation water differed little from normal milk to concentrated milk. Sediment pellets separated at 126,000 × g contained more moisture than those of the preceding fractions. Moreover, fractions separated at 68,000 × g and at 126,000 × g were hydrated more than sediment pellets obtained without fractional centrifugation, 2.69 and 3.55 g/g of proteins sedimented against 2.52 and 2.43 g/g of proteins sedimented for normal milk.

Fig. 3 represents the electrophoretic diagrams of the fractions sedimented in different centrifugal fields. They all appeared to have the same qualitative composition. We can see in decreasing order of mobilities: α_{s1}-casein, β-lactoglobulin (present only when milk is heated), β-casein, and κ-casein.

Influence of pH in milk and of the addition of calcium. Fig. 4 shows that the depression of pH provoked a decrease in amount of solvation water down to pH 5.8. A slight increase was then observed, followed by a considerable fall when pH reached the isoelectric point of casein. But the nonsolvent water remained constant since the cryoscopic depressions of pure milk and sucrose added milk increase linearly and in parallel directions. This value was 1.45 g/g of the proteins in the milk.

The addition of calcium has a different effect on solvation, depending on whether calcium chloride is used, which provokes a regular but limited fall in pH (pH 5.8 for 50 mM Ca), or calcium oxide, which raises the pH (pH 8.5 for 12.5 mM Ca). Fig. 5 shows that the solvation water curve is regular if the two types of addition are opposed. This is an example of a variation parallel to that produced by a single variation in pH. As concerns nonsolvent water, an increase may be observed whatever the form of calcium added. Therefore, Ca (II) has a specific effect. Normal milk corresponds to the minimal quantity of nonsolvent water. For heavy additions of calcium chloride there is apparently more nonsolvent water than solvation water.

Influence of heat on milk. Heat provokes an increase in solvation of the phosphocaseinate micelles, the centrifugation being performed immediately after cooling (Table 2). The increase in quantity of nonsolvent water is much more marked than that of solvation water. After heating at 90°C for 2 min there is about twice as much nonsolvent water as there is in raw milk.
Influence of the disappearance of colloidal phosphate. When milk is deprived of colloidal phosphate at 0 °C, sediment is reduced only by about one-half, but its solvation in grams of proteins is slightly higher as is shown in Table 3.

**Discussion**

The decrease in the amount of phosphocaseinate micelles solvation water produced by an increase in the centrifugal field from 68,000 to 198,000 × g is due to elimination through compression of interstitial water which accompanies particles during sedimentation. This is confirmed by the fact that the sum of the different fractions of the solvation waters obtained through fractional sedimentation is always superior to the solvation water determined by a single centrifugation at the highest velocity. The surplus of water is provided by the first two fractions (8000 and 17,800 × g) which produce the least compact sediment pellets. This effect is perceptibly stronger in normal milk than in concentrated milk, the two fractions represent 85 and 42% of the total amount of sediment. The water remaining beyond 68,000 × g is linked to the proteins by forces sufficiently strong to bind it to the phosphocaseinate micelles in spite of their different densities (1.1 for the phosphocaseinate micelles). This solvation water, thus, may be considered, as it was by Creamer and Waugh (6) and Thompson et al. (26, 27), one of the characteristics of milk.

The last fraction obtained at 126,000 × g is more hydrated than preceding ones. Probably a decrease in density and particles size and a variation of their composition can explain this higher value.

The organization of water molecules within

**Table 2. The effect of milk heating on the solvation of phosphocaseinate micelles.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>72 C 30 s</th>
<th>72 C 2 mn</th>
<th>90 C 15 s</th>
<th>90 C 2 mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvation water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/g of sedimented proteins</td>
<td>2.49</td>
<td>2.61</td>
<td>2.60</td>
<td>2.61</td>
<td>2.66</td>
</tr>
<tr>
<td>Nonsolvent water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/g of proteins in the milk</td>
<td>1.42</td>
<td>2.15</td>
<td>2.34</td>
<td>2.59</td>
<td>3.01</td>
</tr>
</tbody>
</table>

*The determinations are immediately after heating and cooling.
Table 3. The solvation of normal and colloidal phosphate free milks.

<table>
<thead>
<tr>
<th></th>
<th>Normal milk</th>
<th>Colloidal phosphate free milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvation water g/1000 g of milk</td>
<td>68</td>
<td>38</td>
</tr>
<tr>
<td>Solvation water g/g of sedimented proteins</td>
<td>2.55</td>
<td>2.68</td>
</tr>
<tr>
<td>Non solvent water g/1000 g of milk</td>
<td>47</td>
<td>54.1</td>
</tr>
<tr>
<td>Non solvent water g/g of total proteins</td>
<td>1.43</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Protein particles and at their surfaces rapidly becomes much less complex upon moving away from the particle, and it appears that this structure concerns only the first molecular layers of water. The more distant water molecules are less and less bound and even can be removed under certain conditions (pH, calcium additions, etc.). It seems, in fact, that micelle intermediary space, sufficiently permeable to admit large molecules as shown by Ribadeau Dumas and Garnier (21), is normally filled with water. We can hypothesize that nonsolvent water measured by cryoscopy is one part of the total solvation water and is composed by the layers of water bound to the protein molecules, either within or at the surfaces of micelles, and part of following water molecules which still may be joined to the protein molecule through the intermediary of the first layers of water. In other terms, nonsolvent water measured by cryoscopy should group "bound water", "unfreezable water", and "water which does not dissolve sucrose", each water fraction possessing partially or totally these different properties.

According to Nemethy and Sheraga (15) and Sheraga (23) 56% of the water molecules still seem to exist, at 70 C, in the form of fluctuating masses called "flickering cluster". Their existence and disintegration are dependent upon local energy fluctuations, and if they are short-lived in pure water, we are led to believe that they hold out longer in contact with the solute (16). Therefore, we may assume they exist longer within the micelles and that some of the clusters (carried along with the phosphocaseinate micelles during centrifugation) form another part of the solvation water, and perhaps even part of the nonsolvent water. The fact, brought to light by Thompson et al. (26, 27), that the amount of solvation water increases when centrifugation temperature is lowered favors this hypothesis since the number of these clusters increases with lower temperatures (15).

The solvation water seems to be sensitive to pH variations above 6.0 and below 5.0. We may assume that the protein particles are increasingly surrounded by water in proportion to the increase in their charge, and that, because of this, they exercise their attraction at longer distances. The increase in solvation when pH decreases towards 5.8 to 5.6 must correspond to a modification in the charge of the casein micelles, rather in the size of the protein-solvent contact surface. Indeed, the observed stability of the nonsolvent water is incompatible with an increase in size of this surface.

When calcium chloride is added, the amount of nonsolvent water rapidly increases and even surpasses that of the solvation water whereas pH diminishes. It seems likely that interactions take place between the solvent and the added ions which produce hydrated ions. The water molecules are distributed around the ions as they are around the protein particles, and though they are not permanently linked to the ion, the time during which they are immobilized is much longer than in pure water. It may be presumed that they are measured by the cryoscopic method along with water bound to the proteins. On the contrary, they are not sedimented by the centrifugal fields which would explain our results.

The increase in quantity of solvation water when calcium hydroxide is added seems to depend more on pH shift than on Ca. It is linked to the increase in strength of the charge on the proteins, which tend to be dispersed. The resulting increase in the surface area favors solvation. On the contrary the grouping of micelles into large clusters, which occurs when calcium chloride is added to milk, is unfavorable to solvation. The results obtained with phosphate colloidal-free milk confirm this since the use of dialysis in this case also produces a dispersion of the phosphocaseinate micelles and an increase in solvation.

Variations in the amount of nonsolvent water when the quantity of total solids in milk is increased are produced by a similar phenomenon, but the pH and ionic force variations are much weaker. The growth in size of the micelles and micelle clusters results in their fixing less nonsolvent water, and we conclude that the slight increase in solvation water is due to a purely mechanical confinement within the clusters.
The increases in volume due to heating, slight in the case of solvation water and large in the case of nonsolvent water, seem to imply that the links between the protein clusters and the water molecules are multiplied and stabilized by the heat treatment. Likewise, the $\beta$-lactoglobulin-$\kappa$-casein complex formation and serum proteins denaturation which occurred during heating must interfere in the nonsolvent water increase.

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References


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