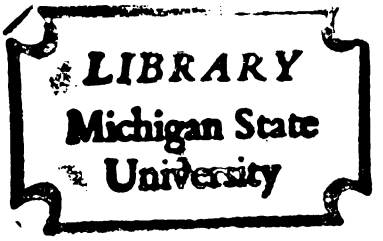

This is to certify that the

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CHEMICAL, PHYSICAL AND FUNCTIONAL PROPERTIES
OF SELECTED MILK PROTEIN CO-PRECIPITATES

presented by

DAVID WARREN TOBELMANN

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J. Robert Brunner
Major professor

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CHEMICAL, PHYSICAL AND FUNCTIONAL PROPERTIES OF SELECTED MILK PROTEIN CO-PRECIPITATES

By

David Warren Tobelmann

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ABSTRACT

CHEMICAL, PHYSICAL AND FUNCTIONAL PROPERTIES OF SELECTED MILK PROTEIN CO-PRECIPITATES

By

David Warren Tobelmann

Acid (ACP), pH 3.5 (P3C), hexametaphosphate (HMP) and calcium (CAC) co-precipitates were prepared from fresh skimmilk. Standard proximate, calcium and phosphorus analyses were performed on all samples. Functional properties examined were solubility, viscosity, water hydration capacity, emulsifying capacity and whippability. HMP exhibited greatest solubility, low viscosity and good water hydration. ACP and P3C displayed moderate solubilities, with ACP exhibiting higher viscosity and water hydration than P3C. CAC was extremely insoluble at neutral pH, and displayed low water hydration. None of the co-precipitates formed a stable foam. ACP, P3C, and CAC exhibited emulsifying capacities similar to sodium caseinate, whereas HMP was similar to nonfat dry milk. Co-precipitates were examined by electrophoresis in urea and sodium dodecyl sulfate (SDS). Urea had no effect on the insoluble fraction of the samples. SDS gels of P3C revealed β-lactoglobulin and α-lactalbumin bands in addition to the casein bands. SDS alone failed to solubilize the insoluble fractions of ACP and CAC.

To my mother and father--

who always encouraged me to strive for the best.

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INTRODUCTION

Milk protein products represent an important source of ingredients to the food industry. These products offer nutritional enrichment, desirable functional properties and are highly accepted by the consumer. Typical products used extensively include nonfat dry milk, sodium caseinate and whey solids.

Co-precipitates are unique in that they contain both casein and whey proteins in a concentrated form, making them of high nutritional quality. Because of their low lactose content, co-precipitates can provide total milk protein in applications where lactose constitutes a problem, e.g., sandiness in ice cream, caking in dry mixes and excessive browning in baked goods. Furthermore, the biological oxygen demand of whey from co-precipitate manufacture is lower than that for most casein and cheese wheys, thereby providing less of a waste disposal problem.

The demand for functional protein by the food industry continues to increase. While such milk protein products as nonfat dry milk, sodium caseinate and whey protein concentrate offer many important functional properties, little work has been conducted on the functionality of co-precipitates. Assessment of these properties could provide insight into new applications for these products.

In this study several types of co-precipitates were prepared in order to assess their functional properties and compare these to nonfat

dry milk, sodium caseinate and whey protein concentrate. Materials were blended in liquid model systems, and solubility, viscosity, water hydration capacity, emulsifying capacity and whipping properties were examined.

In addition, the nature of the protein complement contained in co-precipitates was examined in polyacrylamide gels in several dissociating systems.

REVIEW OF LITERATURE

Milk protein products represent an important and valuable source of protein ingredients to the food industry. The success of these products is due to their recognized nutritional, organoleptic and functional properties (Morr, 1979). As seen from Table 1, nonfat dry milk and dried whey are the major sources of milk protein ingredients currently used in the food industry, although many new products are now available which can contribute special advantages for certain food applications. Table 2 reviews the composition of some selected dry milk protein products.

Nonfat dry milk (NFDM) is the most popular source of milk protein (Hugunin and Ewing, 1977). In addition to imparting excellent flavor, functional properties and nutritional quality, NFDM is available in a convenient, storage-stable form. NFDM can be produced by spray drying or roller drying, although the former is the most satisfactory method. NFDM is manufactured by heating centrifugally separated skimmilk to meet the desired heat treatment, pasteurizing, concentrating to 45-50% solids and drying. Dry whole milk and dry buttermilk are similar to NFDM except that their fat contents are higher.

Muller (1971) reviewed the manufacture of caseins and caseinates, an industry heavily concentrated in New Zealand and Australia. Casein curd is produced from pasteurized skimmilk by treating with rennet,

Table 1. Estimated production of milk protein products in the USA, 1977.

Product	Amount, 1000 metric tons	Protein Content, % dry basis
Nonfat dry milk	421	36
Casein/caseinates	66	95
Co-precipitates	5	90
Partly delactosed whey	14.5	20
Partly demineralized whey	11.8	13
Whey protein concentrates	3.6	50
Whey solids in dry whey	210	13

Source: Morr (1979)

Table 2. Approximate compositions of selected dry dairy products.

Product	Moisture %	Protein %	Fat %	Lactose %	Ash %
Dried Whole Milk	2.0	26.4	27.5	38.2	5.9
Nonfat Dry Milk	3.0	35.9	0.8	52.3	8.0
Dried Buttermilk	2.8	34.4	5.3	50.0	7.6
Casein	7.0	88.0	1.0	--	4.0
Caseinates	4.0	92.0	0.8	--	1.5
Dried Whey	4.5	12.9	1.1	73.5	8.0
Whey Protein Concentrates	2.0	20-60	2-9	18-60	3-18
Co-precipitates	4.0	83.0	1.5	1.0	10.0

Source: Hugunin and Ewing (1977)

direct acidification with acid (usually hydrochloric, sulphuric, or lactic), or by culturing with lactic-producing micro-organisms. Generally, a pH of 4.3-4.5 is achieved to precipitate the casein. At this pH a product with a lower ash content is produced than if higher pH values are used. The curd is easily separated from the whey fraction, washed and dried. Isoelectric caseins are insoluble and are useful only in applications where solubility is not needed.

Preparation of Na, K, and Ca caseinates, which are simply salts of casein, is routinely achieved by neutralizing casein curd with the corresponding base to pH 6.8-7.5, pasteurizing, and spray drying the solubilized protein sol. Caseinates are excellent food ingredients, possessing good storage stability and imparting many unique functional properties.

In 1976, over 34 billion pounds of whey (both sweet and acid), equivalent to 2.2 billion pounds of whey solids, were produced in the United States (Clark, 1979). However, only 56.3% of these solids were further processed. As seen in Table 1, dry whey solids represent the second most popular source of milk protein. This product can be used as a source of crude lactose, milk solids, milk proteins or total solids. Processing of whey involves centrifugal separation and clarification to remove fine casein particles and fat, pasteurization, concentration to 45-50% solids, a holding period to permit a portion of the lactose to crystallize and spray drying. To produce free-flowing powders of low hygroscopicity, a two-stage drying process is used to ensure that the lactose is in the alpha-hydrate form.

Conventional lactalbumin represents a concentrated source of whey protein. It is produced by adjusting the pH of whey to 4.5-5.2 and heating to denature and precipitate 70-80% of the whey proteins (Robinson et al., 1976). However, this results in a yellowish-brown, completely insoluble, gritty powder which greatly limits its use in the manufacture of foods. Recently, Modler and Emmons (1977) developed a novel approach for preparing a heat precipitated whey protein product. This involves heating whey at pH 2.5-3.5 at 90° C, then adjusting the pH to 4.5 to effect precipitation. The resulting product exhibits high solubility, but yields are only 35-53% of total protein in the whey.

Over the last 20 years, there has been considerable interest in implementing processes for recovering whey protein concentrates which retain their native and functional state (Morr, 1979). Whey protein concentrates (WPC) contain higher than normal concentrations of whey proteins. These products can be manufactured by many different processes. Cold precipitation techniques involve the use of polyelectrolytes, including carboxymethylcellulose (Hansen et al., 1971), ferripolyphosphate (Jones et al., 1972), polyacrylic acid (Sternberg et al., 1976), and sodium hexametaphosphate (Richert, 1972). Other processes for the manufacture of WPC include electrodialysis (D'Souza et al., 1973), ultrafiltration/reverse osmosis (Fenton-May et al., 1971), and gel filtration (Morr et al., 1967). These WPC have excellent solubility and diverse functional properties, although their compositions vary considerably depending upon the process used in their manufacture.

Co-precipitates

Procedures for the precipitation of whey protein along with casein were developed in the 1950's and 1960's. Co-precipitates contain combinations of both caseins and whey protein, although the exact nature of this combination is not well understood (Beeby et al., 1971). According to Muller (1971) development of the commercial manufacture of co-precipitates was motivated by several considerations: (1) to increase the yield of protein recovery from milk; (2) to enhance the range of functional properties in foods; and (3) to increase the nutritional role of milk protein due to the contribution of the casein-whey protein combination. In addition, co-precipitates contain lower lactose levels when compared to NFDM.

The initial developments in the production and uses of co-precipitates were carried out in the United States and Russia. However, the Australian Commonwealth Scientific and Industrial Research Organization (C.S.I.R.O.) was the first group to fully exploit the commercialization of co-precipitates (Southward and Goldman, 1975).

Manufacture

Much of the early information concerning co-precipitates is found in patent literature. Muller (1971) notes that in the 1950's, it was observed that protein precipitated by acid or $CaCl_2$ from heated milk contained both casein and whey protein. However, Rowland (1937) had observed this years earlier when he found that 76% of the total soluble nitrogen was coagulated in heated milk when the denatured whey proteins were co-precipitated with casein at pH 4.7.

Heat-treated co-precipitates are produced by heating skimmilk sufficiently to denature the whey proteins, which appear to complex with the caseins (Southward and Goldman, 1975), followed by the addition of some precipitating agent. Scott (1952) described a batch process for making a co-precipitate. Alkali was added to skimmilk to reduce the acidity so as to inhibit initial precipitation of the protein when heating the milk to 90° C. Acid was added slowly to effect precipitation and the resulting curd was separated from the whey, washed and dried. Howard et al. (1954) used a similar batch method but without first adjusting the acidity of the milk. They also dispersed the product with alkali at pH 6.6-7.2 and dried it in that form. Scott (1958) dispersed the co-precipitate described in his earlier process at pH 6.6-6.7 with alkali, then add NH_4OH to pH 8.0-8.5 before heating at 77-85° C and drying. During drying ammonia was evaporated and the pH of the powder was reduced to 6.6-7.3. Loewenstein (1961a) produced a soluble co-precipitate from Scott's product by mixing it with a diglyceride and lecithin and homogenizing the mixture in skimmilk. This powder had a protein content of 40-85% and improved solubility and dispersibility. In another patent, Loewenstein (1961b) described the manufacture of a co-precipitate with high water binding capacity, involving the addition of hydrocolloids such as alginates before drying. To obtain a product with low water binding capacity for use in baked goods, Loewenstein (1965) neutralized Scott's co-precipitate with 1% of lime-in-water to a pH of 7.0-7.4. Following this neutralization and the addition of a calcium salt, the dispersion was spray dried. Engel and Singleton (1968) produced a co-precipitate by heating skimmilk for 10 min at

82-99° C, concentrating to about 30% solids, applying a heat treatment of 138° C for 15 sec, cooling to 101° C and acidifying to produce a curd.

In the early American methods for co-precipitate manufacture, no provision was made for controlling the calcium content. In the U.S.S.R. D'yachenko et al. (1953) described the preparation of a co-precipitate containing 95% of the total skimmilk protein. This was accomplished by adding $CaCl_2$ to milk heated to 85-95° C, yielding a co-precipitate with calcium and phosphorus contents of 2.65% and 1.40%, respectively. D'yachenko (1957) described a continuous process for the manufacture of this product. Calcium chloride was added to milk which was then heated to precipitate the protein. The curd was separated from the whey in a continuous, horizontal solid-bowl centrifuge. Arbatskaya et al. (1962) developed a two-stage heating system for the manufacture of co-precipitates. Skimmilk was first warmed in a heat exchanger by regeneration with hot whey. Calcium chloride was injected and the mixture of milk and $CaCl_2$ was subsequently heated to 95-97° C by steam injection. Rostrosa and D'yachenko (1968), while studying conditions for co-precipitation of milk proteins, concluded that the whey proteins and the casein-calcium phosphate complex coagulated jointly at 80-95° C at a $CaCl_2$ concentration above 0.83 g/l. In another study, Rostrosa et al. (1968) demonstrated that when milk containing 1.25 g $CaCl_2$/l was heated to 80-95° C up to 50% of the added calcium was bound to the curd in low acid milk and about 25% in high acid milk.

Australian work on co-precipitates began with Buchanan et al. (1965) when they modified the procedure of Arbatskaya et al. (1962) to

produce a calcium co-precipitate while reducing losses of curd particles during processing. Manufacture was carried out on commercial acid casein equipment. A combination of heat (90° C) and $CaCl_2$ (0.24%) was used to precipitate the proteins. Following washing, the curd was dispersed in 2% sodium tripolyphosphate and spray dried. Muller et al. (1967) introduced the concept of producing a range of co-precipitates with different functional properties by varying the calcium content. Co-precipitates with a high (2.5-3.0%), medium (1.5%), and low (0.5-0.8%) calcium contents were made using a continuous process. These workers found that by varying the time of heating milk at 90° C, $CaCl_2$ concentration and pH of precipitation, different ranges of calcium content could be obtained. More recently, Southward and Aird (1978) conducted a thorough investigation into the optimum conditions necessary to produce a variety of co-precipitates. They conducted experiments to determine the effect of pH of skimmilk before heating, heat treatment of milk, quantity of acid or $CaCl_2$ added and coagulation temperature on the characteristics and yield of curd. The results of this study are summarized in Table 3 on the following page.

Polish workers have developed a new form of co-precipitate which was summarized by Mann (1976). Raw milk with added $CaCl_2$ was separated, the skimmilk pasteurized at 90-92% C for a few seconds and the proteins precipitated at 40° C by the addition of HCl to pH 4.5. The curd was washed twice and dissolved with NaOH at 70-75° C to a pH to 6.6. Finally, after the addition of NH_4OH, the suspension was spray dried, yielding a product ranging in composition from 3.9-4.6% moisture, 0.20-0.73% fat, 74.4-84.4% protein, 5.2-13.2% lactose, 4.6-5.9% ash, and

Table 3. Optimum conditions for production of selected co-precipitates.

Condition	High Ca	Med. Ca	Acid
Initial pH of milk	6.4	6.4	6.4
Heat treatment	85 C/6 min	85 C/6min	85 C/6 min
Coagulant	$CaCl_2$	sulphuric acid	sulphuric acid
Coagulation temperature	77° C	65° C	58-60° C
pH of whey	5.8-5.9	5.1-5.5	4.8-5.1

Source: Southward and Aird (1978)

0.10-0.53% calcium. Production of various salts of this co-precipitate (sodium, calcium, ammonium and sodium-calcium) has been described by Chojnowski et al. (1975).

Aird (1971) discussed the preparation of co-precipitates from combinations of whey with skimmilk or casein products. A patent was issued to Wakodo Company Ltd. (1969) in which whey was heated and mixed with sodium caseinate or skimmilk. After further heating, the mixture was acidified to pH 4.5-4.6, and the curd was dissolved in alkali and dried. A co-precipitate for use in baked foods (Netherlands, Bedrijven van het Nederlands Institut voor Zwivelonderzock, 1973) was prepared by mixing four parts whey with one part buttermilk or skimmilk. The pH was adjusted to 6.5-7.1, and the mixture was subsequently washed, mixed with polyphosphate and dried. Raeuber et al. (1977) produced co-precipitates using acid whey and skimmilk, or skimmilk, acid whey and blood serum.

Co-precipitates can also be prepared from mixtures of milk and other proteins. Pokrovskii and Levyant (1968) produced a co-precipitate of milk and blood proteins using heat and calcium lactate. Sato and Ito

(1970) found that egg-white protein could be precipitated with casein from heated mixtures of egg-white and skimmilk. Using defatted soya flour and cottage cheese whey, Loewenstein and Paulray (1972) prepared a co-precipitate. The acidic (pH 4.7) mixture was heated for 30 min at 90° C and the precipitated curd was dried. Thompson (1977) co-precipitated rapeseed and cottage cheese whey proteins by heating at 95° C for 15 min at pH 4.6. Thompson (1978) prepared co-precipitates using cottage cheese whey and either soybean or cottonseed proteins, again using acid and heat treatment.

The use of other precipitating agents to make co-precipitates has been cited by Beeby et al. (1971) as an area requiring further research. Hansen (1966) found that some casein is precipitated from milk by carrageenin in the concentration range 0.01-0.10%. Several workers have studied the interactions of carboxymethylcellulose and milk proteins (Asano, 1969; Hansen et al., 1971; Cluskey et al., 1969). Also, sodium hexametaphosphate has been used to precipitate whey protein at acid pH (Gordon, 1943; Hartman and Swanson, 1966; Hidalgo et al., 1972; Richert, 1972). These precipitating agents may also be feasible for the production of co-precipitates.

Compositional Properties

The composition of co-precipitates is affected by several factors. The most obvious of these, of course, is the method of production. Muller et al. (1967) noted that the calcium content of a co-precipitate is determined mainly by pH of precipitation. As the pH is decreased the calcium content is reduced. The use of other precipitating agents will

also affect the composition of a co-precipitate (e.g., use of polyphosphates will increase the phosphorus content). D'yanchenko et al. (1953) noted that co-precipitates with higher calcium contents also had correspondingly higher phosphorus levels. Southward and Aird (1978) found that the fat content increases as coagulating temperature and pH of precipitation are decreased.

The extent of washing has also been shown to affect the composition of co-precipitates (Buchanan et al., 1965). As the number of washes increased, fat, calcium, lactose and ash levels were shown to decrease. Southward and Aird (1978) noted a similar decrease in the lactose, calcium and ash levels with repeated washes, but found the fat content was unaffected.

Neutralization and dispersion procedures will also affect compositional properties. Buchanan et al. (1965) and Muller et al. (1967) dispersed co-precipitates in sodium tripolyphosphate at levels from 2-6% (w/w). This practice will increase the phosphorus content of the dried protein concentrate. Neutralization with lime will increase the calcium content, whereas use of other alkali (e.g., NaOH) will increase the levels of ash and the particular salt used.

The exact nature of the proteins in co-precipitates has not been thoroughly studied. Southward and Aird (1978) conducted an electrophoretic analysis of a high-calcium co-precipitate. Most of the caseins were accounted for, while only half the β-lactoglobulin, most of the bovine serum albumin, and none of the α-lactalbumin or immunoglobulins were accounted for. The authors noted that 25% of the co-precipitated whey proteins could not be detected in the analysis. This discrepancy

was due to the insoluble protein which could not penetrate the gel. It should be pointed out that a greater proportion of whey protein is precipitated in a high-calcium co-precipitate than either the low- or medium-calcium forms.

Nutritional Properties

Casein has long been recognized as being a high quality protein, although the nutritional superiority of whey protein has been established (Mitchell and Block, 1946; Riggs et al., 1955). Lohrey and Humphries (1976) measured PERs for several milk products and found that casein, co-precipitate, skimmilk powder, WPC and lactalbumin had values of 2.50, 2.78, 2.67, 3.03 and 2.92, respectively. The nutritional superiority of co-precipitates over casein is probably due to the higher concentration of sulfur-rich amino acids from the whey proteins. Southward and Goldman (1978) measured the PERs of high-, medium- and low-calcium co-precipitates at 2.91, 2.78 and 2.72, respectively.

Examination of amino acid composition may also be useful in assessing nutritional value. Amino acid analyses of co-precipitates have been determined by Resmini et al. (1971) and Lohrey and Humphries (1976). Resmini et al. claimed the biggest change in amino acid patterns from casein to co-precipitate was in the percentages of cysteine/cystine, alanine, proline and aspartic acid. Southward and Goldman (1975) though, point out that the analysis of the amino acids cysteine/cystine, which are low in both casein and co-precipitate, is subject to some error in determination.

Applications

The use of co-precipitates in food products is well-documented in the literature. Co-precipitates combine a wide range of physical and functional properties with superior nutritive properties which allow for tremendous potential as an ingredient in a variety of food products.

In baked cereal foods, co-precipitates can supplement lysine-deficient cereal proteins. A high-calcium co-precipitate was selected as the milk protein source for the Australian milk biscuit (Townsend and Buchanan, 1967) and for a milk biscuit "pre-mix" (Henderson and Buchanan, 1970). Some workers (Marston, 1971; Craig and Colmey, 1971) have indicated that co-precipitates have potential for use in the bread baking industry either for protein fortification or as a replacement for nonfat dry milk. Goldman (1973) found that insoluble and dispersible co-precipitates with relatively low farinograph water absorption produced doughs with better consistency than did soluble co-precipitates.

Presently, sodium caseinate and NFDM are the main milk protein products added to meat systems. Thomas et al. (1973) evaluated the use of co-precipitates in sausages. Replacement of 40% of meat protein with high-calcium co-precipitate produced sausages that were not significantly different from controls in general acceptibility. Thomas et al. (1976) produced a meat loaf type canned product using a low-calcium co-precipitate. The product resembled meat in chewiness, sliceability, appearance and flavor. Thomas et al. (1978) found that beef patties extended with 5% medium-calcium co-precipitate and 2.5% wheat flour had better appearance, flavor, texture and acceptibility than all-meat controls.

Co-precipitates have also found some applications in other dairy products. Modler (1974) notes that co-precipitates of casein and whey can be used to increase cheese yields. Thomas (1969, 1970) reported that the incorporation of co-precipitates in processed cheese prevented browning reactions, and improved the body and texture. Stavrova and Mochalva (1978) produced a spread-type dairy product using high-calcium insoluble co-precipitate.

Functional Properties

Functional properties are physico-chemical properties which give information on how a protein will behave in a food system (Hermansson, 1979). For protein ingredients, these properties can be useful in determining fields of application. Table 4 summarizes some specific functional properties and applications for proteins. Functional properties are influenced by protein source, methods of isolation, precipitation and drying, concentration, modification, and environmental conditions like temperature, pH and ionic strength. Chou and Morr (1979) have noted that most functional properties are dependent upon the protein's conformation, solubility, and water-binding properties.

Solubility

Many protein functional properties such as solubility, viscosity, gelation, foaming and emulsification are directly related to the manner in which the protein interacts with water. According to Chou and Morr (1979), the most critical step in imparting a protein's functional property to a food system is its interaction with water to rehydrate, swell

Table 4. Functional properties of proteins and applications.

Functional Property	Food Application
Emulsification	coffee whitener, soups, meat products
Stabilization	soups, meats, desserts
Fat absorption	meat products
Water absorption/retention	meat products, bread, cakes
Viscosity	soups, gravies
Gelation	simulated meats, cheese
Fiber/texture	simulated meats
Dough formation	baked foods
Adhesion/cohesion	meat products, baked foods
Aeration	whipped toppings, chiffons, desserts

Source: Morr (1979)

and solubilize the protein. Mattil (1971) noted that studies of the functional properties of proteins can be made more efficient if one first investigates the solubility properties of the protein in different ionic environments. The solubilization of a protein molecule is a simultaneous process involving wetting, swelling, solvation and dissolution, and is dependent upon conformation, pH, ionic strength, concentration, temperature, mechanical disruptive forces and a number of other factors.

A solubility profile over a range of pH values under standardized conditions can be used as a good guide to protein functionality. Although there are many terms used to describe solubility of food proteins (e.g., water-soluble protein, nitrogen solubility index) there are several basic steps in determining this property. Generally, the

protein is dispersed in water, followed by adjustment of the pH, centrifugation, and determination of the nitrogen or protein content of the supernatant.

Many workers have studied the solubility behavior of sodium caseinate and WPC. Hermansson and Akesson (1975a) examined the solubility of WPC and sodium caseinate in water at room temperature and after heat treatments ranging from 70-100° C. Unheated samples of sodium caseinate and WPC had solubilities of 80.8 and 78.3%, respectively. Heat treatment had little effect on the solubility of either sample. Hermansson and Akesson (1975b) also studied the effects of NaCl on the solubilities of these two proteins. Concentrations of up to 1.0 M NaCl had little or no effect on the solubility of either sodium caseinate or WPC. However, when WPC was heated to 80° C in the presence of salt, solubility decreased. This was thought to occur from increased protein-protein interactions favored by the presence of salt.

Morr et al. (1973) studied the solubility behavior of WPCs prepared by metaphosphate complex, electrodialysis, ultrafiltration, gel filtration, dialysis, CMC complex and iron complex. The solubilities for iron, CMC and metaphosphate complex WPC were highly dependent on pH. McDonough et al. (1974) studied the properties of WPC produced by ultrafiltration. They found that the solubility of this WPC remained constant over a variety of treatments, e.g., pasteurization, evaporation and spray drying. Sternberg et al. (1976) reported that the solubility of whey proteins isolated with polyacrylic acid was independent of pH over the range of 3.0 to 9.0. The solubility of this product at pH 6.5 decreased at concentrations greater than 10%. Modler and Emmons (1977)

prepared a WPC from sweet whey by adjusting the pH to 2.5-3.5, heating at 90° C for 15 min and adjusting the pH to 4.5. This product had a minimum solubility of 78%. Jost and Monti (1977) used a partial enzymatic hydrolysis by trypsin to increase the solubility of ultrafiltered WPC. Monti and Jost (1978) compared the efficiency of pancreatic trypsin, papain and bacterial protease for solubilization of heat-denatured whey, and found that trypsin worked best.

Buchanan et al. (1965) noted that a high-calcium co-precipitate, unlike casein, would not dissolve or disperse at neutral pH without the addition of a calcium sequestering agent. Consequently they dispersed this product in 4% sodium tripolyphosphate (STPP). Smith and Snow (1968) examined the factors affecting aqueous dispersions of co-precipitates. They found that a low-calcium co-precipitate could be dispersed by pH adjustment alone. In order to dissolve a medium-calcium co-precipitate at neutral pH, STPP was added at a 2-4% level to the mixture of product and alkali. High-calcium co-precipitate was dissolved at neutral pH by addition of 6% STPP. None of the co-precipitates were as soluble as sodium caseinate at pH 7, with the insoluble fraction consisting mainly of whey proteins. Southward and Aird (1978) examined the solubilities of several co-precipitates at pH 7 by stirring 3.2% solutions for 60 min at 60° C. Acid co-precipitate was found to be 95-99% soluble. The medium-calcium product was 70-90% soluble, but increased to 95-98% in the presence of 2% STPP. High-calcium co-precipitate was only 10-20% soluble, but increased to 90-95% with the addition of 6% STPP.

Whipping Properties

Foaming or whipping pertains to the capacity to form stable foams with air. Food foams generally consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant (Kinsella, 1976). In protein foams, the surfactant protein reduces surface tension and forms structural, cohesive films around the air droplets. Generally whipping properties are measured as foam expansion, foam capacity or overrun. These terms relate to the volume increase of a protein dispersion following the incorporation of air. Foam stability refers to the ability of a formed foam to retain its maximum volume over time.

Webb (1941) made one of the first attempts to utilize milk protein foams by studying overrun and stability of whipped, reconstituted NFDM. High total solids, low temperature and addition of acid all increased foam stability. Also, high heat-treated powders produced superior foam capacity on reconstitution. El-Rafey and Richardson (1944) studied the foaming properties of the casein, lactoglobulin and lactalbumin fractions of milk protein. For the casein fraction, best foam stability was achieved at temperatures between 0 and 55° C and at fat levels below 0.15%. Foam stability increased with concentration to a maximum value, then decreased. The lactoglobulin fraction exhibited limited stability. Foam stability for the lactalbumin fraction increased with concentration to a maximum value, then leveled off. Optimum temperature was between 5 and 25° C.

The whipping ability of whey proteins has been studied prolifically over the last ten years. Hansen and Black (1972) examined the whipping properties of whey protein-CMC complexes. Best foams were achieved at a

4% level of the complex in water. Heat treatment (75° C/15 min) proved detrimental to foam stability, but sugar addition was found to stabilize foams when added immediately after whipping. Jelen (1973) found that delactosed cheese whey produced poor overruns and stability. Heat treatment and higher total solids improved stability. Morr et al. (1973) investigated the functional properties of WPC (metaphosphate complex, electrodialysis, ultrafiltration, gel filtration, dialysis, CMC complex and iron complex). None of these products produced as high an overrun as sodium caseinate. The CMC complex WPC produced the most stable foam.

Richert et al. (1974) studied the effect of heat treatment, pH, calcium concentration, redox potential and sodium lauryl sulphate upon the foaming properties of WPC. Heating temperature and redox potential affected overrun, while foam stability was affected by pH. Heat treatment of 70° C at pH 7.0 under oxidizing conditions gave the best overrun and most stable foam. Kuehler and Stine (1974) hydrolyzed WPC with pronase, prolase or pepsin. A limited amount of hydrolysis increased the foaming but stability was greatly decreased. This was thought to be due to an increased polypeptide content which allowed air to be incorporated. The polypeptides, though, did not have the strength to give a stable foam. McDonough et al. (1974) found that the total solids concentration was critical in ultrafiltered WPC foams, with 25% solids providing improved whippability. DeVilbiss et al. (1974) attempted to bake angel food cakes using WPC in place of egg white. The WPC did not function well, as the foam structure was not strong enough to retain loaf volume during baking. Morr (1979) related this failure in angel

food cake to lower sulfhydryl and disulfide content when compared to egg white protein.

Little work has been reported on the whipping properties of co-precipitates. Goldman and Toepfer (1978) compared the overrun and foam stability of egg albumin, soluble high-calcium co-precipitate, sodium caseinate and WPC. Sodium caseinate and co-precipitate whipped alone and with sugar gave lower overruns than egg albumin, whereas WPC gave higher overruns. The most stable unsugared whips were fresh egg albumin and co-precipitate. In the presence of sugar all milk protein products exhibited maximum stability. Southward and Goldman (1978) found that soluble co-precipitates had whipping properties slightly better than sodium caseinate but not as good as those of egg albumin.

Emulsifying Properties

In food processing it is often necessary to blend edible fats and oils with various hydrophilic materials in an emulsion system (e.g., ice cream, salad dressings, gravies, meat emulsions). The use of many emulsifying agents is restricted due to food hygiene and food legislation requirements. Consequently the ability of ingredient proteins to form and stabilize oil/water emulsions in food systems is extremely important (Cante et al., 1979).

The formation of an emulsion proceeds as follows. Oil is split into minute droplets (e.g., by a mixer, homogenizer, etc.) in the bulk phase (usually water). Protein molecules then diffuse from the bulk phase to the oil/water interface where adsorption occurs. This leads to a lowering of the interfacial tension and hence lowers the mechanical

energy required to produce a given emulsion particle size. The particle size of the oil droplets depends on the splitting mechanism or process, as shown by Tornberg and Hermansson (1977). After emulsion formation, the protein film at the interface serves to stabilize the emulsion by retarding coalescence of the oil droplets.

Emulsion capacity and emulsion stability measurements are often used in functional characterizations of proteins. Emulsifying capacity denotes the maximum amount of oil that is emulsified under specified conditions by a standard amount of protein. Emulsion stability relates the ability of an emulsion with a certain composition to remain unchanged.

A brief description of factors involved in the measurement of emulsion capacity (EC) follows. The usual procedure for determining EC involves addition of oil to a protein dispersion until the emulsion inverts to a water/oil emulsion. The registering of the inversion point, however, varies from visual appearance of the viscosity decrease (Swift et al., 1961) to change in the amperage required to drive the mixer (Crenwelge et al., 1970) to change in electrical resistance of the emulsion (Webb et al., 1970). It is not surprising to find variability in EC data among different laboratories. Saffle (1968) has noted that factors such as equipment design, container slope, rpm of blending, rate of oil addition, temperature, pH, protein source, type of oil used and ionic strength all affect EC determinations. It is also worthwhile to note that the relationship between EC and the amount of protein required to produce a satisfactory emulsion is unclear since most emulsions

contain an amount of oil less than that required for phase inversion (Pearce and Kinsella, 1978).

Richert (1972) found that a WPC provided better emulsion stability than either sodium caseinate or egg albumin when 2.5% dispersions were mixed with corn oil. The WPC emulsion was stable for more than two weeks. Morr et al. (1973) studied the EC of seven different WPC. Those prepared by metaphosphate complex, electrodialysis, ultrafiltration, gel filtration, dialysis and iron complex had similar EC values. A CMC complex WPC had an EC value about double the others. Kuehler and Stine (1974) found that the EC of WPC decreased upon enzymatic hydrolysis. Tornberg (1978b) measured the creaming stability of several protein stabilized emulsions and found WPC to be a better stabilizer than sodium caseinate. Pearce and Kinsella (1978) examined the emulsifying activity index for whey protein powder and several isolated milk proteins using a turbidimetric technique. This index has units of area of interface stabilized per unit weight of protein. Bovine serum albumin demonstrated the best emulsifying activity, followed by sodium caseinate, β-lactoglobulin and whey protein powder. Cante and Moreno (1975) patented an edible whippable emulsion which contained the proteose-peptone fraction of cows' milk as the proteinaceous emulsifier. The authors felt that the proteose-peptone moieties were linear with hydrophilic and hydrophobic groups distributed along the length of the molecules. Thus, the protein could fit itself to the curve of the oil droplets in the emulsion.

Pearson et al. (1965) studied the EC of potassium caseinate and NFDM. Potassium caseinate was most effective as an emulsified at pH10.5

and low ionic strength. The EC of NFDM was greatest at pH 5.6 regardless of ionic strength. Protein solubility was found to be closely related to EC. NFDM emulsions were most stable at pH 5.4 and ionic strength of 0.3 while potassium caseinate emulsions were quite stable at all pH values at ionic strength 0.3. Sabharwal and Vakaleris (1972) studied the emulsifying properties of sodium caseinate in oil/water systems containing 4% coconut fat both with and without other emulsifiers. In the absence of emulsifiers, emulsion stability increased up to 0.4% sodium caseinate, then decreased. With 0.5% emulsifier (HLB 11) added, stability increased up to 0.5% sodium caseinate, then decreased. Smith and Dairiki (1974) studied emulsion stability with sodium caseinate and NFDM. In 25% milk fat emulsions, sodium caseinate alone acted as an emulsifier with optimum activity at 0.5%, while the NFDM gave best emulsion stability at a 6% level.

Emulsifying properties of co-precipitates have been studied to some extent. Thomas et al. (1974) examined the emulsifying properties of calcium co-precipitate in a model meat system. Three types of calcium co-precipitate improved the emulsifying capacity of the system when replacing 20% of the meat at pH 5.6. Emulsion stability was improved when 20% of the meat was replaced with a soluble high-calcium co-precipitate at pH 5.6. Chojnowski et al. (1975) found that calcium co-precipitate and milk protein concentrate (co-precipitate not neutralized) showed good emulsion stabilizing properties. The emulsifying capacity of sodium caseinate, and high-, medium- and low-calcium co-precipitates were investigated by Gronostaiskaya and Kholvdova (1978). Sodium caseinate possessed a greater EC than the co-precipitates. When used as

emulsifiers with sunflower seed oil, emulsions were produced with fat phase content at protein concentration of 80% at 0.2%, 80% at 0.25-0.30%, 79% at 0.4% and 74% at 0.94% for sodium caseinate, low-, medium- and high-calcium co-precipitates, respectively. Southward and Goldman (1978) noted that high-, medium- and low-calcium co-precipitates had emulsion stabilizing properties similar to sodium caseinate.

Water Holding Capacity

The ability of proteins to bind and immobilize water is one of the most important functional properties in food applications (Chou and Morr, 1979). Water holding capacity is a quantitative measure of the amount of water retained within a protein matrix under certain defined conditions, and usually includes entrapped water. Other terms frequently seen in the literature are water adsorption, which refers to the water adsorbed by a dry protein powder after equilibration against water vapor of a known relative humidity, and swelling, which refers to the spontaneous uptake of water. The degree of water binding in a food system is much more complex, and is influenced by pH, ionicity, temperature and protein concentration.

The mechanism of water binding has been reviewed by Kinsella (1976) as it applies to pure proteins. Pure proteins sorb water by binding at specific hydrophilic sites (OH, NH_2, COOH, C=O) at low water activities, followed by multimolecular adsorption and clustering at higher activities. These polar sites bind an average of four to eight water molecules. Other factors can affect the water binding in food systems. For instance, salts can have a dramatic effect. Bull and

Breese (1970) found that monovalent cations decreased the amount of water bound to egg albumin in the order $Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$.

The water adsorption of several pure milk proteins has been measured. Bull (1944) measured the amount of water sorbed by lyophilized β-lactoglobulin and bovine serum albumin to be 28.25 g water/100 g dry protein and 28.70 g water/100 g dry protein, respectively. Ruegg et al. (1974) found the water binding of casein to be 0.435 g water/g dry mass. Berlin et al. (1970) used differential scanning calorimetry to estimate the extent of water binding of casein, bovine serum albumin and β-lactoglobulin at 0.553, 0.498 and 0.553 g water bound/g protein. Hagenmaier (1972) measured the water adsorption of casein, and determined this to be 21.6 g water/16 g N. Water adsorption was independent of pH.

Pure proteins (those free of salts, fat, carbohydrates) are generally not used in food systems, and hence the water bound by protein concentrates is more useful to know. Berlin et al. (1973), while studying the water adsorption properties of WPC, noticed that products containing more low molecular weight materials (e.g., lactose, salts) bind more water. The authors also noted that thermal denaturation did not significantly change water adsorption. McDonough et al. (1974) studied the water affinity of ultrafiltered WPC. Degree of entrapped water was measured by adding the protein to skimmilk and heating at 85° C for 5 min. WPC gave better entrapment of water than egg albumin via a classical gel structure. Delaney (1976) measured the water absorptive capacities of casein, sodium caseinate, NFDM and ultrafiltered WPC to be 68%, 250%, 70% and 50%, respectively, by a Farinograph method. The water

uptake of ultrafiltered WPC was measured by de Wit and de Boer (1975) and was found to increase after heat treatment to about twice the powder weight. Sternberg et al. (1976) compared the water hydration capacities of coagulated WPC (isolated with polyacrylic acid) and egg white, and found that the WPC gave slightly lower values. Quinn and Paton (1979) examined the water hydration capacity of a number of protein concentrates. Sodium caseinate and WPC had values of 2.33 and 0.97 ml water/g sample, respectively. Hermansson and Akesson (1975a, 1975b) investigated the effects of heat and salt on swelling properties of sodium caseinate and WPC. In this study, swelling was defined as the ability to absorb water in amounts smaller than those producing low viscosity dispersions. The swelling ability of casein decreased slightly with increasing temperature, whereas the swelling ability of WPC increased. The WPC swelled to a lesser extent than sodium caseinate, though. Swelling was also measured at ionic strengths ranging from 0 to 1.0 at 25° and 80° C. Sodium caseinate showed a decrease in swelling ability with increasing ionic strength at the lower temperature, while swelling increased slightly with ionic strength at 80° C. WPC showed no change in swelling with ionic strength at 25° C, but showed a slight decrease at 80° C.

Viscosity

Viscosity is a flow property and often reflects changes in hydrodynamic properties due to absorption of water. Knowledge of the viscosity of protein dispersions is of practical importance in relation to processing, new product development, designing of quality control tests,

and mouth-feel and physical appearance. Flow properties are governed by the molecular size, shape, charge, solubility and swelling capacity of proteins. These are affected by temperature, concentration, pH, ionic strength and previous processing history (Hermansson, 1975).

A knowledge of the solubility and swelling properties of a protein enables one to predict viscosity. Highly soluble, nonswelling proteins (e.g., albumins, globulins) have low viscosity. Soluble proteins with high initial swelling (e.g., caseinate) show a concentration dependent viscosity, reflecting the amount of swelled, but not fully solvated particles. Proteins with high limited swelling capacity show a relatively high viscosity at low concentrations (Hermansson and Akesson, 1975a).

Dolby (1961) studied the viscosity of lactic casein and found that the pH of coagulation was important. The viscosity index increased as the precipitation pH increased due to increase retention of calcium in the casein. Washing temperature and drying time also affected viscosity slightly. Hayes and Muller (1961) investigated viscosity-pH relationships of acid casein in various alkalis. Confirmation of the logarithmic relationship between concentration and viscosity was made. Heated casein showed increased viscosity. The authors also confirmed the role of calcium in determining viscosity. In casein solutions near neutral pH, increased calcium had only a minor effect on viscosity, but at values greater than 8, a sharp rise in viscosity occurred with increases in calcium. Towler and Dolby (1970) studied the effects of precipitation pH, temperature of precipitation, acidulation time and wash conditions on viscosity of casein. High precipitation pH and temperature, short

acidulation time and washing in $CaCl_2$ solution favored a high viscosity casein product. These conditions also favored high calcium retention in the curd.

Richert (1972) produced WPC by precipitation with sodium hexametaphosphate followed by neutralization with $Ca(OH)_2$ to remove the phosphate. Viscosity was extremely low for 2.5% solutions. However, a 15% WPC solution had a higher viscosity than a 15% egg albumin solution. The viscosity of 10% protein solutions of ultrafiltered WPC at different pH values and different heat treatments was studied by de Wit and de Boer (1975). At pH 6.7, the viscosity was constant up to 70° C, but showed a sharp increase at higher temperatures. At high and low pH values, the viscosity measurements showed sharp increases at lower temperatures. Hermansson (1975) examined the flow properties of caseinate and WPC solutions as affected by concentration, ionic strength and pH. At concentrations below 12%, caseinate exhibited Newtonian flow characteristics, whereas higher concentrations yielded pseudoplastic behavior. Addition of salt caused an increase in viscosity, although no changes in swelling or solubility occurred. Caseinate had a very complex, pH-dependent viscosity which was maximum at pH 9.8-10.0. In contrast, pH and ionic strength had little effect on the viscosity of WPC. WPC exhibited Newtonian flow behavior at concentrations below 12%.

Viscosities of co-precipitates were studied by Hayes et al. (1969). A low-calcium co-precipitate exhibited non-Newtonian flow, and viscosities were somewhat higher than casein. With added calcium, viscosities for the co-precipitates increased at pH values above 7.0. This response to added calcium was different than that observed with casein, and was

attributed to the influence of protein-protein linkages formed during the manufacture of co-precipitates. Chojnowski et al. (1975) found that viscosity varied for milk co-precipitates neutralized by different salts. Southward and Goldman (1978) measured viscosities of high-, medium- and low-calcium co-precipitates. Apparent viscosities decreased with increasing shear rate, indicating pseudoplastic flow behavior at concentrations of 150 g/kg and 100 g/kg. At each concentration the viscosity of the co-precipitates decreased in the order of high-, medium- and low-calcium preparations.

Effect of Heat on Milk Proteins

Casein

The caseins exist as random coils in comparison with other protein secondary structure (Payens and Schmidt, 1965). Hence, in the chemical sense, caseins are considered to be "denatured", having little, if any, α-helix or β-structure. However, caseins do exhibit a native quaternary structure due to self-association with other caseins. Because the casein micelles are stabilized by hydrophobic, electrostatic and to a limited extent hydrogen bonding, temperature can drastically affect micelle alterations.

Casein is not regarded as a heat-coagulable protein. In normal fluid milk, it is very stable to heat and may resist coagulation for as long as 14 hr at boiling temperatures and 1 hr at 130° C. Heat coagulation of casein in normal milk occurs as a result of increased acidity, the conversion of soluble calcium and phosphates to colloidal forms

and interactions (e.g., denaturation, hydrolysis) between protein components.

Prolonged heating or heating above 100° C causes decomposition of caseins with the release of inorganic phosphorus and nonprotein nitrogen (Pyne, 1962). This release of phosphorus is related to acidity development in heated milk. Belec and Jenness (1961a, 1961b) studied dephosphorylation of sodium caseinate and skimmilk casein heated at 110-140° C. Dephosphorylation conformed to first-order kinetics with an energy of activation of 25-29 kcal/mole. The release of phosphate was independent of pH in the range 6.0-7.0 and was greater for α- than for β-casein. At a given temperature, dephosphorylation was slower in skimmilk than in sodium caseinate sols. Alais et al. (1967) studied the effect of heating at 120° C for 20 min on α_s-, β-, and κ-casein solutions. All three casein fractions were affected, with β-casein being the most resistant to heat action and α_s-casein the most labile. For κ-casein, the action of rennin and heat were somewhat similar, indicating the existence of labile linkages which are split by different processes. Zittle (1969a) showed that κ-casein heated at 96-100° C for 5 min in 0.05 M NaCl lost its ability to stabilize α_s-casein against precipitation by calcium ions, possibly by aggregation through disulfide bonds. The presence of α_s-casein in a 1:1 weight ratio when the κ-casein is heated prevents the heat lability of kappa (Zittle, 1969b). This fact plus the stability of κ-casein heated in 0.15 M $CaCl_2$ indicate that the aggregation of κ-casein in heated milk is not significant.

Fox et al. (1967) examined the nonsedimenting nitrogen of milk heated for 30 min at various temperatures. They found that heat

treatments greater than 110° C resulted in greater amounts of nonsedimenting nitrogen, and that this fraction consisted primarily of casein. This effect was attributed to disaggregation of the casein micelles. Josephson et al. (1967) noted that heating milk at 80° C for 30 min produced no noticeable alterations in the size, shape or charge density of casein micelles. Morr (1969) supported the finding of Fox et al. (1967) by the observation that small amounts of nonsedimenting casein components were observed with UHT milk.

Serum Proteins

Serum proteins are quite soluble, even at their isoelectric pH values. These proteins are not precipitated nor rendered nondispersible by routine heat treatments at normal pH. Whey proteins are sensitive to heat at temperatures above 65° C. Harland et al. (1952) studied the effects of heating time and temperature on serum protein denaturation in heated skimmilk. In the temperature range of 62-80° C the relationship of temperature to time for a constant level of serum protein denaturation is semilogarithmic. The heat treatments required for pasteurization of milk were below serum protein denaturing conditions. Larson and Rolleri (1955) examined the heat denaturation of specific serum proteins in milk by a quantitative electrophoretic analysis of the serum from heated skimmilk. Their classical denaturation curves place the immune globulins, bovine serum albumin, β-lactoglobulin and α-lactalbumin in order of increasing resistance to heat denaturation. Complete denaturation of the serum proteins was achieved by heating at 77.5° C for 1 hr or 90° C for 30 min. Morr and Josephson (1969) note that there are

three stages in whey protein aggregation. The first is denaturation, in which the proteins unfold from a compact, globular conformation to a random configuration. Following this, the proteins aggregate to form intermediate sized complexes which are not sedimented at 1000 x g. Finally, aggregation in the presence of calcium ions forms precipitate particles that are sedimentable at 1000 x g.

β-Lactoglobulin is the major whey protein of cows' milk, and many heat-induced changes in milk have been correlated to the reactions of this protein. At neutral pH, β-lactoglobulin (β-lg) is thought to exist as a 36,000 molecular weight dimer containing two sulfhydryl groups and four disulfide linkages. The SH-groups are buried in the dimer complex and exhibit low reactivity. Sawyer (1968) has proposed a probable pathway for the heat denaturation of β-lg near pH 7. The initial effect of heat treatment is an increase in dimer dissociation to 18,000 molecular weight monomers. This is followed by molecular unfolding, in which the SH-group is fully exposed. The primary aggregation reaction occurs as a result of the unfolded monomer units associating via disulfide interchange or sulfhydryl oxidation. The final stage is a secondary reaction involving a nonspecific aggregation (not resulting from the formation of intermolecular disulfide bonds) and a heavy, 29S component is formed. This heavy component undergoes further aggregation and opalescence develops in the solution. The susceptibility of the three genetic variants to denaturation was found to be in the order C > B > A.

Watanbe and Klostermeyer (1976) examined heat-induced changes in sulfhydryl and disulfide levels of β-lg. They found that SH levels decreased and SS levels increased in β-lg solutions heated at 95° C, and

concluded that SH-initiated-SS exchange reactions were important in the formation of high molecular weight polymers in the presence of air. Hillier and Lyster (1979) studied the denaturation of whey protein in heated milk and determined that the denaturation of β-lg followed second-order kinetics.

The aggregation and precipitation of heat-denatured β-lg is dependent on pH and the presence of calcium ion. Zittle and DellaMonica (1956) observed a progressive decrease in the calcium ion concentration required to precipitate heated β-lg as the pH was lowered. The calcium bound at negatively charged carboxyl sites on the protein, reducing the net charge to zero, and bringing about isoelectric precipitation.

The second major whey protein of bovine milk is α-lactalbumin (14,400 daltons) and it contains no SH groups and four disulfide linkages. This protein is the most heat stable of all the serum proteins. The denaturation of α-lactalbumin appears to be first-order, but is felt to be a second-order reaction displaying pseudo first-order kinetics (Hillier and Lyster, 1979). There is a lack of information available on the effects of heat on α-lactalbumin.

There have been reports of an interaction between α-lactalbumin and β-lg in heated systems. Hunziker and Tarassuk (1965) obtained chromatographic evidence for this heat-induced interaction in phosphate buffer at pH 6.7. Lyster (1970) implicated the SH group of β-lg as being important in the denaturation of α-lactalbumin. Elfagm and Wheelock (1977a) reported that α-lactalbumin interacts with an aggregated form of β-lg when heated in synthetic milk ultrafiltrate. Melo and

Hansen (1978) obtained electrophoretic evidence for this complex in UHT milk.

κ-Casein and β-Lactoglobulin Interaction

The complex formation between β-lg and κ-casein during heat treatment of milk is believed to be of importance in the manufacture of co-precipitates consisting of whey proteins and casein (Southward and Goldman, 1975). Therefore, examination of the existing literature on this subject is in order.

The first information regarding the interaction of casein and β-lg was provided by Tobias et al. (1952) and Slatter and van Winkle (1952), who detected a possible interaction by moving-boundary electrophoresis of heated skimmilk. DellaMonica et al. (1958) studied the effects of heat and $CaCl_2$ on solutions of β-lg and casein. Heated β-lg solutions were very sensitive to precipitation by calcium, while a heated mixture of 1% β-lg and 2% casein would not precipitate until the calcium concentration was close to that required to precipitate casein alone. It was concluded that no complex was formed and that β-lg precipitated independently of casein. Trautman and Swanson (1958) observed that in skimmilks heated to 180° F for 30 min, two-thirds of the original β-lg disappeared from the electrophoretic patterns. However, skimmilks containing sulfhydryl-blocking agents gave electrophoretic patterns similar to unheated skimmilk. Trautman and Swanson (1959) later related this complex formation to heat stability in evaporated milks. Kannan and Jenness (1961) suggested that formation of a complex between β-lg and casein in heated milk interferes with the primary action of rennet,

causing an increased coagulation time.

Zittle et al. (1962) heated a mixture of κ-casein and β-lg at 90° C for 15 min and noted a component of intermediate electrophoretic mobility and a S_{20} value three times greater than that of κ-casein. They also observed that mixing heated β-lg and unheated κ-casein at room temperature caused complex formation. Evidence implicating the possibility of intermolecular disulfide bond formation between β-lg and κ-casein was presented by Sawyer et al. (1963). Kresheck et al. (1964) calculated a weight average molecular weight for the complex formed from heated mixtures of β-lg and κ-casein to be 6.54×10^6. Hartman and Swanson (1966) used polyacrylamide gel electrophoresis (9% T) to investigate this complex. Complex formation was complete when equal parts by weight of β-lg and κ-casein were heated at 85° C for 30 min. The complex was able to enter the spacer gel, but not the running gel. No complex was formed when κ-casein was heated with either bovine serum albumin or α-lactalbumin.

Grindrod and Nickerson (1967) reported that the heat-induced complex of β-lg and κ-casein did not enter polyacrylamide gels (7% T). However, in urea and 2-mercaptoethanol, the proteins migrated the same as unheated controls, indicating possible complex dissociation. It was also noted that hydrogen peroxide would not induce complex formation. Purkayastha et al. (1967) did not observe complex formation when β-lg was heated with alkylated κ-casein, or when β-lg was heated with κ-casein in the presence of N-ethylmaleimide. These workers also observed complex dissociation in polyacrylamide gels containing urea and 2-mercaptoethanol, and concluded that thiol-disulfide interchange was

important in complex formation. In further model system work, El-Negoumy (1974) studied the β-lg/κ-casein interaction in various salt solutions using a heat treatment of 110° C for 30 min. A minimum interaction of 13.8% β-lg and 17.1% κ-casein occurred in de-ionized water, while a maximum of 76.6% β-lg with 83.3% κ-casein occurred in milk dialysate.

The effect of pH on protein aggregation in heated milk was investgated by Creamer et al. (1978). Milk samples at pH 6.50, 6.65 and 6.80 were heated at 100° C for 30 min. Electron microscopy of the heated sample of pH 6.65 milk revealed small protuberances attached to the micelle surface. The pH 6.50 milk had small irregular attachments on the micelles, whereas the pH 6.80 milk had fewer attachments on the micelles and many interspersed particles that were irregularly shaped. The heated pH 6.65 sample was chromatographed over Sepharose 4B and the void volume was found to contain κ-casein and whey proteins, but no other proteins. Mercaptoethanol and 6 M urea were necessary to resolve the void volume fraction by gel electrophoresis. Amino acid analysis of this fraction was not markedly different from a 4:1 mixture of whey protein and κ-casein. In a study on gel formation in yoghurt, Davies et al. (1978) also noted the appearance of appendages on the micelles of milk heated to 95° C for 10 min. These appendages appeared to give rise to a firmer curd with lower tendency to syneresis.

The exact nature of the β-lg/κ-casein complex is still unknown. Many of the earlier studies were performed in buffers employing isolated milk proteins at protein concentrations different from that found in milk. Tessier et al. (1969) were unable to demonstrate the existence of such a complex in heated milk. Thus, the involvement of sulfhydryl

groups in complex formation in heated milk remains unanswered. Moreover, if a complex is formed between β-lg and κ-casein in heated milk, it probably is not identical to that found in heated model systems. El-Negoumy (1974) noted that various salts or salt mixtures could enhance or suppress thiol group reactivity, which would lead to a variable degree of interaction and complex formation.

Beeby et al. (1971) pointed out that sulfhydryl groups may not be necessary for the formation of the β-lg/κ-casein complex, although they may be involved in the primary reaction of β-lg aggregation. Lyster (1970) noted that three forms of denatured β-lg existed: one involving disulfide interchange and two others involving hydrophobic, hydrogen and ionic bonding. Long et al. (1963) observed that heated β-lg formed a complex with heated or unheated κ-casein, suggesting the possible involvement of noncovalent interaction. McKenzie et al. (1971) has suggested that disulfide bonds may be a factor in the stability of the complex, but some less specific associations (e.g., physical entanglement of polypeptide chains) may also occur. Morr and Josephson (1968) proposed that denatured whey proteins are stabilized against heat-induced gross aggregation in skimmilk by complexing with casein micelles through calcium-dependent linkages.

Recently, Shalabi and Wheelock (1976) demonstrated that α-lactalbumin inhibited the primary phase of chymosin action on casein micelles, thus implicating this protein as part of a heat-induced comples. Baer et al. (1976) were able to show a heat-induced interaction between α-lactalbumin and β-lg in a model system. Elfagm and Wheelock (1977a) also demonstrated this, and felt that the α-lactalbumin interacted with

an aggregated form of β-lg. Elfagm and Wheelock (1977b) found that in heated milk, casein facilitates the interaction of α-lactalbumin and β-lg, supporting their view that a complex between β-lg, α-lactalbumin and κ-casein occurs. They proposed a mechanism of action which involves the initial formation of a complex between α-lactobumin and β-lg, followed by an interaction of this complex with κ-casein.

EXPERIMENTAL

Chemicals and Materials

Chemicals

The principal chemicals used in this study and their sources are listed in the Appendix, Table A1. All were reagent grade unless otherwise indicated. Urea solutions were passed over a mixed bed ion exchange column (bed dimensions, 19 mm x 35 cm) consisting of 50% Dowex 50x8 and 50% Dowex 2x8 to remove residual cyanate. Column bed was regenerated with 1.0 M NaCl. Distilled, deionized water was used in all experiments unless otherwise indicated.

Commercial Products

Grade A, low heat NFDM was obtained from Michigan Dairy Producers Co. Sodium caseinate and EnrPro 50, a whey protein concentrate, were obtained from Stauffer Chemical Co., Westport, Connecticut. Sodium Protolac, a metaphosphate precipitated whey protein concentrate, was obtained from Borden, Inc., Columbus, Ohio.

Milk

The milk used in this study was obtained from the Michigan State University dairy herd which consisted of Holstein cows. All milk was obtained immediately after milking and was separated as soon as possible at 37-40° C on a Westfalia separator. The skimmilk was held at 4° C overnight and used the next day.

Equipment

All equipment used regularly during the course of this study will be discussed here. Apparatus specific for certain experiments will be referred to in the appropriate section.

All experiments were performed using stainless steel, plastic or Pyrex containers. An Instrumentation Laboratory pH/mV Electrometer, model 245, was used for all pH measurements. Protein samples were dried from the frozen state by a laboratory-constructed lyophilizer. For most laboratory weighings, a top-loading digital Sartorious type 3716 balance was used. For analytical weighings, a Sartorious type 2433 balance was used.

Low-speed centrifugations were performed with International Clinical or International Model U centrifuges. Intermediate-speed centrifugations were performed on a Sorvall, type RC2-B centrifuge equipped with temperature control.

A Bausch and Lomb Spectronic 21 was used for spectrophotometric determinations.

Vertical acrylamide gel electrophoresis was performed with electrophoretic apparatus manufactured by Buchler Instruments, and using a Bio-Rad model 400 power supply. Gels were destained in a Bio-Rad model 170 diffusion destainer mounted on a Cole-Parmer model 4815 magnetic stirrer. Pictures of the gels were taken using a Polaroid MP-3 Land Camera.

Preparative Procedures

Acid Co-precipitate

Three liters of skimmilk were placed in a stainless steel container and brought to a temperature of 95° C. After heating for 30 min, the milk was cooled to 30° C and the pH adjusted to 4.6-4.8 with 1 N HCl. The precipitate was collected by centrifugation at 2000 rpm for 15 min. The precipitate was washed once with a small amount of water, then dispersed at pH 7.0, lyophilized and stored at 0° C.

pH 3.5 Co-precipitate

Three liters of skimmilk were acidified to pH 3.5 with 1 N HCl. This milk was then heated in a stainless steel container at 95° C for 30 min. After cooling to 30° C, the pH was adjusted to 4.8 with 1 N NaOH. The precipitate was collected by centrifugation, washed once with water, dispersed at pH 7.0, lyophilized and stored at 0° C.

Hexametaphosphate Co-precipitate

Three liters of skimmilk were brought up to room temperature and the pH adjusted to 3.0 with 1 N HCl. Sodium hexametaphosphate was then added to give a final concentration of 0.5% (w/v). The precipitate was collected by centrifugation, washed once with water, dispersed at pH 7.0, lyophilized and stored at 0° C.

Calcium Co-precipitate

Three liters of skimmilk were heated in a stainless steel container at 95° C for 30 min. Immediately after heating, 20.5 ml of a 30% $CaCl_2$

solution were added to give a $CaCl_2$ concentration of 0.20% (w/v). The precipitate was collected by centrifugation, washed once with water, dispersed at pH 7.0, lyophilized and stored at 0° C.

Chemical Methods

Nitrogen

A semi-micro Kjeldahl method was used for the nitrogen analysis (Swaisgood, 1963). The digestion mixture consisted of 5.0 g SeO_2 plus 5.0 g $CuSO_4 \cdot 5H_2O$ in 500 ml of concentrated sulfuric acid. Approximately 10 mg of dried sample was digested in 4 ml of the digestion mixture over a gas flame for about 1 hr. The flasks were then cooled for 15 min followed by the addition of 1 ml of 30% hydrogen peroxide. Digestion was then continued for an additional hr. Each flask was cooled and rinsed with water. The flask was then placed on distillation apparatus and neutralized with 25 ml of 40% NaOH. The released ammonia was steam distilled into 15 ml of 4% boric acid containing 5 drops of indicator. The indicator contained 400 mg of bromocresol green plus 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was complete when a total volume of 80 ml was collected in the receiving vessel. The ammonia-borate complex was titrated with 0.02 N HCl. A reagent blank and a tryptophan standard were analyzed to determine the average percent recovery of nitrogen, which ranged from 95-99%. Nitrogen analyses were performed in triplicate. Nitrogen was calculated as follows:

$$\%N = \frac{(\text{ml HCl} - \text{ml blank})\ (\text{Normality of HCl})\ (\text{meq. wt. N})}{\text{g of sample}} \times 100$$

Fat

The method of Hood (1973) was used for the determination of fat. Specific details of the method can be found in the Appendix, Table A3.

Moisture

Moisture was determined by a standard AOAC (1970) procedure. Approximately 0.5 g of sample was weighed into a previously tared metal dish and covered. This was placed into a vacuum oven at 95° C and left for 5 hr. This dish was cooled in a dessicator and reweighed. All moisture analyses were done in duplicate.

Lactose

Lactose was determined by the phenol-sulfuric acid method of Dubois et al. (1956) as modified by Barnett and Tawab (1957). Fifty to 100 mg of dry sample were ground in a mortar with about 5 ml of water. This mixture was transferred to a 50 ml volumetric flask and made to mark with water. A 10.0 ml aliquot was placed in a 25 ml volumetric flask and made to mark with 20% tricholroacetic acid. This mixture was then centrifuged at 7700 x g for 20 min. One ml of the clear supernatant was pipetted into a clean test tube and mixed with 1.0 ml of water. To this mixture was added 1.0 ml of 5% (w/v) phenol and 5.0 ml of concentrated sulfuric acid with thorough mixing after each addition. The test tubes were allowed to stand at room temperature until cool. Absorbance was read at 490 nm. All lactose analyses were performed in triplicate.

Ten ml of a solution consisting of 0.5135 g of lactose in 1000 ml of water were diluted to 100 ml. A standard curve was then prepared covering the range of 0 to 72.0 µg of lactose.

Ash

Ash was determined by the standard AOAC (1970) method. Porcelain crucibles were heated to 550° C in a muffle furnace, cooled and weighed. Two hundred to 300 mg of sample were weighed into the crucibles and a small amount of a 1% $MgCl_2$ solution was added to prevent volatilization of phosphate. The crucibles were then returned to the furnace and ignited at 550° C for approximately 12 hr. The crucibles were cooled in a dessicator and weighed. Ash was defined as the percent of the original sample weight remaining as the residue (after correction for added Mg). Ash analyses were performed in triplicate.

Calcium

Calcium was determined by the EDTA method of Jenness (1953). A 100 to 200 mg sample was accurately weighed into a clean mortar and ground in 2 ml of water. This mixture was washed into a 25 ml volumetric flask, followed by the addition of 15.0 ml of 20% tricholroacetic acid. The flask was made to mark with water and the contents centrifuged at 7700 x g for 20 min. A 10 or 20 ml aliquot of the clear supernatant was passed over a Amberlite IR-4B ion exchange column (bed size, 15 mm x 7 cm). The column was washed twice with 10 ml portions of water. To this 30 ml was added a little less than the expected amount of 0.02 M or 0.005 M EDTA. Sodium hydroxide pellets were dissolved in the mixture to bring the pH to about 13. Approximately 0.2 g of

of murexide indicator was added. The indicator consisted of 0.2 g of murexide plus 100 g of NaCl ground together in a mortar. The mixture was titrated with EDTA to a purple color that did not change with further additions. A blank and calcium standard were periodically analyzed to check recovery. All analyses were performed in triplicate.

Phosphorus

Phosphorus was determined by the method of Sumner (1944) as modified by Swope (1968). Ten to 20 mg of sample were digested with 2.2 ml of 50% sulfuric acid in test tubes. Digestion was carried out on a sand bath heated by an electric heater at a temperature of 160-170° C until the sample was thoroughly charred. The tubes were cooled, and 8 drops of 30% H_2O_2 were added. Heating was then resumed until all the H_2O_2 was driven off. After cooling, the digestion mixture was transferred to a 25 ml volumetric flask. Five ml of a 6.6% ammonium molybdate solution and enough water were added to give a volume of approximately 15 ml. Then 4.0 ml of a freshly prepared ferrous sulfate solution was added. The ferrous sulfate solution was prepared by dissolving 0.5 g of $FeSO_4 \cdot 7H_2O$ in 50 ml of water and adding 1.0 ml of 50% sulfuric acid. The volumetric flask was made up to volume with water and mixed. After standing 30 min to allow for color formation, the absorbance was read at 660 nm. All phosphorus analyses were performed in triplicate.

A stock solution containing 0.2742 g K_2HPO_4 dissolved in 200 ml water was prepared, and 10.0 ml of this solution were diluted to 100 ml (.024 mg P/ml). This solution was used to make a standard curve covering the range of 0 to 0.15 mg of phosphorus.

Amino Acids

Amino acid analyses were performed on HCl hydrolysates of protein using a Beckman Amino Acid Analyzer, Model 120 C, according to the procedures of Moore et al. (1958). Samples consisting of approximately 4 mg of protein were weighed into 10 ml ampoules. Five ml of 6 N HCl were added to the ampoules. The contents were frozen in a dry-ice-ethanol bath and evacuated with a high vacuum pump. As the contents slowly melted, the gases were removed. The contents were then refrozen and the ampoules were sealed using an air-propane flame. The sealed ampoules were placed in an oil bath in a forced draft, recirculating oven regulated at 110° C for 24 or 72 hr.

After hydrolysis, the ampoules were opened and 1.0 ml of norleucine solution (2.5 moles/ml) was added as an internal standard. The hydrolysate was then quantitatively transferred from the ampoule to a 25 ml pear-shaped flask. The hydrolysate was evaporated to dryness on a rotary evaporator. The dried sample was washed with a small amount of water and again taken to dryness. In all, three washings were performed to remove residual HCl. The washed and dried hydrolysate was dissolved in 0.067 M ditrate buffer (pH 2.2) and diluted to a volume of 5 ml. The solution was then filtered with a 0.22 m Millipore Filter, and 0.2 ml aliquots were used for analysis. The chromatograms were quantitated by peak integration using a Spectra Physics Autolad System AA. Standard amino acid mixtures were analyzed using the same ninhydrin solution within a four-day period.

Methionine and Cystine

Since methionine and cystine undergo a variable amount of oxidation during acid hydrolysis, they must be analyzed separately. The methods of Schram et al. (1964) and Lewis (1966) were used. These methods involve performic acid oxidation of methionine and cystine to methionine sulfone and cysteic acid, respectively. Approximately 5-8 mg of protein was weighed into a 25 ml pear-shaped flask. The protein was oxidized for 24 hr with 10 ml of performic acid at 4° C. After oxidation, 1.0 ml of norleucine (2.5 moles/ml) was added. The performic acid was removed on a rotary evaporator. The dried sample was quantitatively transferred to a 10 ml ampoule with 5 ml of 6 N HCl. Hydrolysis and amino acid analysis were performed as previously discussed. Since the yield of cysteic acid is only 94 $\pm$ 2%, the amount of cysteic acid obtained was divided by 0.94 to give a corrected value.

Tryptophan

The method of Spies (1967) was used. Approximately 3 mg of protein were weighed into vials. To this was added 0.1 ml of a pronase solution (10 mg pronase/ml of 0.1 M phosphate buffer, pH 7.5), and vials were incubated for 24 hr at 40° C. Following incubation, samples were placed in ice. To each vial was added 0.9 ml of phosphate buffer (see Appendix, Table A2), and the vials were placed into flasks containing 9.0 ml of 21.2 N sulfuric acid and 30 mg of p-dimethylaminobenzaldehyde. Flasks were placed in the dark for 6 hr to allow the production of a chromophore resulting from the action to free tryptophan with the p-dimethylaminobenzaldehyde. Following this 0.1 ml of sodium nitrite

was added, and the absorbance was read after 30 min at 590 nm. A pronase blank was run to correct for the tryptophan content of pronase. A standard curve was prepared with tryptophan. Analyses were performed in duplicate.

Sulfhydryl (SH) and Disulfide (SS)

The SH and SS content of milk samples were determined by modifying the procedure of Beverage et al. (1974). For total SH, milk was diluted 1:1 with 0.2 M sodium phosphate buffer, pH 8, containing 8 M urea (for buffer preparation, see Appendix, Table A2). To a test tube containing 2.5 ml of the above sodium phosphate buffer and 0.02 ml of Ellman's reagent (40 mg of 5,5'-dithiobis-2-nitrobenzoic acid in 10 ml of 0.2 M sodium phosphate buffer, pH 8) was added 0.5 ml of the diluted sample. Color was developed for 15 min, and the absorbance was read at 412 nm against a blank containing protein and buffer, but no Ellman's reagent.

The SS groups were determined by adding 0.1 ml of the milk sample to a test tube containing 1.0 ml of 0.2 M phosphate buffer, pH 8, containing 10 M urea and 0.02 ml of 2-mercaptoethanol. The tube was incubated for 1 hr in a 25° C water bath. Ten ml of 12% trichloroacetic acid solution were added, and the mixture was incubated an additional hour at 25° C. The mixture was centrifuged at 1000 x g for 50 min. The precipitate was twice resuspended in 5 ml of 12% trichloroacetic acid and centrifuged to remove all traces of the 2-mercaptoethanol. The precipitate was dissolved in 3.0 ml of 0.2 M phosphate buffer, pH 8, containing 8 M urea, and 0.03 ml of Ellman's reagent was added.

Color was allowed to develop for 15 min, and the absorbance was read at 412 nm against an appropriate blank.

Total solids were determined from a standard procedure (AOAC, 1970). Calculation of SH in μmoles/g solids was done according to the following equation:

$$\mu\text{moles SH/g solids} = \frac{73.53 \times A \times D}{C}$$

where A = absorbance at 412 nm

D = dilution factor (12.08 for SH, 30.0 for SS)

C = concentration in mg solids/ml (8.84 for milk, 8.53 for pH 3.5 milk)

73.53 is derived from $10^6/13{,}600$, where 13,600 is the molar absorptivity and 10^6 is for conversion from the molar basis to the μmoles/ml and from mg solids to g solids.

Soluble Protein

Soluble protein was measured by the method of Lowry et al. (1951). Solution A was prepared by dissolving 100 g Na_2CO_3 in 0.5 M NaOH and making to one liter. Solution B was prepared by dissolving 1.0 g $CuSO_4 \cdot 7H_2O$ in water and making to 100 ml. Solution C consisted of 2.0 g potassium tartrate dissolved in water and made to 100 ml. Solution D consisted of 15 ml A, 0.75 ml B and 0.75 ml C. Solution E consisted of 50 ml water plus 5 ml of the Folin-Ciocalteau reagent.

To each tube was added 1.0 ml of a protein solution, followed by 1.0 ml of solution D. This was mixed and allowed to incubate for 15 min. Solution E was prepared during this period. After the incubation, 3.0

ml of solution E were forcibly pipetted into the tube, and the tube was vortexed immediately. This was then incubated an additional 45 min, and the color read at 540 nm.

A standard curve was also constructed with a protein range of 0-300 μg. Bovine serum albumin was the standard protein. The protein content of this protein was determined by Kjeldahl to be 85.3%.

Functional Tests

Solubility

Solubility was determined by a centrifugation method similar to the one used by Mattil (1971). Enough sample to give a 1% protein solution (based on Kjeldahl) in 25 ml was weighed out and ground in a mortar with about 5 ml water. The contents were washed into a small beaker and the volume brought up to 15-20 ml. The pH was then adjusted to either 7.0, 4.5 or 3.0 using 0.5 N HCl or 0.5 N NaOH. The total volume was then made to 25 ml in a volumetric flask. Following stirring for 5 min, the solution was centrifuged at 4300 x g for 20 min. The supernatant was filtered through Whatman #1 filter paper. A 10 ml aliquot was taken and diluted to 500 ml. One ml of this solution was used to determine protein content by the Lowry method. Solubility was determined as follows:

$$\% \text{ solubility} = \frac{200 - \text{g protein in aliquot}}{200} \times 100$$

Duplicate trials were performed for each sample at pH 7.0, 4.5 and 3.0.

Water Hydration Capacity

To determine water hydration capacity (WHC) the method of Quinn and Paton (1979) was modified. WHC, as determined in this manner, represents the amount of water held by a centrifuged pellet without solubilization of the sample. Two g of a sample were weighed into a tared, transparent centrifuge tube. Water was added in small increments and the mixture stirred vigorously until a paste-like consistency was obtained. The tube was centrifuged at 8700 x g for 15 min, and the slight supernatant was discarded. The tube was reweighed immediately, and the difference in weight represented the approximate WHC in ml water/g sample. To get a more exact number, a series of four tubes, each containing 2 g of sample, were mixed with a range of measured volumes of water that encompassed the estimated WHC. For example, if the estimated WHC is 3.0, then 5.0, 5.5, 6.0 and 6.5 ml of water would be added to the 4 tubes. Each mixture was stirred vigorously for about 2 min and centrifuged at 8700 x g for 15 min. Immediately after centrifugation, the tubes were examined for the presence of a supernatant. At least one tube should have a supernatent and at least one should not, providing the estimated WHC was a good approximation. WHC was reported as the midpoint of the range between the tubes with and without supernatant water layers.

Viscosity

For the measurement of viscosity, samples were dispersed in water at concentrations of 3.0, 5.0, 7.5, 10.0 and 15.0% (w/v). The pH was checked, and was between 6.8-7.2. One ml of sample was placed in the

chamber of a Brookfield Viscometer, Model LVT, and allowed to equilibrate for several minutes. Temperature was maintained at 25° C with a Colora Ultra Thermostat circulating water bath. Shear stress was measured as a function of shear rate over the range 0-240 sec^{-1}.

Emulsifying Capacity

The procedure of Webb et al. (1970) was modified slightly for the determination of EC. To a 600 ml beaker containing 100 ml of 1.0 M NaCl solution was added 5.0 ml from a protein sol equivalent to 10 mg protein. This mixture was weighed, and covered with a rubber stopper fitted with holes for a propeller, two electrodes and an oil delivery tube. The propeller was driven by a Talboy T Line variable speed, laboratory stirrer. The electrodes were attached to a Triplett Model 630 voltmeter. Refined corn oil was added from a 500 ml separatory funnel at a constant flow rate of 12 ml/min while stirring the mixture at an initial speed of 4400 rpm. Oil delivery continued until the inversion point was reached, at which time the voltmeter showed infinite resistance. The beaker was then reweighed to determine the amount of oil added. The EC was calculated as the g of oil required to reach an infinite resistance minus a blank (100 ml of the 1.0 M NaCl plus 5.0 ml water) divided by the amount of protein. Analyses were performed in triplicate.

Whipping

Whipping properties of the various proteins were determined by whipping 50 ml of a 3.2% protein sol at pH 7.0 for 6 min at speed 8 in a Kitchen Aide Mixer, Model 3-C, equipped with a wire whip. Specific volumes (ml/g) were determined by transferring the foam to a tared

beaker of known volume and reweighing the beaker. Foam stability was measured by inverting the beaker over a half inch stainless steel mesh screen and collecting the liquid draining from the foam. The time required for collection of liquid equal to one-half of the weight of the original foam was designated as foam stability. Stability times of less than 5 min were reported as zero. All analyses were performed in triplicate.

Physical Methods

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was run according to the method of Melachouris (1969) in a discontinuous buffer system. All electrophoresis was performed in 6 mm I.D., 2 mm walled, and 75 mm length glass tubes. The tubes were detergent washed, immersed in chromic acid and treated with Photoflo after use.

Running gels of 10% total acrylamide concentration (10% T) and 5% crosslinking (5% C) were prepared using a 0.380 M Tris-HCl buffer, pH 8.9 (see Appendix, Table A2, for buffer preparation) with the ratio of acrylamide to bisacrylamide being 19:1. Spacer gels of 5% T and 5% C were prepared in 0.062 M Tris-HCl, pH 6.7. Polymerization was initiated using a 5% ammonium persulfate solution and Temed (N,N,N',N'-tetramethylethylenediamine). The electrode buffer was a pH 8.3 solution of Tris-glycine. Samples of 0.5% protein were prepared in the pH 6.7 buffer, and sucrose was added to increase density. Bromophenol blue was added as a marker dye.

PAGE was also run in gels containing 7 M urea. All buffers remained the same, except for the presence of 7 M urea.

A constant current of 3 mA/tube was applied to the gels until the marker dye reached the bottom of the tube, usually 45-60 min. Gels were then removed from the tubes and stained.

SDS PAGE

SDS gels were run according to the method of Weber et al. (1972). Gels of 10% T with a acrylamide to bisacrylamide ratio of 37:1 were prepared in 0.1 M phosphate buffer, pH 7.2, 0.1% SDS (see Appendix, Table A2, for buffer preparation). Polymerization was initiated using ammonium persulfate and Temed, as above. Samples were prepared in 0.01 M phosphate buffer, pH 7.0, 1% SDS, 1% 2-mercaptoethanol, with a final protein concentration of 0.2%. This sample was placed in a boiling water bath for 5-10 min. Some samples were prepared without mercaptoethanol. After cooling, sucrose and Bromophenol blue were added, as above. The electrode buffer consisted of 0.1 M phosphate buffer, pH 7.2, .01% SDS.

A constant current of 8 mA/tube was applied to the gels until the marker dye reached the bottom of the tubes, usually about 4-5 hr. Gels were then removed from the tubes and stained. Relative mobilities were calculated by measurement of the protein zone migration distance, dye migration distance, and the length of the gels from the following relationships:

$$\text{Relative mobility} = \frac{\text{distance protein migrated}}{\text{distance dye migrated}} \times \frac{\text{length of gel before destaining}}{\text{length of gel after destaining}}$$

A standard curve was constructed using an electrophoresis calibration kit for molecular weight determination of low molecular weight proteins (Pharmacia). The kit contained the following proteins: phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400.

Staining

Gels were stained by the method of Reisner et al. (1975) using Coomassie Brilliant Blue G250 in 3.5% perchloric acid (see Appendix, Table A2, for stain preparation). Staining was usually carried out overnight, and gels were destained in a diffusion destainer in 7% acetic acid.

Statistics

Analysis of variance was completed on all functional test data in addition to the yield and sulfhydryl information. If significant differences were found, the Tukey technique was used to pinpoint the difference (Hays, 1973).

RESULTS AND DISCUSSION

Preparation of Co-precipitates

Four co-precipitate samples were prepared in order to vary their functional properties and to study some physical properties of the products. Abbreviations for the various products referred to can be found in Table 5.

Products produced in this study were not made under the same conditions as commercial co-precipitates. For example, the heated co-precipitates (ACP, P3C, CAC) all had heat treatments of 95° C for 30 min, followed by precipitation. American-made co-precipitates are made from milk heated at 90-95° C for 20-30 min, concentrated to 10-30% total solids and heated to 140° C for 20 s prior to precipitation (Noyes, 1969). Australian and New Zealand processes typically involve heat treatments of 85° C for 6-8 min by steam injection followed by precipitation (Southward and Aird, 1978). Muller et al. (1969) noted that the heat treatment required to precipitate most of the whey proteins by acid was much greater (92° C for 15 min) than that required when calcium chloride was used as the precipitant (92° C for 5 min). Thus, although the acid co-precipitate (ACP) and calcium co-precipitate (CAC) were intended to simulate commercially available products, the similarities may only be approximate.

Table 5. Abbreviations for products referred to in this study.

ACP - Acid co-precipitate
P3C - pH 3.5 co-precipitate
HMP - Hexametaphosphate co-precipitate
CAC - Calcium co-precipitate
NAC - Sodium caseinate
NDM - Nonfat dry milk
SWC - Whey protein concentrate (gel filtration)
BWC - Whey protein concentrate (metaphosphate)

The preparation of the pH 3.5 co-precipitate was based on a paper by Modler and Emmons (1977) in which sweet whey was heated at 90° C for 15 min at pH 2.5-3.5, cooled and precipitated at pH 4.5. Protein recovery was 20-30% higher when $FeCl_3$ was added prior to heating. By heating skimmilk at pH 3.5 for co-precipitate preparation, it was hoped that unique functional properties would be achieved.

The use of sodium hexametaphosphate (SHMP) to produce a co-precipitate represents a method for producing a protein concentrate without resorting to high temperatures. This method was used for recovering whey proteins (Hidalgo et al., 1973; Richert, 1972). Zittle (1966) showed that caseins were precipitated by divalent or polyvalent anions whereas β-lg was precipitated by polyanions only under acidic conditions. In acidic environments, the protein acts as a polycation and the SHMP acts as a polyanion. Insoluble complexes form as the result of polycation-polyanion interactions. Likewise, long-chain polyphosphates promote intermolecular crossbonding and aggregation.

Composition

Compositional properties of the four co-precipitates studied are summarized in Table 6. The pH 3.5 co-precipitate (P3C) has an unusually high moisture content. This characteristic may be a ramification of a relatively low ash content which could allow for more available water binding sites on the proteins. Also, the high lactose content could account for a portion of this moisture. Specimens were dried by lyophilization, therefore one must consider the possibility of incomplete dehydration in the system, as a large number of samples were dried at one time.

Table 6. Proximate compositional analysis of co-precipitates.

		ACP	P3C	HMP	CAC
Moisture	(%)	7.3	15.2	5.2	5.6
Fat	(%)	3.0	2.1	2.0	2.5
Ash	(%)	3.8	2.1	13.0	9.2
Protein (N x 6.38)	(%)	78.5	73.0	67.7	69.5
Protein (dry basis)	(%)	84.7	86.1	71.4	73.6
Lactose	(%)	5.8	3.9	8.7	8.1
Calcium	(%)	0.2	0.2	0.7	2.3
Phosphorus	(%)	0.7	0.6	3.3	1.6
pH of whey after separation		4.6	4.6	3.1	6.2

Washing has been shown to affect the composition of isoelectric casein and co-precipitate (Buchanan et al., 1965). On the commercial scale, washing is accomplished with pH and temperature controlled

(pH 4.6, 30-35° C) water and is repeated 2-4 times. In this study, only one wash was utilized, and there was no pH or temperature adjustment of the wash water. Consequently, some of the values for lactose and ash may be slightly higher than normally encountered. For example, commercial calcium co-precipitate offered for sale by New Zealand Milk Products, Inc. (undated) have lactose and ash levels of 0.6% and 8.0%, respectively. The CAC prepared in this study had levels of 8.1% and 9.2% for these components, respectively. Similarly, the other co-precipitates may have been affected by the washing procedure.

The fat contents of all four samples were higher than found in commercial products (0.6-1.0%). Buchanan et al. (1965) attributed the higher fat content of co-precipitates when compared to casein to fat-protein complexes formed during the manufacturing process which, presumably, are retained in the curd. In this study, co-precipitates were produced from inefficiently separated skimmilk which could account for the relatively high fat content.

The calcium and phosphorus contents are similar to those found in commercial samples. The hexametaphosphate co-precipitate (HMP) has a very high phosphorus level, resulting from the use of SHMP as an aggregation agent. The slightly higher calcium level may have resulted from SHMP sequestering. CAC had higher calcium and phosphorus levels than the other co-precipitates. D'yachenko et al. (1953) also observed higher calcium and phosphorus levels in calcium co-precipitate.

The amino acid composition of the protein in each of the co-precipitates is shown in Table 7. Data for the commercial co-precipitates and casein were obtained from technical brochures. Under the conditions of

Table 7. Amino acid composition of co-precipitates and selected commercial products.

Amino Acid	g amino acid/100 g protein						
	ACP	P3C	HMP	CAC	HICAL2[a]	COP[b]	Casein[b]
Lysine	7.13	7.76	8.29	6.51	7.49	7.39	8.08
Histidine	2.39	2.44	2.90	2.16	2.90	2.77	2.90
Arginine	3.17	3.27	3.01	3.48	4.58	4.11	3.87
Aspartic acid	7.12	6.45	7.25	7.50	6.69	6.83	7.17
Threonine[e]	4.35	4.18	4.28	4.53	4.00	3.86	4.29
Serine[e]	6.71	6.34	4.52	6.61	4.93	5.01	6.14
Glutamic acid	16.65	17.57	20.62	18.99	19.80	18.53	22.67
Proline[e]	9.80	8.86	7.11	9.83	8.20	14.54	13.09
Glycine	1.43	1.89	1.75	1.35	2.11	2.03	2.14
Alanine	2.66	3.35	2.95	2.60	3.02	3.60	3.12
Half-cystine[c]	1.01	0.80	0.59	1.09	0.92	1.10	0.42
Valine	6.30	7.01	7.14	5.68	5.50	6.53	6.35
Methionine	3.07	2.21	1.82	2.71	2.92	2.61	2.83
Isoleucine	4.59	4.82	5.17	4.16	4.66	5.06	5.31
Leucine	8.38	8.67	9.19	7.88	8.80	9.34	9.60
Tyrosine[e]	6.44	5.95	4.98	7.22	5.04	5.60	5.63
Phenylalanine	4.73	4.35	4.97	3.82	4.72	5.36	5.33
Tryptophan[d]	2.25	2.22	1.79	2.07	1.39	1.57	1.28

[a]High-calcium co-precipitate from Erie Casein Co., Inc., Erie, Ill. (undated).

[b]Calcium co-precipitate and casein from New Zealand Milk Products, Inc., Rosemont, Ill. (undated).

[c]Methionine and cysteine/cystine were determined as methionine sulfone and cysteic acid, respectively.

[d]Tryptophan was determined by the method of Spies (1967).

[e]Values for these amino acids were extrapolated to zero hydrolysis time.

acid hydrolysis, quantities of threonine, serine, proline and tyrosine are lost with longer hydrolysis times. Therefore, the values of these amino acids were extrapolated to zero time of hydrolysis, according to:

$$\log A_0 = \left[\frac{t_2}{t_2 - t_1}\right] \log A_1 - \left[\frac{t_1}{t_2 - t_1}\right] \log A_2$$

where A_0, A_1 and A_2 are the quantities of amino acids after t_0, t_1 and t_2 times of hydrolysis, respectively. Hydrolysis times of 24 and 72 hr were used.

All the co-precipitates contain a well-balanced amino acid pattern with respect to the essential amino acids, although HMP is slightly low in the sulfur amino acids. This could reflect a lower whey protein content, and will be discussed later.

A comparison of the essential amino acids and chemical scores of co-precipitates, casein, NFDM and WPC is shown in Table 8. Only HMP, P3C and casein have chemical scores less than 100, and each is limiting in the sulfur amino acids. Comparison of the amino acid patterns of the various co-precipitates with NFDM reveals clear similarities, thus demonstrating the high quality of concentrated, total milk protein as represented by co-precipitates.

Yields

Yields reported for the manufacture of calcium co-precipitates have been as high as 96% of the total milk protein (D'yachenko *et al.*, 1953; Buchanan *et al.*, 1965). The amount of the total milk protein precipitated from skimmilk for each type of co-precipitate is presented in Table 9. These values were determined by assaying the protein

Table 8. Comparison of essential amino acids and chemical scores of selected milk protein products.

Amino Acid	Reference Protein[a]	g amino acid/100 g protein							
		ACP	P3C	HMP	CAC	Casein[b]	HICAL[c]	NFDM[d]	WPC[d]
Isoleucine	4.0	4.54	4.82	5.17	4.16	5.31	4.66	6.06	5.58
Leucine	7.0	8.38	8.67	9.19	7.88	9.60	8.80	10.64	13.00
Lysine	5.5	7.13	7.76	8.29	6.51	8.08	7.49	8.42	8.74
Sulfur amino acids	3.5	4.08	3.01	2.41	3.80	3.25	3.84	3.76	5.89
Phenylalanine/ tyrosine	6.0	11.17	10.30	9.95	10.04	10.96	9.76	9.95	7.62
Threonine	4.0	4.35	4.18	4.35	4.53	4.29	4.00	4.64	5.65
Tryptophan	1.0	2.25	2.22	1.79	2.07	1.28	1.39	--	2.93
Valine	5.0	6.30	7.01	7.14	5.68	6.35	5.50	7.04	5.38
Chemical score	100	100	86	69	100	93	100	100	100
Limiting amino acid	--	--	S	S	--	S	--	--	--

[a] FAO/WHO (1973)

[b] From New Zealand Milk Products, Inc. (undated).

[c] From Erie Casein Co., Inc. (undated).

[d] From Lohrey and Humphries (1976).

Table 9. Yield and whey protein content of co-precipitate samples.

Product	% of Total Milk Protein[4]	Whey Fraction[2]	% Whey Protein[3]
Casein[1]	--	1.64	--
Whey[1]	--	11.21	100
ACP	92.8[a]	2.36	27.1
P3C	94.2[b]	2.95	19.3
HMP	89.1[c]	3.72	12.1
CAC	94.4[b]	2.43	30.4
HMP (0.6%)	90.8[a]	--	--
HMP (0.7%)	93.6[b]	--	--

[1]From Resmini et al. (1971).

[2]Calculated according to Resmini et al. (1971).

[3]Calculated according to de Koning and van Rooijan (1971).

[4]Within this column, yields without a common superscript are significantly different at the 1% level.

content of the aqueous phase resulting from the preparation of the co-precipitates from skimmilk. The skimmilk had an initial protein content of 3.40%. Protein contents were determined by the method of Lowry et al. (1951).

Yields of ACP, P3C, HMP and CAC were 92.8, 94.2, 89.1 and 94.4%, respectively. Southward and Aird (1978) reported yields of 92.7 and 95.8%, respectively, for an acid and high-calcium co-precipitate. Chojnowski et al. (1975) obtained yields of 90-92% characterizing their co-precipitate production. Thus, the yields obtained for ACP and CAC were comparable to those obtained by others. Southward and Aird (1978) claimed that the decrease in yield for an acid compared to a calcium co-precipitate resulted from a reduced amount of whey protein precipitated with the casein and a reduction in ash content as the pH of precipitation was decreased. The pH values of the aqueous phases were presented in Table 6. In this study, this relationship between pH of coagulation and yield was apparent for ACP and CAC. However, the other heat-treated co-precipitate, P3C, had a yield similar to the CAC preparation. P3C is an acid precipitated product, and it appears that heating skimmilk at low values of pH produces yields similar to a calcium precipitated product, but with a substantially lower ash content. The HMP had a yield of less than 90%. Hartman (1966) found that 0.5% SHMP at pH 2.5 represented optimum conditions for the precipitation of whey proteins with SHMP. However, for total milk protein recovery, this level was found to be too low. When the concentration of SHMP was increased to 0.6% and 0.7% at pH 3.0, protein recovery increased to 90.8% and 93.6%, respectively. Thus, optimum recovery of total milk protein using SHMP

is achieved at concentrations of SHMP of approximately 0.7% at pH 3.0.

The aqueous phases separated from the four co-precipitates were dialyzed to remove lactose and salts, then lyophilized. The residual protein was examined on polyacrylamide gels using a discontinuous buffer system. The results of this analysis are presented in Figure 1. Although an exact component assessment cannot be made from these gel patterns, it appears that the major portion of protein remaining in the aqueous phases is from the proteose-peptone fraction, a group of proteins defined as a mixture of heat-stable and acid-soluble (at pH 4.6) phosphoglycoproteins insoluble in 12% trichloroacetic acid (Whitney et al., 1976). Kaspar (1978) noted that the heat stability of proteose-peptone components of serum origin could be accounted for by postulating that they are rendered heat-stable by virtue of glycosylation and/or phosphorylation. The residual proteins from the HMP preparation showed two heavy bands about two-thirds of the way down the gel. These may be residual β-lg which was not precipitated completely. This preparation was made using 0.5% SHMP, and, as mentioned previously, the yield of total milk protein was only 89.1%.

Resmini et al. (1971) examined the amino acid composition of casein and whey proteins and proposed that the fraction,

$$\frac{P \times Q}{R},$$

be used to determine the whey protein content in co-precipitates, where,

P = % aspartic acid in the co-precipitate

Q = % alanine in the co-precipitate

R = % proline in the co-precipitate.

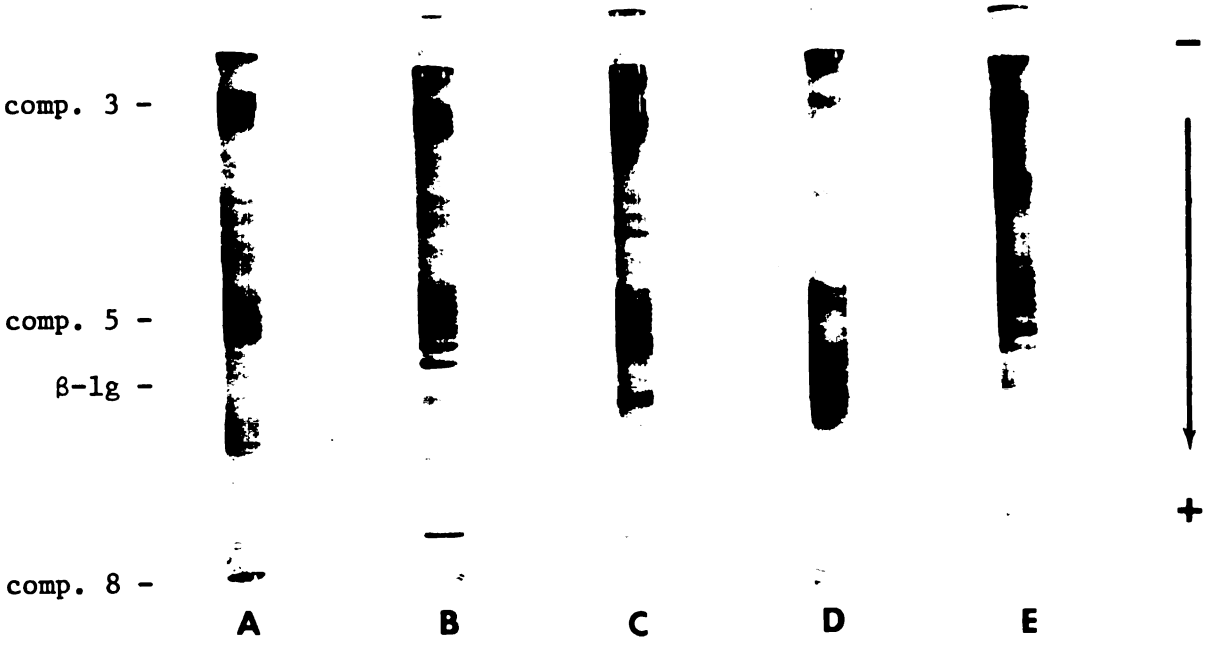

Figure 1. Electrophoretic patterns of proteose-peptone specimen from skimmilk (A), and co-precipitate aqueous phases of ACP (B), P3C (C), HMP (D) and CAC (E). Total acrylamide concentration was 5% and 10% in the spacer and running gels, respectively.

This whey fraction is presented in Table 9. Casein and whey were reported to have values of 1.65 and 11.21, respectively. Ratios for ACP, P3C, HMP and CAC were 2.36, 2.95, 3.72 and 3.43, respectively. The amount of whey protein in a co-precipitate could be found from a graph of whey fraction vs. proportion of whey protein in a casein-whey mixture. However, the original publication could not be located, so estimation of the whey protein content was not possible using this procedure. These values do indicate a moderate amount of whey protein in the co-precipitates based on the values for casein and whey.

The whey protein content of co-precipitates was also estimated by de Koning and van Rooijen (1971), using the cysteine plus cystine contents. Cystine was reduced to cysteine with dithiothreitol and total cysteine was determined. The content of whey protein in a co-precipitate was calculated by the formula:

$$\% \text{ whey protein} = \frac{x - 0.26}{3.06 - 0.26} \times 100$$

where x equals the percentage of cystine plus cysteine in the co-precipitate. Using the amino acid data from this study, the amount of whey protein in each co-precipitate was estimated and results presented in Table 9. The percentage of whey protein in ACP, P3C, HMP and CAC was 27.1, 19.3, 12.1 and 30.4, respectively. The low value of HMP is probably due to incomplete precipitation of β-lg, which results in a reduced cysteine content, as reflected in the amino acid data and chemical score. The value for P3C of 19.1 seems reasonable when considering that skim-milk protein normally contains 15-22% whey protein. The higher values for ACP and CAC could reflect losses of fine casein particles when

separating the curd and whey. Also, some error in the amino acid data is possible. de Koning and van Rooijen obtained percentage whey protein values ranging from 10.4-16.0% for different co-precipitate samples. Southward and Aird (1978) reported casein:whey protein ratios of 6:1, 7:1 and 8:1 for high-calcium, medium-calcium and acid co-precipitates, respectively.

Functional Properties

Southward and Goldman (1975) noted that little work had been reported on the functional properties of co-precipitates. Recently, some literature has appeared regarding functional properties (Chojnowski et al., 1975; Southward and Goldman, 1978). However, several factors complicate the comparison of data from different laboratories. Variation in how functionality is assessed creates a problem, since currently there are no standardized functional tests available. In addition, the term "co-precipitate" has never been defined.

Functional evaluations in this study were performed in simple model systems. It must be emphasized that model systems do not necessarily simulate more complex food systems. Therefore, it should not be unexpected to find that a protein preparation behaves differently in real food systems than when evaluated in a model system.

Solubility

Solubility is regarded as the principal property that precludes the overall functionality of proteinaceous materials and provides a good index for specific applications (Kinsella, 1976; Chou and Morr, 1979).

Operationally, solubility represents the percentage of total protein retained in a supernatant after centrifuging a protein solution at a standardized speed and time.

The solubility profiles of four commercially available milk protein products and the four co-precipitates prepared in this study are shown in Figures 2 and 3. The three heat-treated co-precipitates (ACP, P3C, CAC) have profiles similar in shape to that of NAC, although CAC is partially insoluble at neutral pH. The solubility curves for HMP and BWC are essentially similar. At pH 7.0, the solubilities of SWC, BWC and HMP were similar, whereas NAC, NDM, HMP and CAC differed at the 1% significance level. ACP and P3C were not significantly different from each other, but were different from all the other products at the 1% level. At pH 4.5, NAC, ACP, P3C and CAC were completely insoluble, whereas the remaining products had solubilities that were significantly different at the 1% level. At pH 3.0, the solubilities of all the products were significantly different from each other at the 1% level, with the exception of CAC and NDM, which were not different, and ACP and CAC were different at the 5% level.

Southward and Aird (1978) determined the solubilities of high-calcium, medium-calcium and acid co-precipitates at pH 7.5 and noted that their solubilities were not as complete as that of acid casein due to the inclusion of heat-denatured whey proteins. Smith and Snow (1968) examined the solubilities of high-, medium- and low-calcium co-precipitates over the pH range 4-8. Solubility profiles were all similar to that of sodium caseinate.

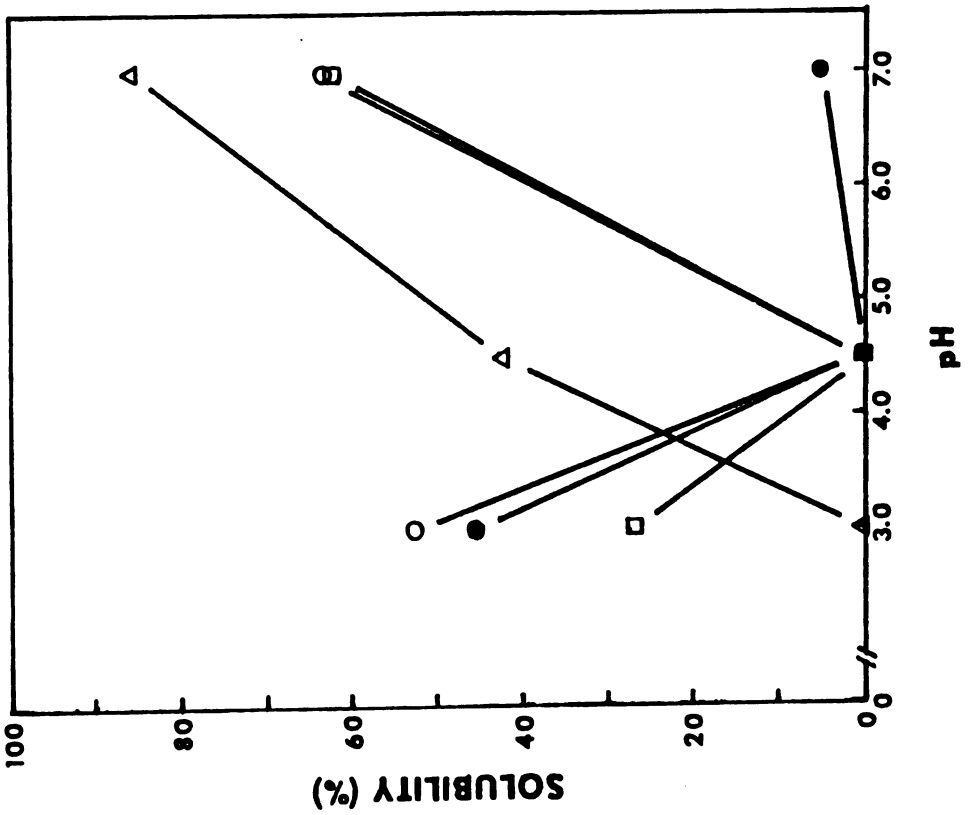

Figure 2. Solubility profile at pH 3.0, 4.5 and 7.0. SWC (○), BWC (□), NAC (△), NDM (●).

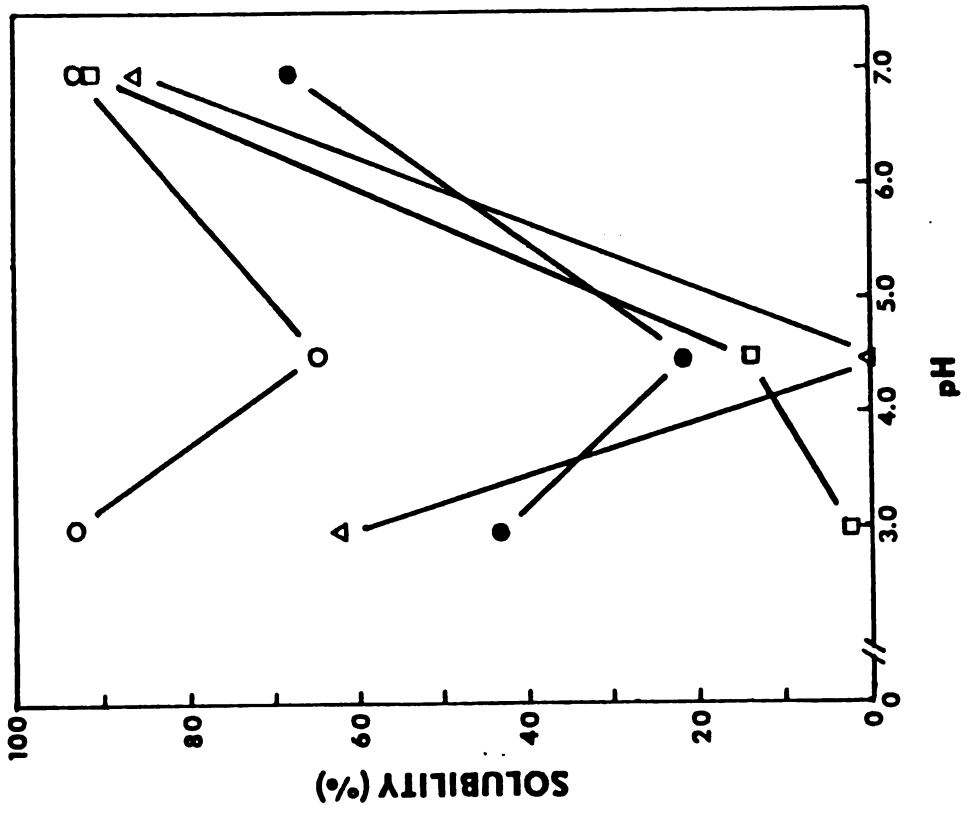

Figure 3. Solubility profile of co-precipitates at pH 3.0, 4.5 and 7.0. ACP (○), P3C (□), HMP (△), CAC (●).

The variation in solubility as the pH increased or decreased from pH 4.5 is due to increased net charge of the protein molecules, resulting in greater repulsion between protein molecules and greater relative attraction for water molecules. For the products precipitated with SHMP, solubilities decreased with pH, with complete insolubility observed at pH 3.0. At this lower pH, the SHMP-protein complex precipitates, due to maximum polycation-polyanion interaction. CAC exhibits little solubility at neutral pH due to its high calcium content. El-Negoumy (1971) attributed the instability of caseinate sols at high calcium concentration either to a lower zeta potential or to crosslinks established by the divalent cation. At lower pH, CAC has an increased solubility due to the dissociation of calcium ion from the proteins at this pH. High-calcium co-precipitates are often rendered more soluble by adding complex polyphosphates such as sodium tripolyphosphate to sequester the calcium (Hayes et al., 1969).

Viscosity

The viscosity of protein dispersions can be used to assess the thickening power of proteins, a property of practical interest in fluid foods, soups, beverages, batters and other formulations. A knowledge of viscosity behavior is important to both processing and process design and in new product development.

The effect of concentration on absolute viscosity for eight milk protein products are shown in Figures 4 and 5. A comparison of the profiles for the co-precipitates (ACP, P3C, HMP, CAC) with NAC shows similarities in shape between the curves. For the shear rates examined,

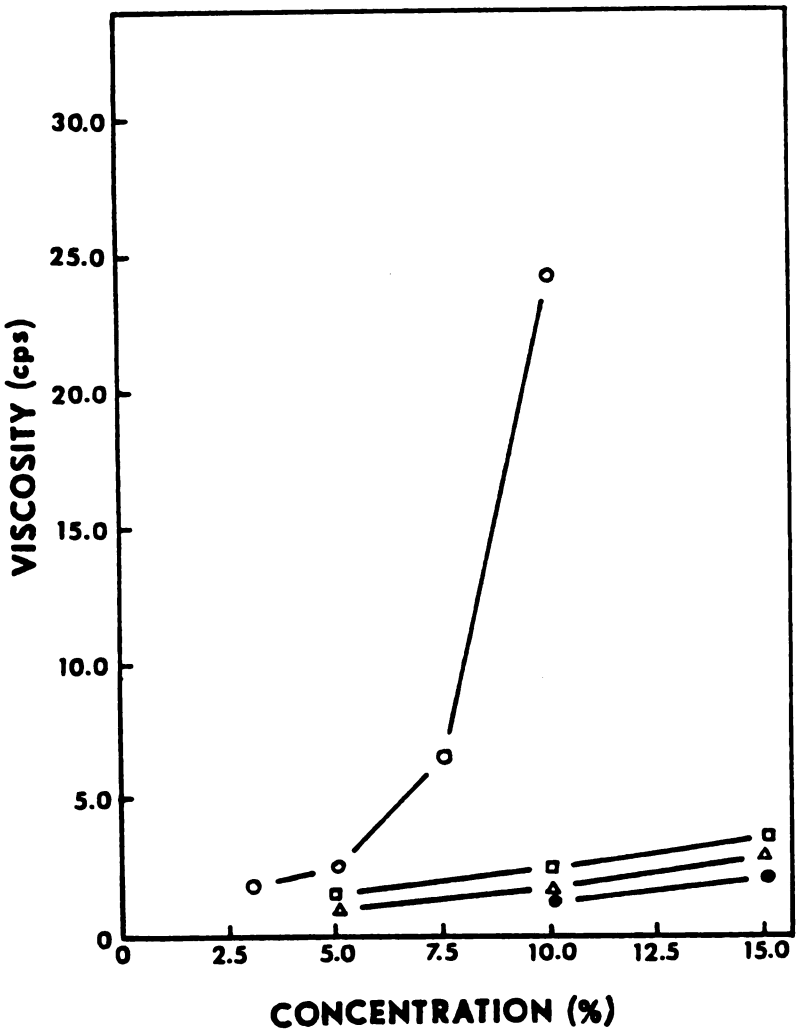

Figure 4. Effect of concentration on absolute viscosity of different milk protein samples. NAC (○), SWC (□), BWC (△), NDM (●).

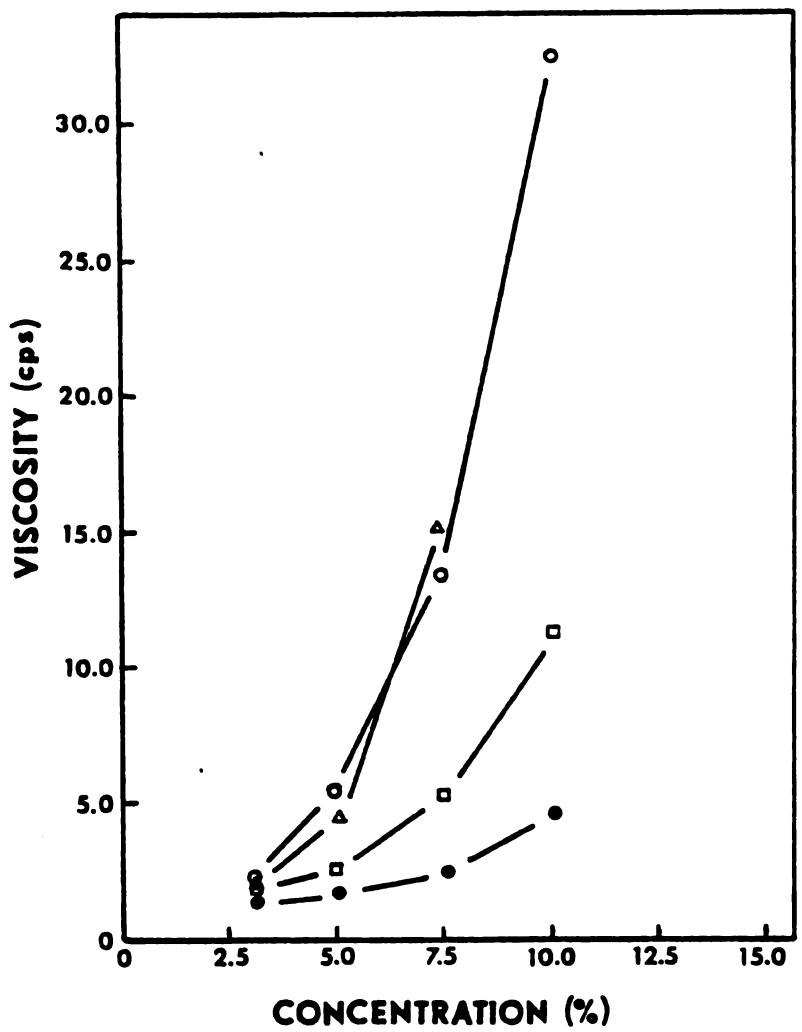

Figure 5. Effect of concentration on absolute viscosity of different co-precipitates. ACP (○), P3C (□), HMP (△), CAC (●).

all samples displayed Newtonian flow behavior in the concentration range up to 15%. Hermansson (1975) studied the viscosity of casein in distilled water. An almost Newtonian flow behavior was a common feature of caseinate dispersions below 12% concentration. The whey protein products (SWC, BWC) and NDM exhibited the lowest viscosities; HMP was also quite low. Whey proteins are highly soluble and exhibit low swelling, and thus display low viscosities. Harte (1978) observed the lower viscosities of whey products when compared to casein products.

HMP had lower viscosities than the other co-precipitates. This product exhibited high solubility and moderate water hydration capacity (discussed in next section), probably a ramification of a reduced water-protein interaction. This characteristic was especially apparent when compared to ACP, P3C or NAC. However, the protein content of HMP was slightly lower than the other co-precipitates. The greater viscosity of ACP over NAC, especially at the higher concentrations, may be partially due to its higher calcium content and heat treatment. Caseinates (excluding calcium salts) typically contain 6-7% ash, but negligible amounts of calcium (Hargrove and Alford, 1974). Hayes and Muller (1961) demonstrated the effect of calcium on the viscosity of casein solutions. Casein solutions with 1% calcium exhibited higher viscosities than those devoid of calcium.

Hayes et al. (1961) ascribed some of the viscosity difference between a low-calcium co-precipitate and casein to the formation of protein-protein linkages during the manufacture of co-precipitate. P3C shows lower viscosities than ACP which may reflect differences in ash content. Hermansson (1975) reported that increasing ionic strength

increased the viscosity of caseinate solutions. Also, the protein content of P3C is about 6% less than ACP. Differences in method of preparing P3C and ACP may also be important. CAC was extremely insoluble, and viscosity was very difficult to measure. In the use of proteins as thickeners, CAC would have no application. Morr (1979) noted that calcium caseinate exhibited a much lower viscosity than sodium caseinate. Southward and Goldman (1978) reported that acid co-precipitate yielded higher viscosities than sodium caseinate. They concluded that the occluded calcium content of co-precipitate and caseines is the prime factor causing variations in the viscosity of these products, whereas co-precipitated whey proteins had little, if any, effect on viscosity.

Water Hydration Capacity

The ability of a protein to bind and hold water is extremely important in many food systems such as meat products, bread and cakes. The term water hydration capacity (WHC) denotes the maximum amount of water a protein material can imbibe and retain under conditions of food formulation.

The WHC for eight milk protein products are presented in Figure 6. There is no significant differences in the WHCs or SWC, BWC and NDM, and in NAC and P3C. All others are significantly different at the 1% level. ACP exhibited the greatest WHC, followed in decreasing order by P3C and NAC, HMP, CAC and SWC, BWC and NDM. The presence of other components in a food system, such as salt and lipid, are expected to change the hydration characteristics of a protein product considerably (Quinn and Paton, 1979). Proximate analysis (Table 6) reveals compositional variations

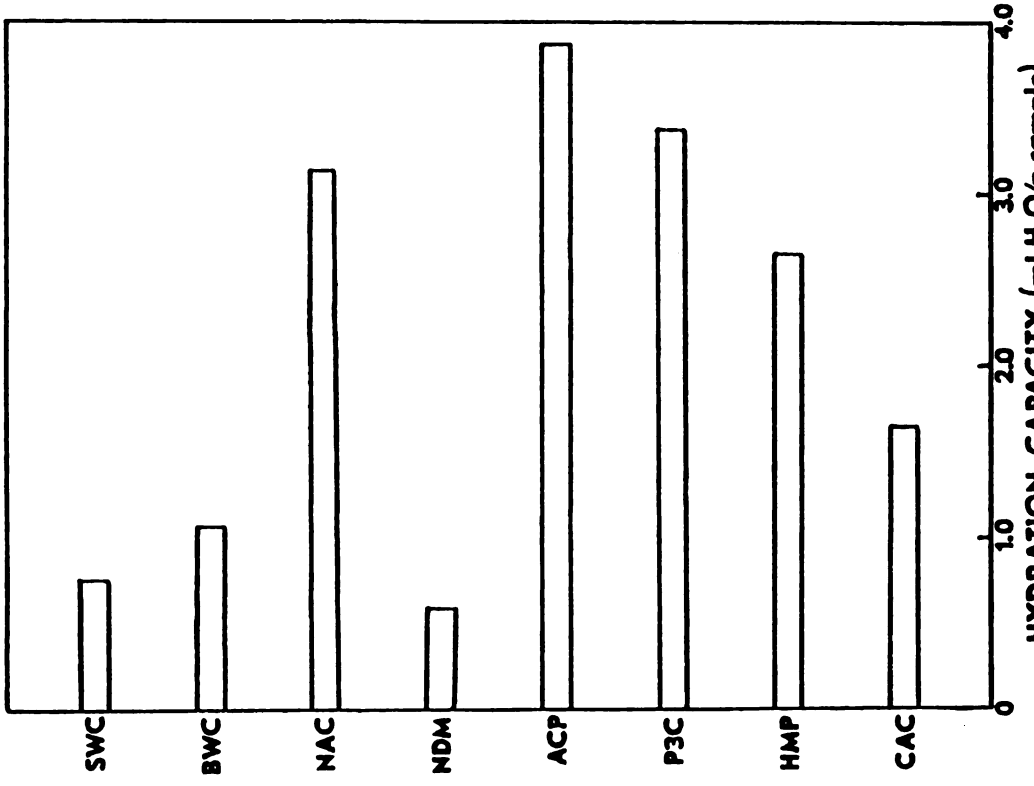

Figure 6. Water hydration capacities of co-precipitates compared to other protein samples.

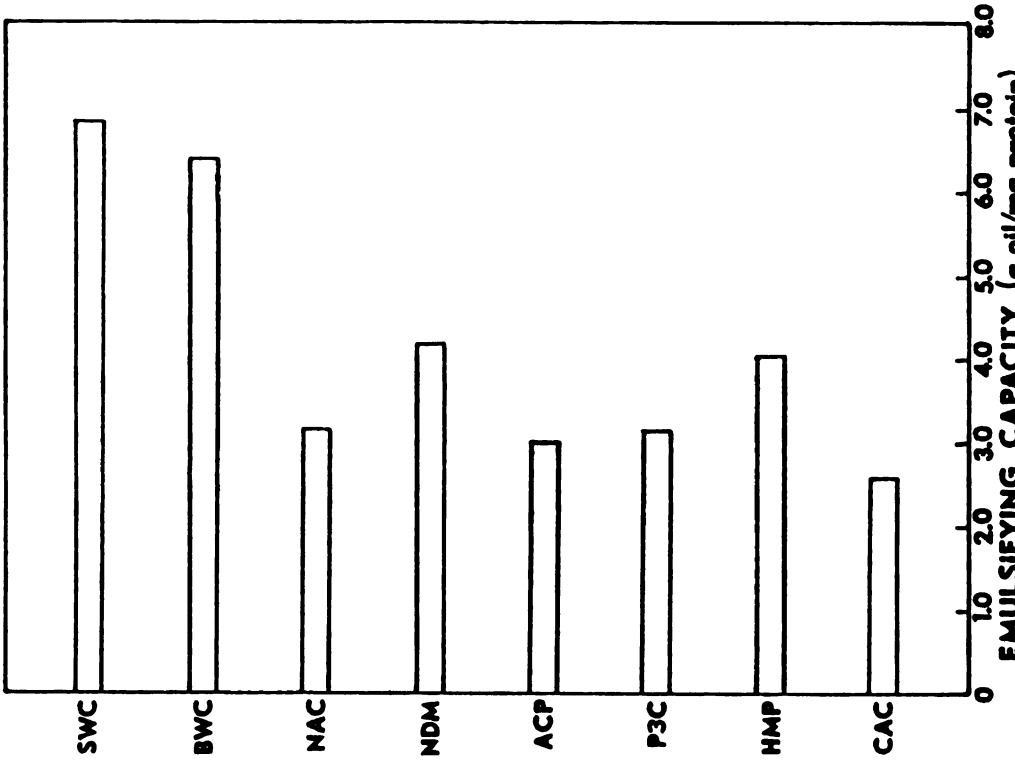

Figure 7. Emulsifying capacities of co-precipitates compared to other protein samples.

between the different products tested. Variation in protein content may account for some of the WHC differences.

The HMP seems to be a rather unique product when compared to the other co-precipitates. It exhibited high solubility, low viscosity and good WHC. SHMP may render the HMP product more hydrophilic in nature. CAC has the lowest WHC among the co-precipitates, a ramification of its high calcium content. Morr (1979) reported that calcium caseinate does not bind as much water as sodium caseinate. Goldman (1973) used a Brabender Farinograph to measure moisture absorption of milk protein products and found that insoluble, high-calcium co-precipitate absorbed three times less water than sodium caseinate. Loewenstein (1965) reported that the water binding capacity of low-calcium co-precipitate was decreased by the addition of calcium before it was dried. The denatured whey proteins incorporated in both ACP and P3C contributed only slightly to the WHC.

Chojnowski et al. (1975) measured the water absorption of sodium caseinate and several co-precipitates. Sodium caseinate had a value of 3.1 g/g sample, whereas values for the co-precipitates ranged from 2.2-3.7 g/g sample. Southward and Goldman (1978) reported that soluble, acid co-precipitate was a more effective water binder in meat products than sodium caseinate or insoluble, high-calcium co-precipitate. The data presented in this study seems to be in general agreement with these observations. Morr (1979) commented that co-precipitates would be most useful in applications requiring high nutritional quality and water absorption, but without significant protein solubility, such as in breakfast cereals, snacks and pastas.

Emulsifying Capacity

The ability of a protein to emulsify fat in a food system is extremely important in such applications as meat products and salad dressings. A protein's effectiveness as an emulsifier depends on its ability to lower the interfacial tensions between the hydrophilic and hydrophobic components in foods (Kinsella, 1976).

Figure 7 shows the emulsifying capacity (EC) of the various products tested. The whey products had the highest ECs of all the protein samples tested. NAC, ACP, P3C and CAC had the lowest ECs; there was no significant differences between these products. NDM and HMP had EC values intermediate between the above two groups. Kuehler and Stine (1974) and Harte (1978) reported that whey protein concentrates had greater EC than sodium caseinate, with NFDM exhibiting a value intermediate between the two.

All the heat-treated co-precipitates closely resemble NAC in their emulsifying behavior. The additional presence of whey proteins seemed to have little effect on the EC of the co-precipitates as this fraction was probably denatured to a large extent. Wingerd (1971) and Morr (1979) pointed to the lack of functionality exhibited by heat-denatured lactalbumin, which is completely insoluble. Soluble caseinates are good emulsifiers, as they possess high surface activity (Tornberg, 1978a). The results obtained with CAC were somewhat surprising in view of its poor solubility. However, the proteins were all dispersed in 1.0 M NaCl prior to the addition of oil. This concentration of NaCl could have exerted a salting-in effect and rendered the protein more soluble by increasing the net charge, allowing for a greater EC than expected. Southward and

Goldman (1978) reported that the emulsion stabilizing properties of high-calcium, medium-calcium and acid co-precipitates were similar to that of sodium caseinate.

Both NDM and HMP had EC values greater than NAC, possibly because of the soluble whey proteins present. Tornberg (1978b) reported that whey protein concentrate produced better emulsions than sodium caseinate. Tornberg (1978a) noted that both caseinate and whey protein concentrate diffuse rapidly to an oil-water interface.

Whipping

The whipping properties of a protein are an essential requirement for any aerated food product, such as sponge cakes, angel food cakes, meringues and whipped toppings. Whipping capacity is affected by a number of factors, such as solids content, pH, ionic strength and the presence of sucrose and lipids (Richert, 1979).

The whipping properties of the eight protein products are presented in Figure 8. None of the co-precipitates formed stable foams. NAC gave the greatest specific volume (ml/g), followed by NDM and SWC. Stabilities of the foams are presented in Table 10. NDM had the most stable foam, with a half-time of 31 min. NAC and SWC had similar half-times of about 16 min. SWC had to be heated to 70° C for 30 min in order to form a stable foam, as noted by Richert et al. (1974).

The failure of the co-precipitates to form stable foams could be attributed to the high fat content of the dry samples. The ability of milkfat to act as a foam depressant was reported by Leviton and Leighton (1935) and more recently by Cooney et al. (1973). During the whipping

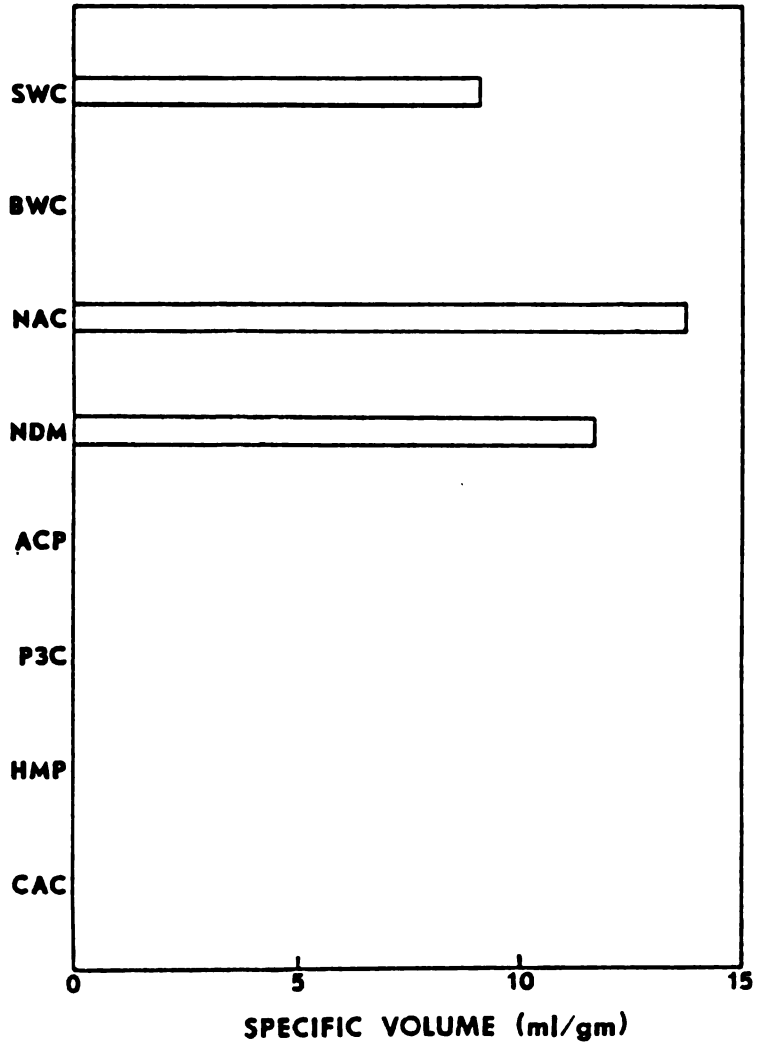

Figure 8. Specific volumes for co-precipitates compared to other protein products.

Table 10. Foam stabilities (1/2 times) for co-precipitates compared to other milk protein products.

Product	1/2 time (min)
SWC	16
BWC	0
NAC	16
NDM	31
ACP	0
P3C	0
HMP	0
CAC	0

tests, some sodium caseinate made from skimmilk which had been separated on the laboratory separator would not form stable foams. The fat contents of the co-precipitates ranged from 2-3%, while good commercial caseinates should have fat contents less than 1%.

Probably, the CAC would not have whipped even if its fat content had been lower because this co-precipitate was highly insoluble at pH 7.0. Likewise, it is improbable that the HMP would form stable foams. Richert (1979) noted that SHMP-precipitated whey protein concentrates failed to produce stable foam, an observation verified in this study with BWC.

Goldman and Toepfer (1978) reported that a soluble calcium co-precipitate produced greater overrun and stability than sodium caseinate. Southward and Goldman (1978) found that high-calcium, medium-calcium and acid co-precipitates, when whipped with sugar, produced overruns similar to dried egg albumin and sodium caseinate, but with greater stability than sodium caseinate. In an unsugared system, calcium co-precipitate formed stable foams with overruns similar to sodium caseinate. In the above study, fat content of the dried products was 0.6-0.7%.

Electrophoretic Analysis of Co-precipitates

PAGE

Figure 9 shows the electrophoretic patterns of the various co-precipitates obtained in 10% polyacrylamide gels. A qualitative interpretation of individual components in gels involves some speculation. However, for purposes of this discussion, tentative identification of major bands was established.

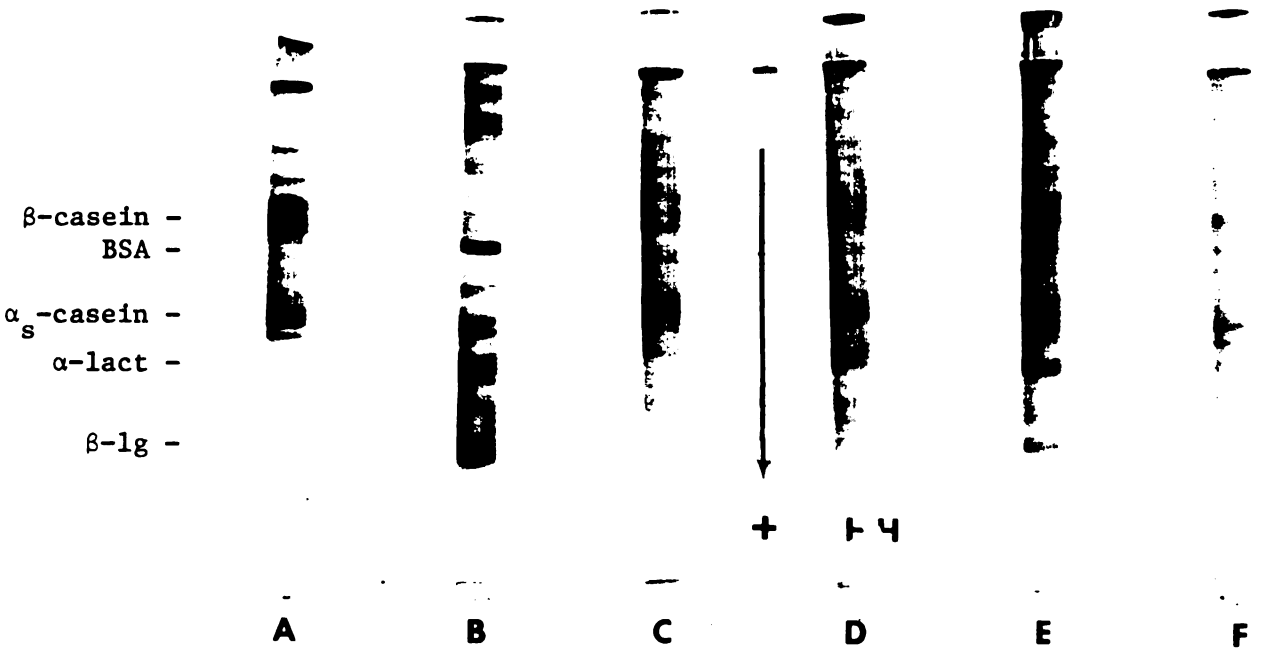

Figure 9. Electrophoretic patterns of whole casein (A), acid whey (B), ACP (C), P3C (D), HMP (E) and CAC (F). Total gel concentration was 5% and 10% in the spacer and running gels, respectively.

CAC was exceedingly insoluble in the spacer gel buffer, and the electrophoretic pattern of this co-precipitate demonstrates this, as only faint bands can be seen. In the electrophoretic patterns of ACP, P3C and HMP, the α_s- and β-casein components are clear. Because HMP represents an undenatured mixture of casein and whey proteins, serum albumin, β-lactoglobulin and α-lactalbumin are visible. The pattern of ACP reveals mainly the presence of casein components. Muller et al. (1967) reported the absence of a β-lg band in starch gel electrophoresis of calcium co-precipitates. In addition to the casein components, an additional band moving ahead of α_s-casein appears in the electropherogram for P3C. Identification of this band remains obscure. It appears to move in a manner similar to α-lactalbumin and the relative thickness of the band is greater than anticipated. However, these are qualitative observations and may present a need for more work on this type of co-precipitate.

Overall, with the heat-treated co-precipitates it appears that most of the whey proteins were insoluble, and therefore could not enter the gel structure. This is in accordance with Southward and Aird (1978) who noted the difficulty in dissolving co-precipitates for gel electrophoresis, and that the insoluble protein was likely to be rich in whey proteins.

Urea PAGE

The electrophoretic patterns of the co-precipitates in 7 M urea are shown in Figure 10. As noted by Morr (1967), the β-lg band displayed reduced mobility in urea in the acid whey pattern.

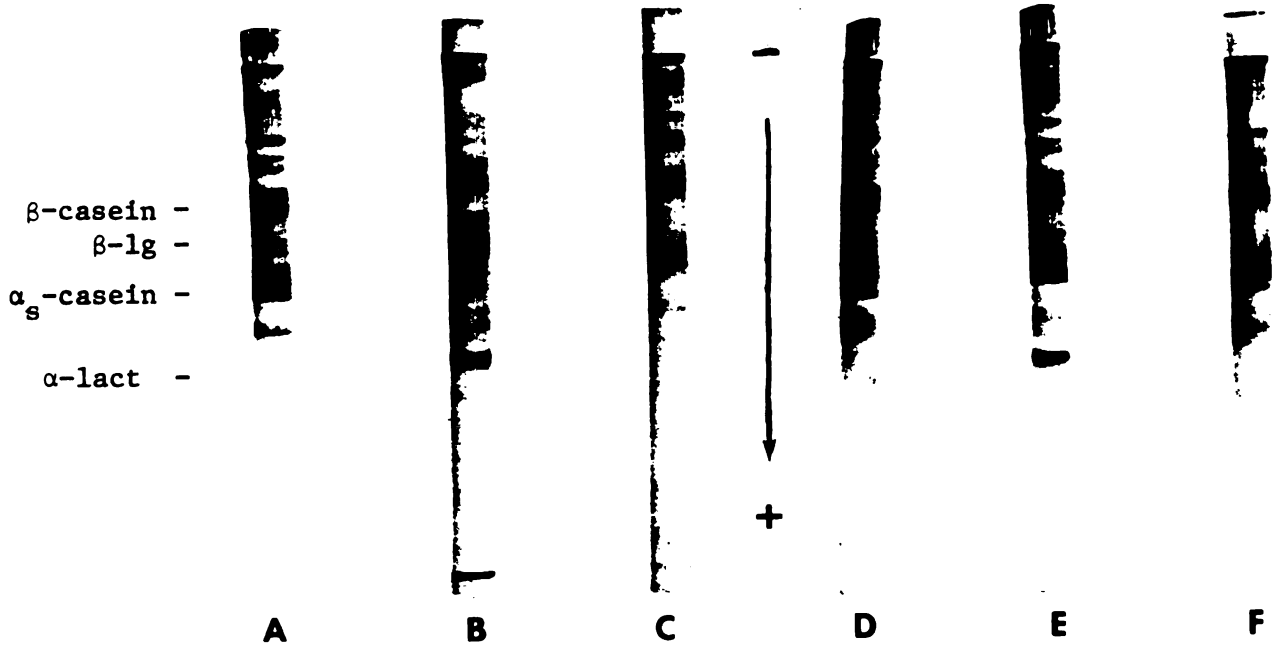

Figure 10. Electrophoretic patterns of whole casein (A), acid whey (B), ACP (C), P3C (D), HMP (E) and CAC (F) in 7 M urea. Total gel concentration was 5% and 10% for the spacer and running gels, respectively.

The CAC appears to be more soluble in urea, which is reflected in the electrophoretic pattern. All of the heat-treated co-precipitates displayed electrophoretic patterns closely resembling that for whole casein. It appears that urea alone has little effect on the insoluble fraction of co-precipitates. These insoluble fractions should be examined more closely in other dissociating systems in order to determine their exact nature. HMP displayed a similar casein profile, as well as a band for α-lactalbumin. The β-lg band may be either too faint or masked by the α_s-casein.

Creamer et al. (1978) reported that the void volume fraction obtained when heated skimmilk was fractionated on a Sepharose 4B column consisted mainly of κ-casein and whey proteins, but that these proteins had to be solubilized by 2-mercaptoethanol in 6 M urea at pH 8.6 to be observed by gel electrophoresis. Thus, it appears that urea has little effect on the electrophoretic patterns of the heat-treated co-precipitates when compared to the patterns obtained without urea.

SDS PAGE

The electrophoretic patterns of the co-precipitates were observed in SDS gel electrophoresis, both with and without the presence of 2-mercaptoethanol. A standard curve was also prepared which is shown in Figure 13.

Figure 11 shows the electrophoretic patterns of the various co-precipitates in SDS alone. The casein components in these gels do not appear as well-defined zones. The HMP patterns revealed additional bands corresponding to molecular weights of approximately 14,400 and

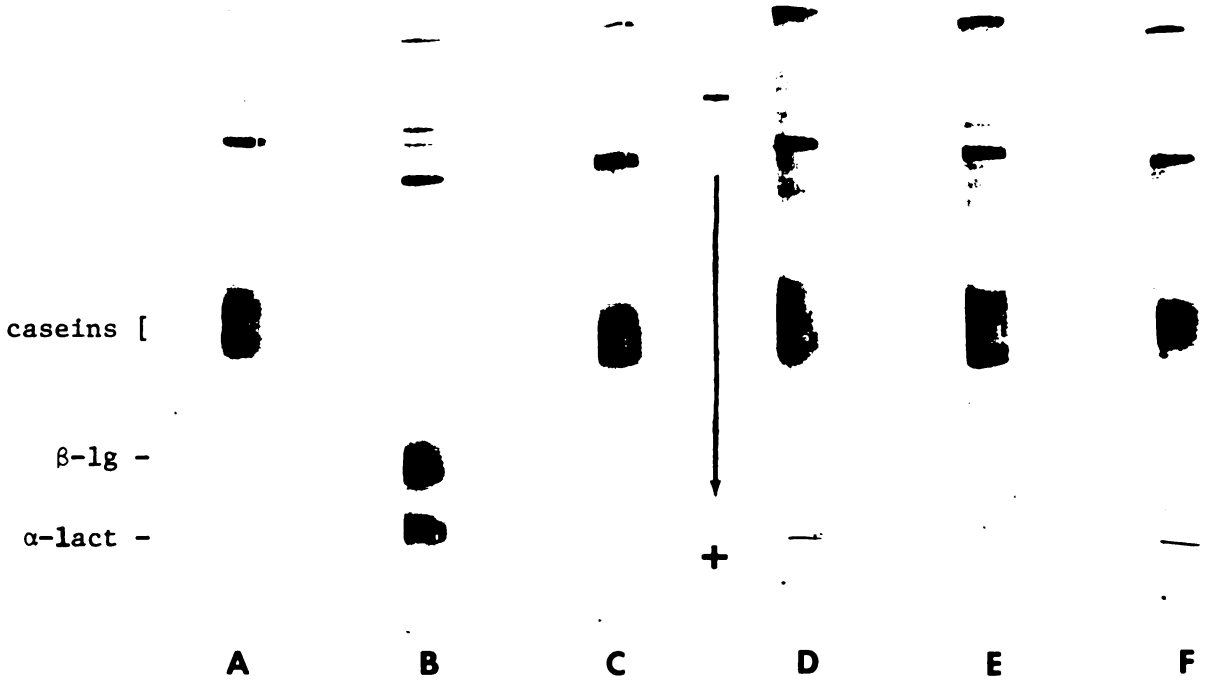

Figure 11. Electrophoretic patterns of whole casein (A), acid whey (B), ACP (C), P3C (D), HMP (E) and CAC (F) in SDS. Total gel concentration was 10%.

19,800 (α-lactalbumin and β-lg) although faint and difficult to photograph. P3C also revealed these two whey proteins bands. The appearance of a κ-casein band was difficult to discern because of the diffuse nature of the casein bands.

Figure 12 shows SDS electrophoretic patterns in the presence of 2-mercaptoethanol. In all patterns, β-lg and α-lactalbumin bands appeared although difficult to photograph. Casein bands appear much sharper and three components can be distinguished with corresponding molecular weights of 37,900, 34,300 and 30,700, representing κ-casein, α_s-casein and β-casein, respectively. This order of casein appearance in SDS gels has been reported (Peterson, 1977). The reported molecular weights for these proteins are 19,000, 23,000 and 24,000, respectively (Whitney et al., 1976). Thus, the values presented here are higher than the reported values. Gordon (1975) noted that glycoproteins generally bind less SDS than the average of 1.4 g/g protein. Cheeseman and Jeffcoat (1970) reported that the maximum binding of SDS to κ-casein, α_s-casein and β-casein was 1.1, 0.9 and 3.4 g SDS/g protein, respectively. Banker and Cofman (1972) noted that anamalous binding of SDS by proteins, atypical conformation of the protein-SDS complex or unusual properties of the protein-SDS complex can affect the protein mobilities.

As mentioned earlier, no one has isolated and characterized a heat-induced complex from milk involving casein and whey protein. Assuming this complex exists, the exact nature of the protein-protein interaction involved also remains a mystery. Furthermore, the stability of such a complex during conditions of co-precipitate manufacture (precipitation, washing, drying) is an area requiring more work.

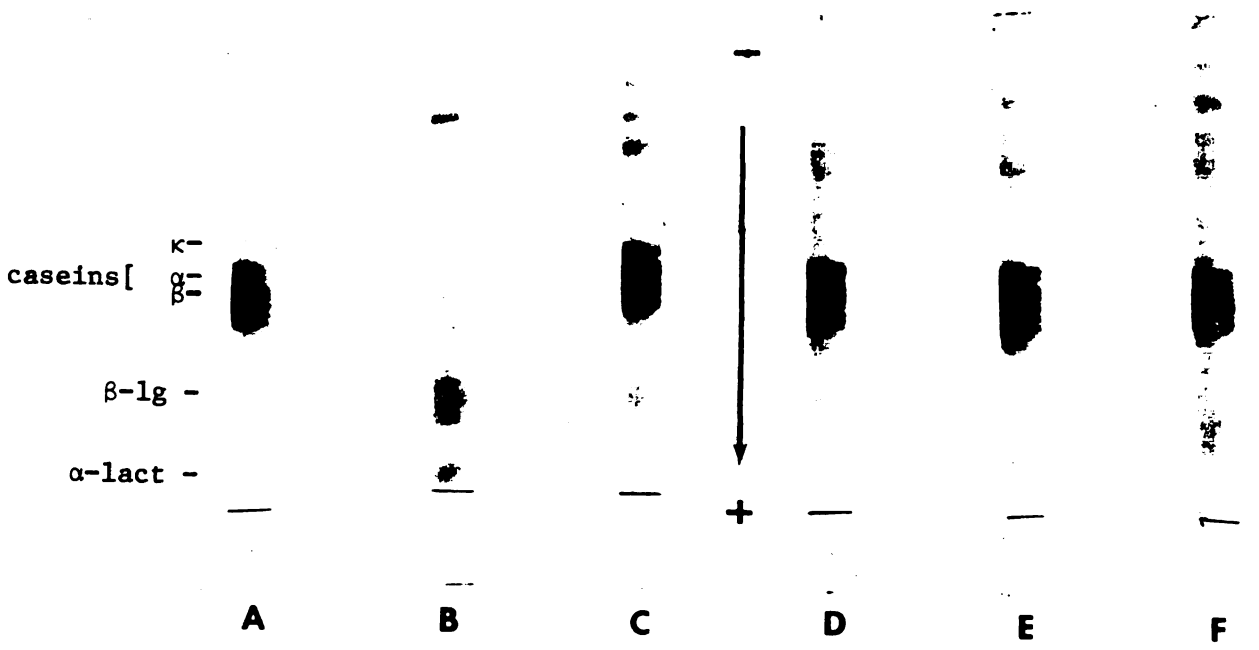

Figure 12. Electrophoretic patterns of whole casein (A), acid whey (B), ACP (C), P3C (D), HMP (E) and CAC (F) in SDS with 2-mercaptoethanol. Total gel concentration was 10%.

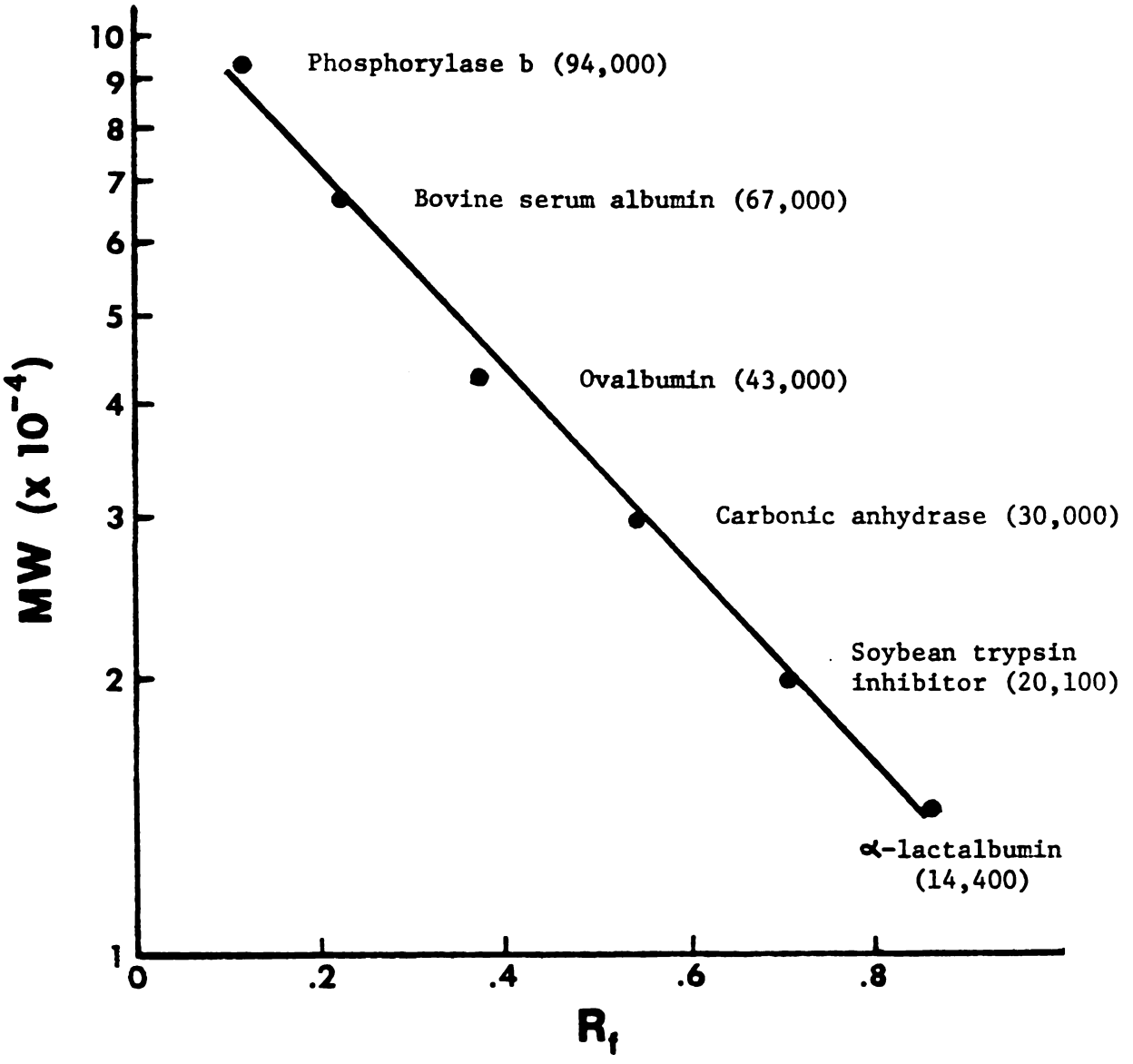

Figure 13. Standard curve for molecular weight determination. Total acrylamide concentration was 10%.

It appears that SDS and mercaptoethanol will resolve the major milk protein components in co-precipitates, whereas SDS alone resolves the components in P3C and HMP only. HMP was prepared without a heat-treatment and its electrophoretic resolution will not be discussed here. P3C was heat treated at pH 3.5, yet whey protein bands were visible in SDS alone. Fox and Hoynes (1975) commented that an interaction between β-lg and κ-casein may not necessarily occur at all pH values. On the other hand, a complex could occur but through a mechanism other than disulfide interchange. Table 11 presents the levels of SH and SS groups in milk heated at pH values of 7.6 and 3.5. No differences were found to exist in detectable SS content. This indicates the possibility of complex formation through noncovalent interactions in the pH 3.5 milk or no complex formation at all.

Table 11. Levels of SH and SS groups in unheated and heated milks at normal pH and at pH 3.5.

Sample	μM/g solids	
	SH	SS
Milk	1.07[a]	4.07[a]
pH 3.5 milk	1.12[a]	3.92[a]
Heated milk 90° C/30 min	0.20[b]	4.92[b]
Heated pH 3.5 milk 90° C/30 min	0.91[a]	5.24[b]

Values within each column with a different superscript are significantly different at the 1% level.

The electrophoretic data for ACP and CAC show the presence of β-lg and α-lactabumin only in the presence of mercaptoethanol. The difficulty in interpreting the SDS gels lies with the resolution of the casein bands which appeared extremely diffuse in the absence of mercaptoethanol. If the κ-casein band is present with and without mercaptoethanol, the possibility exists that either the complex is unstable in the presence of SDS and dissociates (with the whey proteins remaining denatured), or that a complex between κ-casein and the whey proteins was nonexistent. If the κ-casein band is present only in the presence of mercaptoethanol, a covalent interaction involving disulfide groups is likely.

Smith and Snow (1968) reported that in co-precipitate dispersions at pH 7 the insoluble fraction consisted of aggregated whey protein and a sialic-acid-containing fraction. During the study of the viscosity of co-precipitates, Hayes et al. (1969) noted that co-precipitate and casein dispersions behave differently with the addition of calcium. Added calcium had no effect on the viscosity of co-precipitate solutions, whereas it affected casein solutions. Hayes et al. (1968) showed that with added calcium, casein solutions exhibited a rapid drop in viscosity with increasing temperature, presumably due to dehydration. Co-precipitate solutions did not, however, suggesting that these are less prone to dehydration. These results indicate that some type of protein-protein linkage, in addition to protein-calcium-protein linkages, was formed during co-precipitate manufacture and that the same sites involved in protein-protein linkages are also involved in protein-calcium linkages.

Morr (1973) reported the electrophoretic separation of a κ-casein-β-lg complex from milk heated at 90° C for 30 min using urea-starch gel electrophoresis. The band had a mobility between β- and α_s-casein. No information regarding the type of interaction (covalent or noncovalent) was reported. Creamer et al. (1978) isolated a heat-induced complex from milk by gel filtration chromatography, but were unable to observe a similar behavior in gel electrophoresis due to insolubility of the specimen. Urea and mercaptoethanol were required to solubilize it. Both of these studies implicate the involvement of disulfide bonds in some manner. Morr and Josephson (1968), though, proposed that whey proteins were stabilized against heat-induced gross aggregation in heated skimmilk by complexing with casein through calcium-dependent linkages. Lyster (1970) noted that β-lg could exist in several forms involving disulfide bonds, or hydrophobic, hydrogen and ionic bonding. Presumably, a heat-induced complex in milk could involve one or more of these interactions.

CONCLUSIONS

Co-precipitates were prepared by four methods, with three of these involving heat treatment. Yields of total protein for the various co-precipitates varied from 89.1-94.4%. Compositions for the co-precipitates depended on the method of preparation. All samples had high fat contents when compared to commercially available products.

Functional properties were investigated for the co-precipitate samples and compared to nonfat dry milk, sodium caseinate and whey protein concentrate. ACP displayed moderate solubility, high viscosity and high water hydration capacity. P3C exhibited moderate solubility, and lower viscosities and water hydration capacity than ACP. HMP was highly soluble, with low viscosity and moderate water hydration capacity. CAC was extremely insoluble at neutral pH, and exhibited low water hydration capacity. None of the co-precipitates formed stable foams. ACP, P3C, and CAC had emulsifying capacities similar to sodium caseinate, whereas HMP had an emulsifying capacity similar to nonfat dry milk.

Examination of the various co-precipitates was conducted using gel electrophoresis in several dissociating systems. In alkaline PAGE with and without 7 M urea, the heat-treated co-precipitates (ACP, P3C, CAC) had electrophoretic patterns similar to whole casein. In SDS gels without mercaptoethanol, ACP and CAC patterns resembled whole casein, whereas the P3C pattern showed additional bands for β-lg and α-lactalbumin.

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APPENDIX

Table A1. List of chemicals used in this study.

Chemical	Company
Ammonium persulfate	Baker
Phenol	
Potassium phosphate, dibasic	
Selenium dioxide	
Sodium acetate	
Sodium carbonate	
Sodium nitrite	
Sucrose	
Acrylamide	Bio-Rad
N,N'-methylene-bis-acrylamide	
Sodium dodecyl sulfate (SDS)	
Photoflo	Eastman Kodah
N,N,N',N'-tetramethylethylenediamine	
Boric acid	Fisher
Bromophenol blue	
Calcium carbonate	
Calcium chloride	
Folin-Ciocalteau reagent	
Hydrogen peroxide	
Lactose	
Perchloric acid	
Potassium chloride	
Potassium tartrate	
Urea (laboratory grade)	
Sodium hexametaphosphate	FMC
Acetic acid	Mallinckrodt
Ammonium molybdate	
Cupric sulfate	

Table A1--continued

Chemical	Company
Ethyl ether	
Hydrochloric acid	
Methyl red	
Petroleum ether	
Sodium chloride	
Sodium hydroxide	
Sodium phosphate, dibasic	
Sodium phosphate, monobasic	
Sulfuric acid	
Trichloroacetic acid	
Bromocresol green	Matheson, Coleman & Bell
p-Dimethylaminobenzaldehyde	
Ferrous sulfate	
2-Mercaptoethanol	
Bovine serum albumin	Sigma
Coomassie Brilliant Blue G250	
Ellman's reagent (5,5'-dinitrobis-2-nitrobenzoic acid)	
EDTA, disodium salt	
Magnesium chloride	
Murexide	
Pronase	
Tris (tris(hydroxymethyl)aminomethane)	
Tryptophan	

Table A2. Preparation of buffers used in various experiments.

Experiment	Buffer
Tryptophan	0.1M phosphate buffer, pH 7.5 .222 g $NaH_2PO_4 \cdot H_2O$ + 2.253 g $Na_2HOP_4 \cdot 7H_2O$ made to 200 ml with water
SH and SS	0.2M phosphate buffer, pH 8 .147 g $NaH_2PO_4 \cdot H_2O$ + 5.080 g $Na_2HPO_4 \cdot 7H_2O$ + .530 g EDTA made to 200 ml with water
	0.2M phosphate buffer, pH 8, 8M urea .292 g $NaH_2PO_4 \cdot H_2O$ + 4.855 g $Na_2HPO_4 \cdot 7H_2O$ + .530 g EDTA made to 200 ml with 8M urea
	0.2M phosphate buffer, pH 8, 10M urea .528 g $NaH_2PO_4 \cdot H_2O$ + 4.345 g $Na_2HPO_4 \cdot 7H_2O$ + .530 g EDTA made to 200 ml with 10M urea
PAGE	0.062M tris-HCl, pH 6.7 1.877 g Tris + water + HCl to pH 6.7 made to 250 ml with water
	0.380M Tris-HCl, pH 8.9 23.009 g Tris + water + HCl to pH 8.9 made to 500 ml with water
PAGE (urea)	buffers are the same as in PAGE, except that 7M urea is used instead of water
	0.046Tris-glycine buffer, pH 8.3 11.132 g Tris + 57.6 g glycine made to 2000 ml with water

Table A2--continued

Experiment	Buffer
SDS PAGE	0.01M phosphate buffer, pH 7.0, 1% SDS .138 g $NaH_2PO_4 \cdot H_2O$ + 1.0 g SDS to 100 ml with water
	0.1M phosphate buffer, pH 7.2, 0.1% SDS 1.95 g $NaH_2PO_4 \cdot H_2O$ + 9.65 g $Na_2HPO_4.7H_2O$ + 0.5 g SDS made to 500 ml with water
	reservoir buffer is the same as the 0.1M phosphate buffer

Preparation of Coomassie Brilliant Blue G250

Weigh out 5.0 g of 70% perchloric acid and dilute this to 100 ml to give a 3.5% perchloric acid solution. Add 40 mg of CBB G250, and stir overnight. Filter over Whatman #1 filter paper and store.

Table A3. Method for determination of fat.

a. weigh approximately 200 mg of sample into conical 15 ml centrifuge tube
b. add 1.5 ml of 2% KCl and agitate tube
c. add 1.0 ml of 95% ethanol; seal tube with cork stopper wrapped in saran; shake tube 30 sec
d. release pressure and add 2.5 ml ethyl ether, making certain to rinse stopper
e. seal tube and shake 30 sec
f. release pressure and add 2.5 ml petroleum ether, making certain to rinse stopper
g. seal tube and shake 30 sec
h. centrifuge tube 1 min on clinical centrifuge
i. carefully remove upper layer with syringe and place in previously tared dish held on hot plate
j. wash tube with 1.0 ml of ethyl, petroleum ether mixture (1:1)
k. repeat steps c-j twice
l. evaporate pooled washings to dryness and place dish in 100 C vacuum oven for 30 min
m. cool dish in dessicator and reweigh

Table A4. Numerical values, standard deviations and levels of significance for various functional tests.

Product	Value (std. dev.) SWC	BWC	NAC	NDM	ACP	P3C	HMP	CAC
Solubility (%)								
pH 7.0	92.7(.9)^{a}	91.7(1.6)^{a}	86.8(1.2)^{b}	67.3(2.4)^{c}	61.0(1.4)^{c*}	61.2(1.6)^{c*}	87.2(2.6)^{a}	5.5(.8)^{e}
pH 4.5	65.0(.8)^{a}	15.0(0)^{b}	0^{c}	22.0(0)^{d}	0^{c}	0^{c}	42.7(1.2)^{e}	0^{c}
pH 3.0	92.0(1.6)^{a}	3.3(.5)^{b}	62.0(1.6)^{c}	40.3(4.5)^{d}	52.2(.8)^{d*}	25.6(2.4)^{e}	0^{f}	44.7(.9)^{d}
Viscosity (cps)								
Conc. 3%	-	-	1.91(.01)^{a}	-	2.76(.07)^{b}	2.00).03)^{a}	1.38(.02)^{c}	2.52(.02)^{d}
5%	1.34(.02)^{a}	1.08(.04)^{b}	3.67(.03)^{c}	-	5.54(0)^{d}	2.97(.05)^{e}	1.97(.05)^{f}	4.56(.20)^{g}
7.5%	-	-	6.69(.02)^{a}	-	15.14(.12)^{b}	5.65(.12)^{c}	2.77(.01)^{d}	13.52(.31)^{e}
10%	2.72(0)^{a}	1.82(.03)^{b}	24.46(.05)^{c}	1.58(.10)^{b}	32.83(.23)^{d}	11.30(.04)^{e}	4.95(.11)^{f}	-
15%	3.56(.03)^{a}	3.18(.16)^{a}	-	2.09(.05)^{b}	-	-	-	-
Hydration Capacity (ml/g)	.68(.06)^{a}	1.06(.06)^{a}	3.12(.12)^{b*}	.56(.06)^{a}	3.88(.12)^{c}	3.38(.12)^{b}	2.62(.12)^{d*}	1.62(.12)^{e}
Emulsifying Capacity (g/mg)	6.67(.24)^{a}	6.30(.22)^{a}	3.13(.12)^{b}	4.07(.19)^{c}	3.00(.08)^{b}	3.13(.17)^{b}	3.97(.20)^{c}	2.57(.12)^{b}
Whipping								
ml/g	9.0(.3)^{a}	0^{b}	13.7(.1)^{c}	11.0(.4)^{d}	0^{b}	0^{b}	0^{b}	0^{b}
1/2 time(min)	16.0(.3)^{a}	0^{b}	15.7(1.2)^{a}	31.0(4.2)^{c}	0^{b}	0^{b}	0^{b}	0^{b}

Within each row, numbers without a common superscript are significantly different at the 1% level.

* Denotes significance at the 5% level.