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To the Graduate Council;

I am submitting herewith a dissertation written by Jyung Rewng Park entitled "The Influence of Milk Processing upon the Binding of Iron to Various Milk Protein Fractions." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

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THE INFLUENCE OF MILK PROCESSING
UPON THE BINDING OF IRON
TO VARIOUS MILK PROTEIN FRACTIONS

A Dissertation
Presented to
the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

by
Jyung Rewng Park

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ABSTRACT

Fortification of food with iron has been of great interest to nutritionists because anemia is the most prevalent nutritional problem throughout the world. In spite of the fact that milk and milk products are widely available, they are low in natural iron content. Because of the significance of milk in our diet, it is an appropriate food for fortification with iron. Pasteurization and homogenization are the standard dairy industrial processes. The purpose of this study was to investigate the effect of pasteurization and homogenization on the binding of added iron to milk proteins.

Pasteurization was carried out at 62.8 C for 30 min and the pasteurized milk was homogenized through a two stage commercial homogenizer. The effect of processing was determined by measuring iron content in casein, whey proteins, beta-lactoglobulin, proteose-peptone, and fat-globule membrane protein.

The data indicated that most of added iron was recovered in the skim milk portion. Pasteurization changed only the binding capacity of whey proteins. Whey proteins from raw milk bound more added iron than that from pasteurized milk. Homogenization increased the binding capacity of all milk proteins. The order of decreasing binding capacity of milk proteins (microgram Fe/g protein) from raw or pasteurized milk was as follows: fat-globule membrane protein, rennin-coagulated casein, proteose-peptone, acid-precipitated casein, and whey proteins. The order

of binding capacity in homogenized milk (microgram Fe/g protein) was as follows: rennin-coagulated casein, fat-globule membrane protein, acid-precipitated casein, proteose-peptone, and whey proteins.

CRANE & CREST

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CHAPTER I

INTRODUCTION

Nutritional anemia is one of the most prevalent deficiency diseases in the United States and throughout the world (8, 35, 42, 45, 58). The USDA's Agricultural Research Service (36, 79) estimates that infants under 3 years of age and women under 55 years of age consume only about half of the recommended dietary allowance (RDA) of iron.

Milk and milk products are low in iron content, but are good sources of calcium. They are widely available and are consumed in substantial amounts by most people. Dairy products contribute only 2% of total iron intake in the American food supply, whereas meat and cereals provide a high percentage; 36 and 27%, respectively (46). About three-fourths of the calcium in diets of Americans is supplied by milk. The dairy products consumed in the United States make a significant nutritional contribution to the national diet. Milk is of special significance in infant diets, and makes a major contribution to the nutrition of adults as well. Because of the significance of the role of milk in our diets milk appears to be an appropriate food for fortification with iron.

Tennessee law has prohibited the fortification of milk with iron, but in 1970 it was made optional. Most of states in the United States permit fortification of milk with iron at the concentration of 10 mg per quart of milk as a minimum (32, 33).

Pasteurization and homogenization are standard dairy industrial processes. Pasteurization destroys pathogenic organisms and preserves the desirable characteristics of milk. The minimum pasteurization process does not alter the physical behavior of the lipid phase but it changes the characteristics of milk proteins. Homogenization stabilizes the fat emulsion against gravity separation, but the normal characteristics of milk are profoundly altered by this process.

The purpose of this study was to investigate the effect of pasteurization and homogenization on the binding of added iron to milk proteins,

CHAPTER II

REVIEW OF LITERATURE

I. DISTRIBUTION OF IRON AND COPPER IN MILK

The presence of iron in milk was noted by Bunge in 1889 (10) long before any of the other trace elements were detected. Milk always contains iron, usually ranging from 100 to 900 micrograms per liter (19). Higher amounts have been found, but any appreciable increase was due to contamination of the milk from equipment. For example, pasteurized milk was found to contain 2.3 ppm, range 2.2 to 2.5 (52).

The stage of lactation appeared to have only a minor effect on the iron content of milk. Underwood (105) stated that cows' colostrum contained 3 to 5 times as much iron as ordinary milk and that more iron was found in cows' milk toward the end of lactation. But another worker noted a tendency for iron concentration to fall during lactation (3).

King and Williams (64) investigated the distribution of metabolized copper in milk during early lactation by using radioactive Cu^{64} . The amount of copper bound to fat globules was about 15% at the 10th week of lactation. The change in copper distribution in fat-globule membrane was accompanied by a change in susceptibility to oxidized flavor. A combination of cream washing and chelation by ethylenediaminetetraacetic acid (EDTA) removed up to 90% of lipid-bound copper.

According to Corbin and Whittier (19), the feeding of supplemental iron and copper did not increase the iron or copper content of milk. However, Dunkley et al. (29) tested the effect of supplemental copper sulfate at the level of 5.0 g per cow daily on the concentration of copper in the milk and on the oxidative stability of milk. Milk copper was increased significantly by supplements of copper sulfate. Differences in oxidative stability of the milk were not significant.

Murthy et al. (78) analyzed the iron content of market milk collected in alternate months from February 1967 to February 1968 from 63 cities in the United States. Iron content ranged from 0.20 to 1.51 mg/l. They found significant differences among months and locations. Iron content was lowest in summer and highest in winter.

The form in which iron exists in milk has been investigated by several workers. The early concept was that at least half of the iron was combined with the fat, but other workers considered it to be in organic combination, partly with the protein and partly with the fat. Later research confirmed that iron was bound to the protein fraction (54). Horst and Schafer (53), in electrophoretic studies, noted that iron migrated with the immune globulins (protein). Allan (1) concluded that both added and natural iron were nonionic and were associated with the fat globule membrane.

Van der Waarden (106) studied the distribution of natural and added copper and iron in butter and found that the triglycerides contained no metals, the proteins contained all of the iron and 50 to 75% of the copper, the phospholipids contained 25 to 50% of the copper and the

water was free of metals. He concluded that the natural and added forms of copper and iron were both distributed in a similar manner.

Davies (21) reported that added copper and iron were uniformly distributed in gravity-separated milk in direct proportion to the curd nitrogen. But a higher percentage of copper and iron was found in the cream after milk had been subjected to centrifugal separation. The heavy metal entering into butter from cream of different acidities was not affected by the acidity but by the curd-nitrogen content of the butter. Davies (21) also found the presence of protein increased slightly the amount of heavy metal entering into the fat fraction due to adsorptive and buffering effects of the protein at the oil-water interface in the emulsion. The concentration of the ionic form of heavy metal in milk was of a low order, but increases in acidity caused more ionic metal to form,

Menger (74) found that the actual milk fat does not contain any copper but that 15 to 20% of the total natural copper was on the surface of the fat globules. The remainder was in the milk plasma, bound to the proteins. Added copper was only distributed to a small extent (2.5%) on the surface of the fat globules and the remainder was bound to the serum proteins and was not dialysable.

Rice and Miscall (84) found that when milk contaminated with copper was separated into milk and cream, the copper was distributed roughly in proportion to the water. They concluded that the copper was in aqueous solution and that since the copper did not go with the fat, no dissolving action of copper in the fat occurred.

Miller and Tracy (75) observed that butter made by the continuous process contained less copper (0.19 ppm) than butter made by the batch system (0.27 ppm). The centrifugal treatment given cream in the process of making butter by the continuous method resulted in a concentration of copper in the non-fat portion. Hartman (49) reported that among milk proteins the whey proteins had the greatest affinity for copper.

The distribution and chemical state of ferrous iron added to a prepared infant formula was studied by Vaughan and Knauff (108), using Fe^{59} as a tracer. The high level of Fe^{59} activity in the protein fraction showed that most of the added iron was either bound by the milk proteins or by some other substance precipitated by alcohol.

Chien and Richardson (17) fractionated the fat-globule membrane by using ultracentrifugation and found the iron content to be higher in the outer layers of the membrane than the inner layers, while copper was high in inner supernatant aggregates and low in outer supernatant.

When sweet or slightly acid creams containing added copper or iron sulfate were churned, the distribution of the added copper or iron between the buttermilk and the butter indicated that the added metal ions were bound non-specifically to the serum and the fat-globule membrane proteins. When fully ripened cream containing added copper and iron was churned the proportion of the added copper or iron passing from the cream into the butter was much higher than for sweet or slightly acid cream because of the higher proportion of protein retained in the butter (72).

Richardson and Guss (85) attempted to relate the concentration of traces of copper and iron to the density of the component obtained by

centrifugal fractionation of fat-globule membrane. They found that iron was high in the high-density fractions, whereas copper was more evenly distributed. Highest ratios of metal-to-fatty acid indicated one copper and one iron ion per 24 and 21 polyunsaturated fatty acid molecules, respectively.

Koops (65) studied the effect of the pH on the partition of natural and added copper in milk and cream. He found that 1 to 2% of the total amount of radioactive copper added to milk was bound by the fat-globule membrane at pH 6.6. When milk was neutralized after being acidified to a very low pH value (pH 6.6 $\rightarrow$ 1.8 $\rightarrow$ 6.6), the content of natural copper in the fat-globule membrane increased considerably. He also indicated that acidification of milk to pH 4.6 did not affect the partition of natural copper in milk. As a result of acidification of cream to pH 4.6, about 30 to 40% of the added copper shifted from the plasma proteins to the fat-globule membrane.

Chen and Tobias (16), very recently revealed that the naturally occurring copper associated with fat was largely or wholly a part of copper containing components which desorb from the surface of fat-globules during storage. Added copper associated with the inner nondesorbing part of the membrane remained there for at least 144 hr of storage.

Dill and Simmons (25) investigated the binding of copper by milk proteins by adding copper to aqueous suspensions of several milk proteins maintained at constant temperature. Reaction was completed in less than 1 hr for proteins studied. No copper was bound below pH 2.0 by whole milk proteins while a preparation of bovine beta-lactoglobulin

did bind a significant amount below this pH. Binding increased linearly with pH for all proteins to a maximum around pH 8.0, ranging from about 1.7 mole copper per mole of alpha-lactalbumin to 3.8 moles per mole for alpha-casein. Beta-lactoglobulin bound about 12 moles of copper per mole of protein.

The equilibrium dialysis method is commonly used for studying the binding of ions with protein. By using this method, Aulakh and Stine (5) examined the binding of copper with micellar casein, fat-globule membrane protein, alpha-casein, beta-casein and beta-lactoglobulin at pH 6.5. They found micellar casein bound the most copper and the fat-globule membrane had the second greatest affinity. Much less copper was bound by alpha-casein, beta-lactoglobulin and beta-casein.

According to Fiess and Klotz (34) the affinity of proteins for copper at pH 6.5 decreased in the following sequence: alpha-casein, beta-casein, bovine serum albumin, beta-lactoglobulin and lysozyme.

II. EFFECT OF IRON AND COPPER ON QUALITY OF MILK AND MILK PRODUCTS

The addition of metals to nonhomogenized milk depresses or retards lipase activity. Davies (20) reported that traces of heavy metals in amounts up to 25 ppm had the property of inhibiting the action of lipase in butter from unpasteurized sweet cream in varying degrees depending on the concentration of the metals and on the metal itself. He found that iron was less active than copper. The depression of lipase activity by the various metals varied directly with their catalytic power in inducing

fat oxidation. He suggested that destruction of lipase was catalyzed by the traces of heavy metals according to their varying powers of activating oxygen.

Frankel and Tarassuk (38) reported a 7 to 17% inactivation of lipase when raw milk was treated with 5 to 20 ppm of Cu^{++} for 15 min at room temperature. Incubation with 5 ppm of Cu^{++} for 1 hr at 37 C produced 69% loss of milk lipase activity. However, Krukovsky and Sharp (69) showed that copper was incapable of inhibiting lipase activity in nonhomogenized milk in the absence of oxygen. At the same time they also found that oxygen alone was an active inhibitor, its effect being greatly enhanced by the presence of small amounts of copper.

On the other hand, the addition of copper to homogenized milk in concentrations as high as 10 ppm had no effect on subsequent lipolysis (43). A special affinity might have existed between copper and the membrane material. Copper added to nonhomogenized milk may have tended to be adsorbed onto the natural membrane. Since this would be the eventual natural environment for the lipases, inactivation might have taken place through whatever mechanism was involved (e.g., oxidation, binding, or chelation with the active sites of lipase molecule) before the enzyme had an opportunity to penetrate the membrane material. In homogenized milk, on the other hand, the natural membrane was largely removed (11, 12, 13). Lipolysis proceeded rapidly due to the increase in available surface coupled with the elimination of the natural membrane material.

The fact that iron or copper promotes the development of oxidized flavor in dairy products has been well established (39). The cupric ion

exerted a greater catalytic effect than the cuprous (93, 94), and the ferrous ion appeared to be more influential than the ferric ion (47).

The work of Thurston and Barnhart (101), Thurston et al. (102), Swanson and Sommer (97) and Pont (81) showed clearly that spontaneous or copper-induced oxidized flavor in milk arose from oxidation of phospholipid in the fat-globule membrane.

Metallic ions, mainly copper and iron, have been shown to be effective catalysts of oxidized flavor in milk, but the question remained as to whether the small amounts normally in milk was of significance to development of the defect (48).

King and Dunkley (62) obtained a highly significant correlation between the concentration of natural copper content of milk and the incidence and intensity of spontaneous oxidized flavor. They concluded that the copper present in milk as it came from the cow was an important catalyst of oxidized flavor.

Scanlon and Shipe (92) reported on the susceptibility of multivitamin mineral (MVM) milk to oxidation. They indicated that the ferrous iron was responsible for the catalytic oxidation on milk when iron compounds were used for mineral fortification of whole milk.

Williams and Burgwald (111) studied the development of oxidized flavor in milk when copper at a concentration of 2 ppm was added after pasteurization of the milk at 62.2 C for 30 min. Copper was an effective catalyst for the development of oxidized flavor in milk and the intensity of the flavor was greater when copper was added after, rather than before pasteurization.

King (60, 61) revealed that addition of copper in concentrations less than 0.1 ppm inhibited oxidation rates of ascorbic acid as well as the lipid of fat-globule membrane material. Copper addition of more than 0.1 ppm increased ascorbic acid oxidation, but lipid oxidation was further inhibited.

Olson and Brown (80) and Foster et al. (37) concluded that proteins and protein hydrolyzates formed complexes with copper and retarded or prevented the development of oxidized flavors in fluid milk.

Dunkley et al. (28) found that the addition of copper (0.1 ppm) increased light-induced flavor in milk. Addition of neocuproine to chelate the natural copper prevented the development of such an intense light-induced flavor.

When iron, as ferrous sulfate (2 ppm), was added to cream to give a total iron content above 0.5 ppm in the butter, there was usually a metallic flavor in the fresh butter. Iron in the proportions usually present in commercial butters was not found to have any pro-oxidative effect on the butterfat in the butter during storage (72).

The normal amounts of iron in commercial butter, 0.4-1.0 ppm, are too small to cause significant oxidation of the butterfat. The metallic flavor was found only in the butter serum and was thought to be due to the intrinsic flavor of the dissolved iron (57).

Bishop (9) reported tallowy discoloration or white streaks in Cheddar cheese was due to oxidation and the flavor resembled that of tallow. In the tallowy portions of the cheese the copper content was lower and the iron content was higher than adjacent normal cheese. The

copper probably migrated in cheese following oxidation of the sulfhydryl compounds in the tallowy area while the unexpected distribution of iron might be explained by changes that occur during salting of the curd. The ratio of ferrous to ferric iron tended to increase as the cheese matured and tallowy portions of cheese did not contain less ferrous iron than adjacent portions. The addition of iron salts to cheese milk did not increase the incidence of tallowy discoloration, but the addition of sufficient copper salts reduced it.

Organoleptic evaluation of fortified milk with iron has been conducted by several workers. Demott (24) reported that of the iron compounds employed, ferric pyrophosphate and ferric phosphate had the least effect on flavor. Flavor scores on skimmilk were influenced very slightly by the amount of iron added as ferric phosphate or as ferric pyrophosphate, even after 4-day storage. Lab-pasteurized whole milk containing 5.2 or 10.4 ppm added iron in the form of ferric pyrophosphate had less cooked flavor than the control milk containing no added iron, perhaps due to a binding of iron to the protein components responsible for the cooked flavor. Vat-pasteurized skimmilk containing 5.2 ppm iron as ferric pyrophosphate had the same flavor score before and after homogenization at 2500 p.s.i. Using rats he also found that both ferric pyrophosphate and ferric phosphate were biologically available.

Reisfeld et al. (83) stated that the free fatty acid content of commercial vitamin-mineral fortified pasteurized milk (milk containing added iron, iodine, vitamin A, D, and B complex) was found to increase during storage at 4 C. The addition of ferric ammonium salt to the milk

prior to processing was found to be responsible for the apparent lipase activation after homogenization and pasteurization.

According to Edmundson et al. (30) organoleptic evaluation of iron-enriched whole milk showed that ferric iron compounds uniformly resulted in lipolytic rancidity when milk was pasteurized at minimum to moderate temperatures (less than 79 C). This off-flavor was reduced or completely eliminated simply by pasteurizing at 81 C which inactivates lipase. Data showed that milk lipase was made more heat resistant by ferric forms of iron. Ferrous compounds normally caused a definite oxidized flavor when added to raw milk before pasteurization. This off-flavor was markedly reduced by deaerating the milk before adding the iron. Ferrous compounds did not cause rancidity. The degree of rancidity, if present in freshly pasteurized samples, increased with storage time, whereas the oxidized flavor in the ferrous iron fortified samples decreased during storage. They stated that pasteurization should then follow the addition of the iron.

III. EFFECT OF HEAT TREATMENT ON THE DISTRIBUTION OF IRON AND COPPER

Pasteurization and allied forms of heat treatment are the foci of most processing procedures. The objectives of heat treatment are (a) the destruction of pathogenic organisms, and (b) the preservation of the desirable characteristics of milk and its products pending their consumption. Pasteurization (61.8 C for 30 min or 71.8 C for 15 sec) represents a minimum heat treatment designed to destroy all of the pathogenic bacteria

and most of the nonpathogenic organisms. Most of the degradative enzyme systems are also partially or totally inactivated by pasteurization.

Pasteurization does not appear to affect the iron content of milk. Kraus (67) noted no loss of iron when milk was pasteurized. Vasa (107) found no significant difference in iron content between pasteurized whole milk and pasteurized milk standardized to contain 2% butterfat.

Aurand et al, (6) reported that heating of milk at 76 C for 30 min prevented the development of oxidized flavor; whereas, 63 C for 30 min accentuated its development. They explained this phenomena by the production of active protein sulfhydryl groups and the thermal inactivation of an enzyme at 76 C for 30 min.

Ramada (82) found the heating of cream caused migration of the phosphatides from the fat globules to the plasma of the cream. Koops and Tarassuk (66) further reported heating of milk caused the transfer of phosphatides from the fat-globule membrane to the plasma to the extent of about 20%. This evidence indicated about 80% of the fat-bound phosphatides were so firmly bound as to require a greater disruption of the fat-globule membrane material than was usually obtained by plant processing treatment in order to accomplish the migration of the phosphatides from the fat phase to the aqueous phase.

Sargent and Stine (90) investigated the mechanism by which heat imparts resistance to copper-catalyzed lipid oxidation in fluid whole milk. They showed migration of 2 to 4% of the added copper to the cream phase resulted when raw milk treated with 100 to 500 micrograms of Cu/1 was stored at 1 C for 18 hr. Heating this milk to temperatures greater

than 60 C increased the copper content of the cream by as much as 800%. Milk having a total copper content of 40 to 240 micrograms of Cu/1 exhibited maximum and constant absorption of copper by the fat fraction following both flash and 10-min holding at 85.0 to 93.3 C. When the total copper content of the milk was increased to 500 to 700 micrograms of Cu/1, maximum copper adsorption occurred in the cream following both flash heating at 85 C and 10-min holding at 76.6 C. Heating the milk to higher temperatures resulted in desorption of copper from the cream phase. Their results indicated that the extent of migration of residual and added copper to the cream phase as a result of heating was dependent upon the fat content of the milk, total copper content, and temperature history of the milk.

Sargent and Stine (91) further reported that raw milk treated with 500 micrograms Cu/1 and heated at temperatures of 71.1 to 93.3 C showed maximum adsorption of copper by the cream phase at 82.2 C. When this milk was treated with 200 ppm N-ethylmaleimide, the extent of migration of copper to the cream at 82.2 C was reduced by 50%. Washing the cream with distilled water at 2.2 C removed a relatively constant quantity of adsorbed copper from the cream of the milk heated to 71.1 to 82.2 C. Washing the cream of milk heated to 82.2 to 93.3 C removed as much as 50% of the adsorbed copper. Wash water at 26.6 C increased the extent of desorption of copper from the cream of milk heated to 82.2 to 93.3 C. Copper was most tenaciously adsorbed in the washed and unwashed cream of milk heated to 82.2 C. When skim milk treated with 500 micrograms of Cu/1 was heated to 71.1 to 93.3 C and mixed with unheated cream, the

maximum copper adsorption was observed in the cream mixed with skim milk which had been heated to 76.6 C.

Reisfeld et al. (83) found that the addition of ferric iron salts protected the milk lipase against heat inactivation, whereas citrate salts did not protect the lipase against heat inactivation at normal pasteurization temperature (73.3 C for 16 sec). They also made an effort to find the effect of the sequence of processing on the protective effect of ferric iron. Results showed that complete pasteurization before homogenization eliminated the protective effect of ferric iron and that homogenization just prior to holding for 16 sec reduced somewhat the effect of the ferric iron.

Loewenstein and Gould (71) reported fat globules in milk heated to 82 C for 15 min lost membrane material and the loss of proteins exceeded the loss of lipids. They indicated that phospholipid and protein were lost in the same ratio, which suggests that lipoproteins were displaced from the globule surface as a unit and not as free phospholipids.

During high-temperature (82 to 92 C) pasteurization of cream the high concentration of fat caused shearing forces between fat globules and thermal destruction of proteins combine to weaken binding forces between the two membrane layers releasing outer lipoprotein particles into the aqueous phase. Butter produced from high-temperature pasteurized cream had a lower copper content and better keeping quality than that from low temperature-pasteurized (72 C) cream or milk, or high temperature-pasteurized milk (60).

Samuelsson (87) tested the effect of different heat treatments on copper distribution in milk. Tests involving the addition of Cu^{64} showed that when whole milk was heated, the copper content of the cream and butter serum was more than doubled as the 10 min heating temperature rose from 50 to 80 C, the increase being particularly sharp between 65 and 75 C. When milk was pasteurized at 83 C and cooled to 40 C before separation and the cream pasteurized at 95 C, the butter serum gave more than three times higher Cu^{64} activity than milk separated prior to pasteurization.

Samuelsson (88) also studied the effect of chelating compounds on the distribution of copper. Adding a chelating compound resulted in the migration of most of the copper from skimmilk to cream; when this cream was washed with distilled water, relatively small amounts of Cu^{64} were washed off. Addition of EDTA caused the migration of Cu^{64} to the fat-free fraction of the milk.

The effect of heat on the Ca^{++} has been an interesting subject for many researchers. Christanson et al. (18), Baker et al. (7), Tessier and Rose (100), and Demott (22) reported decreases in the concentration of ionized calcium when milk was subjected to heat. Demott (23) reported that the heat treatments (65 C for 30 min and 75 C for 30 min) did not influence the calcium ion concentration of beta-lactoglobulin.

Zittle et al. (114) however, found heating beta-lactoglobulin at 90 C for 30 min in the presence of calcium ions resulted in aggregation but did not affect the amount of calcium bound.

Demott (22) further reported that decreased ionized calcium from heating appeared to be reversible after 24 hr of storage at 4 C.

However, Muldoon and Liska (77) observed a decrease in ionized calcium occurred in heated milk and appeared to be irreversible even after 10 days at 2 C. They also found, in most cases, that a more intense heat treatment caused a greater decrease in ionized calcium.

The isoelectric point of casein was slightly increased in heated milk, presumably due to its association with the denatured serum proteins, and under these conditions the coagulation constitutes a coprecipitate of casein and denatured serum protein (31).

IV, EFFECT OF HOMOGENIZATION ON MILK PROTEINS

Because iron is attached to various milk proteins and homogenization changes the nature of milk proteins, an understanding of these protein changes is critical. The milk fat-globules are reduced in size from an average diameter of 5 or 6 micron to less than 2 micron, increasing the fat surface several fold (104).

Conceivably, this transformation affects the orientation or the composition of the materials most closely associated with the fat-globule at the fat plasma interface. In addition to the components comprising the normal fat-globule membrane of nonhomogenized milk, casein and other plasma proteins have been suggested as components of the newly created fat surface in homogenized milk (11, 12, 13, 59).

Fox et al. (40) reported that a fat-protein complex was produced in milk by homogenization. Fat-protein complex formation was found to increase with increasing homogenizing pressure, with increasing fat content, with an increase in concentration above 31% total solids, and with

increasing calcium concentration up to a limit of 1 mM added calcium. They indicated conditions existing at the homogenizer valve at the instant of homogenizing were responsible for complex formation.

Brunner et al. (12) found the proteins bound on the fat globules of homogenized milk were different from the proteins in nonhomogenized milk. They demonstrated there was an increase in the concentration of lysine and glutamic acid and a marked decrease in the concentration of tryptophan, cystine, and glycine in the fat-membrane protein of homogenized milk. These differences in the amino acid composition indicated that the character of the fat-membrane protein of milk was changed as a result of homogenization and, further, that the main protein constituents of the milk plasma formed a large percentage of the membrane-protein complex in homogenized milk.

Wiegner (110) calculated the amount of casein that could be adsorbed on the fat surface in nonhomogenized and homogenized milk was about 2 and 25%, respectively, of the total available casein. He assumed that casein was the sole substance adsorbed. Doan (26) believed the decrease in heat stability of homogenized milk resulted from an increase in the amount of casein adsorbed on the enlarged fat surface.

Jackson and Brunner (56) isolated an atypical casein fraction in which the alpha-component appeared to be associated with the normally occurring soluble membrane-protein (glycoprotein) from the resurfaced fat globule. The presence of a heat-coagulable protein fraction (whey proteins) was also noted. Henstra and Schmidt (50) concluded that the internal structure of the casein particles in milk was broken down during

homogenization, thus providing a possibility for the casein sub-units to adhere to the surface of the fat globules. By employing ultracentrifugal sedimentation technique, Brunner et al, (11) also identified the proteins which constitute the adsorbed proteins in homogenized milk as casein, whey protein, and an altered beta-lactoglobulin or a complex of two or more of the constituent proteins.

Brunner et al. (13) further reported on the electrophoretic patterns of the fat-membrane proteins in milk. They found that one to three components of different electrophoretic characteristics were observed in the homogenized fat-membrane proteins,

Inhibition of the development of oxidized and tallowy flavors in milk by homogenization is well known. The following explanations have been advanced: (a) migration of the unstable lipid component, presumably phospholipids, from the surface into the interior of fat globules (68), (b) partial removal of phospholipids from the fat globule membrane into the serum phase (103), and (c) irreversible change in the structural configuration of the copper protein lipid complex in such a way that ascorbic acid was no longer able to initiate the formation of lipid free radicals (62).

Homogenization did not increase the phospholipids in the newly formed fat-globule membrane; in fact, they decreased slightly (66). The copper concentration in the membrane per unit weight of fat was somewhat decreased by homogenization, due to adsorption of plasma protein which bind only about 0.04 as much natural copper as the protein of the original membrane (76).

Tarassuk and Koops (99) reported that the site of the oxidative reactions that cause the oxidized flavor in milk was the surface of the fat-globules and the reactants were phospholipids and oxygen, catalyzed by the copper-protein complex. The concentration of the phospholipids and the copper-protein complex per unit of surface area was decreased proportionally to the homogenization pressure.

Cerbulis (15) determined the lipid and chloroform-methanol-extractable protein (CMEP) content of casein miscelles. The CMEP content was decreased from 2% of the total casein in fresh milk to about 0.52% in pasteurized homogenized milk. The lipid content was increased from 1.52% in fresh milk casein to 32.67% in homogenized milk casein. Bound lipid content of homogenized milk was 25.3%, while its content of fresh milk varied from 3.3 to 27.9%. The pasteurization and homogenization of milk probably redistributes the milk lipid, produces new lipid-protein complex, and releases a new extractable phospholipid in milk.

CHAPTER III

MATERIAL AND METHODS

I. TREATMENT OF MILK

Food grade ferric pyrophosphate, $\text{Fe}_4(\text{P}_2\text{O}_7)_3 \cdot 9\text{H}_2\text{O}$, containing approximately 12% iron, 20% P_2O_5 , and 40% citric acid, was used in this study. An analysis of this shipment was made and the amount of the additive necessary to result in 10 ppm elemental iron was found to be 0.833 g/10 l milk. This amount of additive dissolved in 5 ml of double distilled water, was added to 10 liters of raw whole milk and stirred to distribute uniformly. The iron-added milk was set in refrigerator (4 to 7 C) overnight to allow complete interaction. The milk was warmed to 45 C for separation.

Each experiment consisted of six trials. The experiments were designed to determine the influence of pasteurization and pasteurization plus homogenization upon the distribution of iron in the various protein components. In the case of beta-lactoglobulin, five trials were conducted.

Pasteurization. Pasteurization was carried out by heating 10 liters of the iron-treated milk at 62.8 C for 30 min in the laboratory-type vat-pasteurizer and cooled to 45 C for separation.

Homogenization. Twenty liters of pasteurized milk was homogenized through two stage commercial homogenizer by employing 2,500 p.s.i. on the first stage and 500 p.s.i. on the second stage. The homogenized milk was cooled to 45 C for separation.

II. FRACTIONATION OF MILK

The fractionation procedure proposed by King et al. (64) was employed in this experiment. The flow diagram for the fractionation is shown in Figure 1,

The milk was separated into skimmilk and cream at 45 C by use of a laboratory-type DeLaval separator. Due to expected low fat recovery in separation of cream from homogenized milk, this milk was re-separated 5 times,

Total Protein

One hundred ml of skimmilk was mixed with 400 ml of 15% trichloroacetic acid (TCA) and stirred on a magnetic stirrer until all proteins were precipitated. The overall concentration of TCA was 12%. The precipitate was filtered from the suspension by use of Whatman No. 40 filter paper.

Casein

Skimmilk was warmed to 40 C and casein was isolated by two different methods; (a) precipitation at isoelectric point (pH 4.67) by using 10% acetic acid and 1 N sodium acetate, and (b) coagulation by proteolytic enzyme, rennin. Twenty five ml of 5% rennin solution was

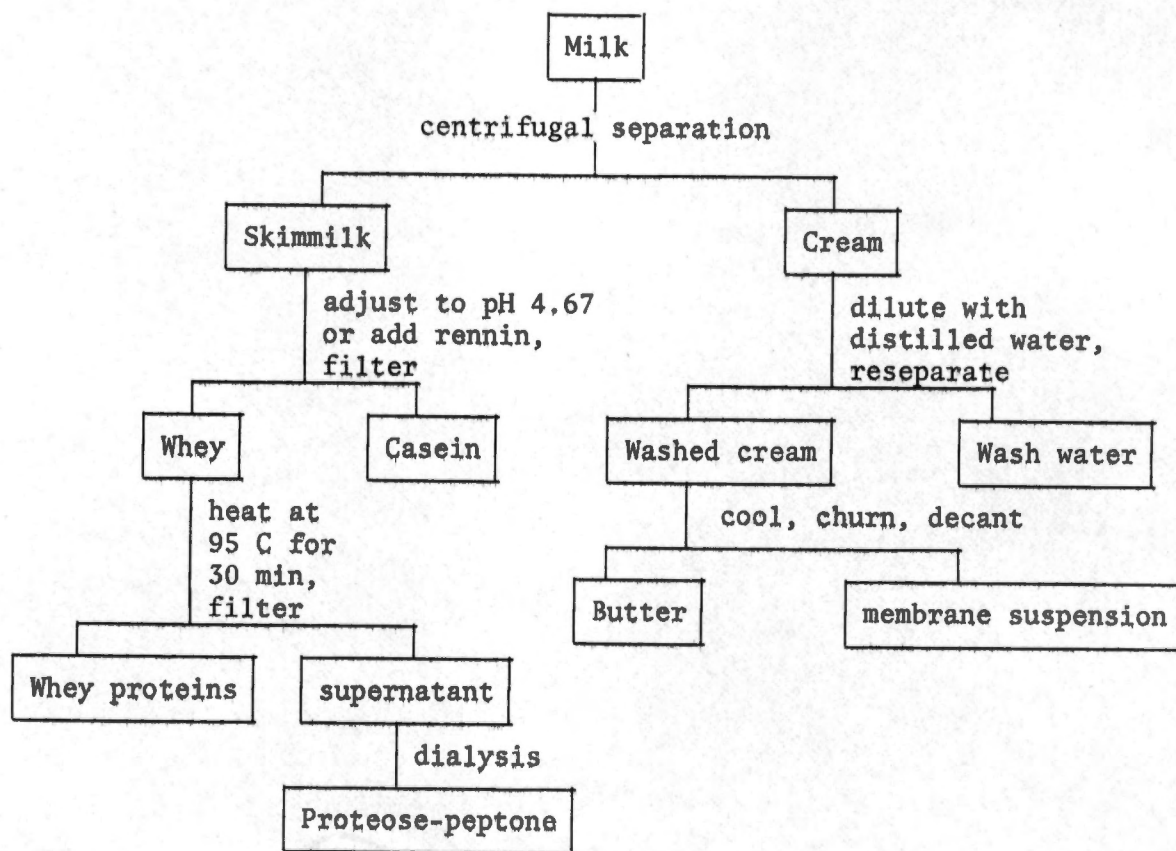


Figure 1. Diagram for the fractionation of milk.

added to 500 ml of skimmilk and incubated at 35 C for 30 min. The coagulum was cut into small pieces to facilitate the removal of whey. The casein was removed by use of Whatman No. 1 filter paper.

Whey Proteins

Whey proteins were precipitated by heating the acid whey at 95 C for 30 min. At room temperature when the denatured proteins were settled, the protein was removed using Whatman No. 1 filter paper (14).

Proteose-Peptide

The filtrate obtained from whey protein precipitation was dialyzed overnight against double distilled water. The proteose-peptide remained inside the dialyzing bag (86).

Fat-Globule Membrane Protein from Raw and Pasteurized Milk

The procedure outlined by Herald and Brunner (51) was used to isolate fat-globule membrane from raw and pasteurized milk. The summarized scheme is presented in Figure 2. Ten liters of milk were separated by laboratory-type DeLaval cream separator. The cream was washed three times with three volumes of distilled water at 40 C. After standing overnight at 4 to 7 C the washed cream was churned at 7 to 10 C until butter granules and serum were separated. The fat-globule membrane complex was concentrated in a dialysis tube (No. S-25275-KA, E. H. Sargent & Co.) at 4 C. An electric fan was used to pass air over the tubing, thus speeding the concentration process. Cleavage of lipoprotein complex was accomplished by the addition of a cold (0 to -5 C) 35% ethanol-in-ethyl ether solution at the rate of 10 ml/5 g of membrane concentrate.

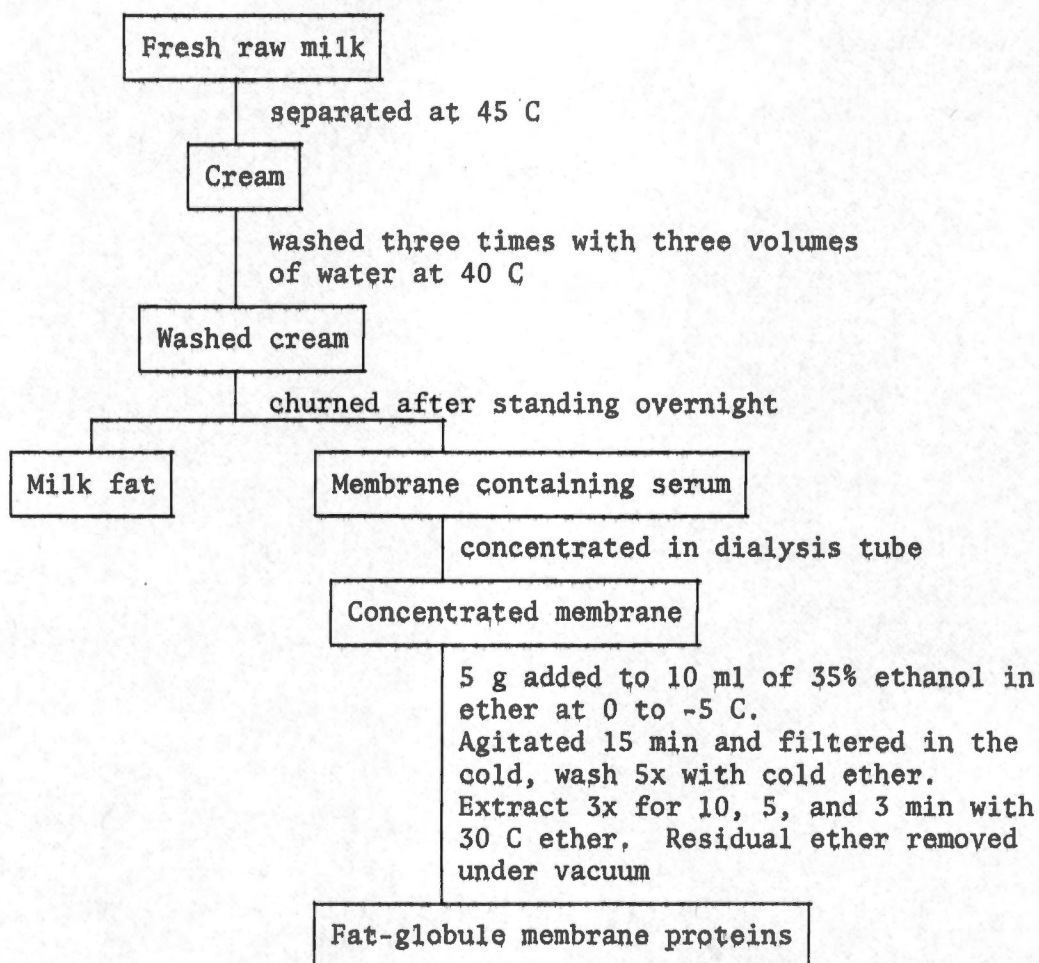


Figure 2. Flow sheet showing preparative procedure for fat-globule membrane protein fraction.

The alcohol and a portion of the liberated lipid materials were removed in five successive washings with cold ethyl ether. The remaining lipids were removed from the ethanol-treated membrane by extraction with several portions of ethyl ether at room temperature. The residual ether was removed under vacuum.

Fat-Globule Membrane from Homogenized Milk

Fat-globule membrane proteins were isolated from the homogenized milk by a method similar to that described by Herald and Brunner (51), but modified as follows: A 3% sucrose solution was employed for all three washings to increase the difference in specific gravity between the fat and the aqueous phase, thus increasing the recovery of fat from the homogenized milk (56). Brunner et al. (12) showed that subsequent to the third washing, practically all of the plasma protein had been diluted out of the system because additional washings failed to lower the nitrogen to fat ratio.

Isolation of Beta-Lactoglobulin

The beta-lactoglobulin was isolated from raw and pasteurized milk by the method of Aschaffenburg and Drewry (4). Two hundred ml of whole raw milk was tempered to 40 C, and 40 g of anhydrous Na_2SO_4 were added with stirring. After the salt was dissolved, the mixture was cooled to 25 C and filtered through a Whatman No. 4 filter paper. One ml of concentrated HCl was added to 100 ml of filtrate and stirred. The resulting precipitate which contained all protein constituents except beta-lactoglobulin was removed by filtration. The filtrate was then adjusted to

pH 6.7 by addition of concentrated NH_4OH (about 0.6 ml per 100 ml). Fifty ml of filtrate was dialyzed overnight against distilled water to remove all sulfate salts as determined by adding a drop of 5% BaCl_2 .

III. ANALYSIS OF IRON

Iron content was determined by a colorimetric method described by Willard et al. (112).

Reagents

- (a) 0.3% 1,10-phenanthroline (w/v).

Dissolved 0.3 g in 100 ml of double distilled water and warmed to dissolve completely (discarded when coloration appears).

- (b) 1% Hydroquinone (w/v).

Dissolved 1 g in 100 ml of 0.05 M sodium acetate buffer at pH 4.5 (discarded when coloration appears).

- (c) 2M Sodium acetate.

Dissolved 17 g of sodium acetate in 100 ml of double distilled water.

Preparation of Standard Curve

Exactly 1 g of iron wire (No. 36 for standardization) was weighed into a clean, dry, iron-free beaker and dissolved in 20% hydrochloric

acid to which 1-2 ml of redistilled nitric acid had been added. The solution containing iron was carefully evaporated on hot plate to dryness and the residue was dissolved in a small amount of hydrochloric acid. The solution was then transferred to a 1000 ml volumetric flask and diluted to volume. A few drops of bromine water was added prior to adjusting to volume. From this stock solution standard solutions containing 1 to 5 ppm of iron were prepared by diluting with water. Two M sodium acetate solution was added to adjust the pH to 3.5 ± 1.0 as measured by a Corning pH meter. The solution was transferred to 100 ml volumetric flask and then 4 ml each of hydroquinone and o-phenanthroline solutions were added, and diluted to volume. The solution was mixed well and allowed to stand for 2 hr to develop color. The optical density was measured at 500 nm by using a Bausch and Lomb Spectronic 20 Colorimeter. The iron content was read by reference to standard curve for known iron concentration (Figure 3). Both ferrous and ferric iron was determined simultaneously.

Sample Analysis

Samples of milk and milk fractions were weighed in porcelain crucibles which had been soaked in nitric acid for 5 min and washed with double distilled water 5 times to remove residual iron if any. Most of the moisture was removed on the steam bath, but the samples were further dried at 85 C in a vacuum oven until there was no change in weight.

Dry ashing procedure was used because of its simplicity, its requirement for little operator attention, and because no reagents were required thus avoiding contamination which they might introduce (113).

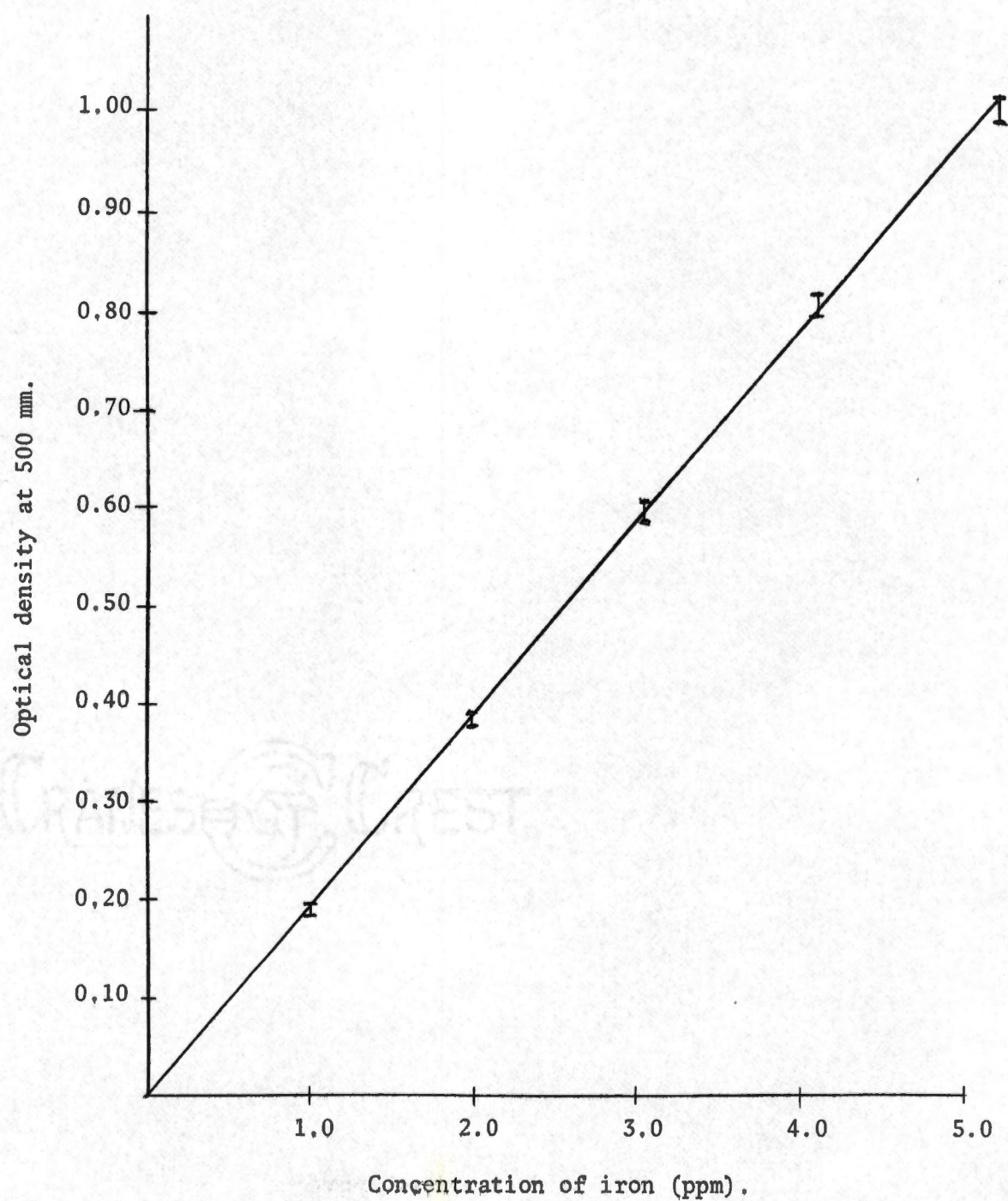


Figure 3. Standard curve for iron determination.

The dried samples were placed in a cold muffle furnace and the temperature was raised slowly over a period of five to six hours to 550 C. The sample was then left at that temperature until it was completely ashed to a white powder. In spite of the fact that the casein samples had been previously dried, these samples tended to foam out of the crucible when the temperature reached about 300 C. A preliminary charring technique using a gas flame was used subsequently to prevent this foaming.

The ashed samples were cooled and dissolved in 5 ml of concentrated hydrochloric acid, allowing the acid to rinse the upper portion of the crucible, then evaporated to dryness on hot plate. The residue was dissolved by adding 2.0 ml of concentrated hydrochloric acid measured accurately and the mixture was heated for 5 min on hot plate with a watch glass on the crucible. The watch glass was rinsed with double distilled water and the solution containing iron was transferred to a beaker. The crucible was rinsed thoroughly with double distilled water and the color developed as noted for the standard curve (2).

IV. DETERMINATION OF PROTEIN AND FAT

The protein content was determined by Kjeldahl method. The weighed sample was placed in 800 ml digestion flask. One packet consisting of 15 g of 87% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 4% HgO , and 25 ml of sulfuric acid were added to each sample. The flask was placed in an inclined position and the procedure completed as described in A.O.A.C. (2). The protein content was calculated by multiplying the nitrogen content by 6.38. The fat content of milk, cream and skimmilk was determined by Babcock test (2).

V. STATISTICAL METHODS

All data were analyzed by the analysis of variance method, using the randomized complete block design. Significant differences between means were determined by Duncan's New Multiple Range Test (96).

CHAPTER IV

RESULTS AND DISCUSSION

I. DISTRIBUTION OF ADDED IRON IN RAW MILK

Natural iron content in raw milk used in these experiments varied from 0.42 to 0.60 ppm (Table 1). Statistical analysis (Table 2) showed that there was no significant difference in iron content in raw milks used for processing at the 0.01 level of probability, but it was significant at the 0.05 level. Since milk contains only a small amount of natural iron, no attempt was made to distinguish natural iron from added iron. The average composition of raw milk used was 3.64% protein, 3.72% fat, and 0.75% ash.

The distribution of added iron in raw milk is shown in Table 3. About 86% of added iron was recovered in the skimmilk portion. When milk was separated about 86% of it was skimmilk and 14% was cream. If the first wash water from the cream could be regarded as a part of the skimmilk, the added iron content of the skimmilk was more than 95%.

In the skimmilk fraction most of the added iron was bound with proteins. Among the proteins in skimmilk the casein fraction bound most of the added iron, about 67%, while only a small amount of added iron was recovered in whey proteins and proteose-peptone fractions.

Table 1. Natural iron content in raw milk
used in these trials.^a

Group I (raw)	Group II (pasteurized)	Group III (homogenized)
0.55	0.48	0.50
0.57	0.48	0.50
0.51	0.52	0.64
0.50	0.48	0.62
0.60	0.46	0.46
0.60	0.46	0.48
0.42	0.52	0.48
0.42	0.52	0.48
0.48	0.44	0.55
0.50	0.46	0.56
0.54	0.58	0.56
0.54	0.62	0.54

^aExpressed as ppm of iron.

Table 2. Analysis of variance of the natural iron in raw milk used in experiments.^a

Source	D.F.	Mean Square	F
Total	35		
Groups	2	0.0011	1.22 (NS)
Milks	17	0.0026	2.89*
Error	16	0.0009	

^aCalculation was based on ppm of natural iron in raw milk.

*Significant at 0.05 level of probability,

Table 3. Effect of processing on the distribution of iron in milk fractions.^{a, b}

	Raw milk	Pasteurized milk	Pasteurized- Homogenized milk
Skimmilk	86.58 ^a	87.28 ^a	83.80 ^b
Unwashed cream	11.21 ^a	11.23 ^a	17.28 ^b
First washed cream	2.26 ^a	3.11 ^b	11.34 ^c
Second washed cream	1.54 ^a	1.95 ^a	10.22 ^b
Third washed cream	0.96 ^a	1.05 ^a	9.87 ^b
First wash water	10.11 ^a	8.19 ^b	4.33 ^c
Second wash water	1.11 ^a	1.53 ^b	2.31 ^c
Third wash water	0.54 ^a	0.75 ^b	0.97 ^c
Casein by acid	67.60 ^a	67.84 ^a	69.97 ^b
Whey by acid	16.58 ^a	16.50 ^a	11.60 ^b
Casein by rennin	70.22 ^a	70.01 ^a	78.22 ^b
Whey by rennin	9.13 ^a	9.29 ^a	6.69 ^b
Whey proteins	3.95 ^a	2.84 ^b	6.85 ^c
Proteose-peptone	4.49 ^a	4.54 ^a	4.88 ^b
Fat-globule membrane	0.21 ^a	0.19 ^a	0.43 ^b
Total protein	79.54 ^a	80.12 ^a	83.26 ^b
TCA-filtrate	5.29 ^a	5.14 ^a	4.16 ^b

^aMeans of 12 observations.

^bExpressed as percentage of added iron.

Note: Means in horizontal rows followed by the same letter are not significantly different at the 0.01 level of probability.

There was a slight difference in binding of iron between casein precipitated at its isoelectric point and rennin-coagulated casein. Rennin coagulated casein bound about 2% more added iron.

To preserve the organization of the fat-globule membrane structure during the course of its isolation, the washing operation was restricted to the minimum number of steps required to eliminate the plasma proteins. Swope and Brunner (98) stated that three washes, consisting of three times the volume of cream was enough to decrease the plasma components 300 to 400-fold. Further washings failed to lower the plasma components in thrice washed cream which indicated no more plasma components were present in the membrane.

About 10% of the added iron remained in the first wash water when cream was washed. The fat content of cream from raw milk was increased by washing. Unwashed cream contained 27.5% fat; first washed cream, 39.5%; second washed cream, 45.7%; third washed cream, 53.4%. Cream which has been washed three times had absorbed 0.96% of the added iron; however, only 0.21% was recovered in the fat-globule membrane suspension. This indicates that iron was associated with butter granules to some extent. Thrice washed cream had less than 1% of the added iron. The small amount of added iron found in fat-globule membrane may indicate that added iron does not attach itself to the phospholipids to any great extent, at least under these conditions.

King et al. (63) and Samuelsson (89) reported that the washing procedure did remove large amounts of iron and copper in the first wash water as a part of the skimmilk portion. They found that only a small

amount of added iron was recovered in thrice washed cream. The amount of added iron found in the casein fraction was above 90%. The results of the present experiment do not agree with King et al. (63), exactly. However, the difference may be due to the different method for fractionation of casein. They isolated the casein fraction by centrifugation.

These experiments did not distinguish whether the iron found in the membrane was largely the naturally occurring iron which was desorbing as part of another component, or whether the added iron exchanged with the natural iron. Chen and Tobias (16) from their experiment with copper explained that the naturally occurring copper was located within the removable layers of the fat-globule membrane while the added copper became a part of the permanent membrane from which it was not easily released. An understanding of the physical and chemical combination of iron with the membrane protein and lipid is lacking.

King et al. (63) reported that natural and added iron and copper were distributed in a similar pattern in various milk fractions. They found that iron had less affinity for the fat globule surface than copper which indicates iron was much less effective than copper as a catalyst for oxidized flavor.

II. EFFECT OF PASTEURIZATION AND HOMOGENIZATION ON THE DISTRIBUTION OF ADDED IRON IN MILK

The distribution of raw-milk-added iron in raw, pasteurized and homogenized skim milk and cream is presented in Table 4. Analysis of variance for the effect of processing revealed that there was a

Table 4. Effect of processing on the distribution of added iron^a in skimmilk and unwashed cream.

Skimmilk			Unwashed cream		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
85.15	84.65	86.74	11.65	10.83	17.63
86.27	87.09	86.49	11.05	11.14	17.13
88.09	87.05	83.40	11.50	10.68	17.48
86.09	86.45	82.18	10.10	10.82	16.93
84.63	86.80	83.64	11.24	11.52	16.30
89.09	88.10	84.35	11.38	11.02	16.69
85.58	87.85	82.40	10.81	11.30	17.25
84.69	85.53	82.66	10.62	11.00	17.69
87.80	88.04	82.48	11.40	11.08	17.45
86.75	89.44	82.18	11.70	10.95	18.86
87.00	88.48	85.00	11.57	11.94	16.80
87.78	87.82	84.08	11.51	12.44	17.12

^aExpressed as percentage of added iron.

significant difference in distribution of added iron in skimmilk and unwashed cream from raw, pasteurized and homogenized milk (Table 5). But when mean values were compared between these fractions, the raw and pasteurized samples were not different (Table 3, page 36).

Table 6 contains data on the iron concentration in washed creams. The analysis of variance (Table 7) showed a significant difference in iron distribution between creams from raw, pasteurized and homogenized milk. Table 3 shows first washed cream to be significantly different due to each processing procedure. In the second and third washed creams the raw and pasteurized samples were not significantly different ($P < 0.01$). However, Sargent and Stine (90) reported that heating the milk above 60 C resulted in 800% increase of added copper in cream phase. They indicated that the extent of migration of added copper to the cream phase as a result of heating was dependent upon the fat content of the milk, total copper content, and temperature history of the milk. The fat content of washed creams from pasteurized milk was about the same as creams from raw milk. First washed cream from pasteurized milk contained approximately 0.9% more added iron than cream from raw milk. The fat content of creams from homogenized milk was increased by washings. Unwashed cream contained 15.5%; first washed cream, 17.2%; second washed cream, 19.2%; and third washed cream, 23.5%. The fat content of skimmilk separated from homogenized milk was 1.3%.

The iron content in wash waters from raw, pasteurized and homogenized milk is presented in Table 8. The analysis of variance (Table 9) indicated that processing caused a significant difference in

Table 5. Analysis of variance of the distribution of added iron in skimmilk and unwashed cream,^a

Source	D.F.	Mean Square	
		Skimmilk	Unwashed cream
Total	35		
Processing	2	40.55**	148.16**
Replicates	5	2.53	0.89
Processing x Replicates	10	3.37	0.20
Residual Error	18	1.29	0.17

^aCalculations were based on the percentage of added iron.

**Highly significant at the 0.01 level of probability.

Table 6. Effect of processing on the distribution of added iron in washed creams.^a

First washed cream			Second washed cream			Third washed cream		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
2.37	2.95	11.08	1.68	1.90	10.45	0.95	1.12	9.40
2.37	2.94	11.02	1.88	1.70	8.91	1.10	1.00	11.24
2.24	3.10	11.25	1.50	2.00	11.12	0.90	0.95	9.27
2.18	3.12	10.20	1.64	1.86	9.79	0.86	0.87	10.00
2.20	3.14	12.34	1.48	2.19	10.36	1.12	0.98	10.05
2.16	3.00	12.66	1.60	1.79	8.20	1.15	0.88	10.05
2.25	3.24	12.20	1.35	1.72	11.54	0.85	1.21	8.85
2.24	3.14	11.25	1.52	2.00	10.28	0.90	1.00	9.50
2.28	3.18	10.94	1.60	1.94	10.09	1.02	1.25	10.40
2.22	3.29	10.10	1.20	2.06	12.00	1.00	1.04	8.62
2.33	3.16	11.89	1.65	2.06	10.10	0.80	1.10	11.00
2.33	3.04	11.09	1.35	2.20	9.84	0.82	1.24	10.30

^aExpressed as percentage of added iron.

Table 7, Analysis of variance of the distribution of added iron in washed creams.^a

Source	D.F.	Mean Square		
		First washed cream	Second washed cream	Third washed cream
Total	35			
Processing	2	301.34**	288.07**	315.83**
Replicates	5	0.32	0.25	0.22
Processing x Replicates	10	0.39	0.40	0.22
Residual Error	18	0.10	0.41	0.23

^aCalculations were based on the percentage of added iron.

**Highly significant at the 0.01 level of probability.

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Table 8. Effect of processing on the distribution of added iron in wash waters. ^a

First wash water			Second wash water			Third wash water		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
10.63 9.61	6.96 7.45	4.32 4.71	1.02 1.12	1.67 1.47	2.35 2.28	0.47 0.53	0.70 0.70	0.95 1.06
9.05 9.50	8.36 7.22	4.26 4.32	0.95 1.20	1.64 1.54	2.23 2.37	0.45 0.50	0.72 0.74	1.09 1.09
10.05 9.13	8.84 8.59	4.86 4.32	1.22 1.08	1.45 1.40	2.38 2.28	0.55 0.55	0.75 0.78	0.90 0.92
11.45 10.16	8.74 9.52	4.05 3.58	1.15 1.11	1.32 1.50	2.20 2.34	0.56 0.60	0.80 0.76	0.92 0.96
10.48 11.74	8.28 7.64	4.70 4.44	1.00 1.16	1.60 1.45	2.34 2.17	0.54 0.58	0.80 0.80	0.95 0.95
9.70 9.82	8.72 7.93	3.82 4.62	1.08 1.22	1.61 1.68	2.42 2.34	0.55 0.60	0.68 0.78	0.86 0.94

^aExpressed as percentage of added iron.

Table 9. Analysis of variance of the distribution of added iron in wash waters.^a

Source	D.F.	Mean Square		
		First wash water	Second wash water	Third wash water
Total	35			
Processing	2	103.84**	4.45**	0.5441**
Replicates	5	0.63	0.01	0.0017
Processing x Replicates	10	0.76	0.01	0.0044
Residual Error	18	0.28	0.01	0.0013

^aCalculations were based on the percentage of added iron.

**Highly significant at the 0.01 level of probability.

CRANE & CREST

iron distribution. Table 3 shows the raw, pasteurized and homogenized samples to be different in all three washings. The washing of cream from homogenized milk removed about 7% of the added iron. Whereas the washing of cream from raw or pasteurized milk removed 11, and 10%, respectively. The first wash water removed less added iron from cream of pasteurized milk than from cream from raw milk. However, second and third wash water removed more iron from cream of pasteurized milk than that from raw milk.

Table 10 shows iron distribution in acid and rennin caseins and their corresponding wheys. The analysis of variance (Table 11) indicated a significant difference in iron distribution between caseins from raw, pasteurized and homogenized milk as well as a significant difference between wheys from these sources. Table 3 shows the caseins and wheys from raw and pasteurized milk to be not significantly different. In the casein fraction, both acid-precipitated casein and rennin-coagulated casein from homogenized milk contained significantly higher ($P < 0.01$) amounts of added iron than caseins from raw and pasteurized milk. Also in the case of homogenized milk rennin-coagulated casein bound more added iron than acid-precipitated casein. Casein from raw or pasteurized milk precipitated at its isoelectric point contained about 67% of the iron, whereas casein coagulated by rennin contained about 70% of the iron. Paracasein has been shown to bind more metal than casein (57) which might explain the finding reported here.

The data on the iron distribution in proteose-peptone, whey proteins and fat-globule membrane are presented in Table 12. The significant differences of iron distribution in these fractions resulting

Table 10. Effect of processing on the distribution of added iron in acid casein, acid whey, rennin casein, and rennin whey.^a

Acid casein			Acid whey			Rennin casein			Rennin whey		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
68.13	68.40	70.25	16.52	17.20	12.65	73.13	70.40	77.25	8.90	9.95	6.38
67.50	67.02	69.85	15.70	17.20	12.23	71.17	68.02	79.85	9.10	10.11	6.38
68.40	68.00	68.49	15.50	17.04	12.40	71.40	71.00	77.00	9.22	10.49	6.34
68.10	68.61	70.00	15.74	17.84	12.08	71.10	71.61	75.49	9.06	9.79	6.01
66.95	68.91	70.47	15.05	16.78	10.00	70.95	69.91	79.49	10.18	8.65	7.40
66.31	68.91	70.37	16.97	15.32	11.46	68.31	69.91	77.39	8.72	8.03	7.84
66.40	67.00	69.00	16.95	16.26	12.14	71.40	68.00	75.00	9.85	9.12	6.92
68.78	67.00	71.53	17.33	16.90	11.23	69.00	70.04	75.53	9.24	8.96	6.60
68.20	68.80	69.84	17.84	16.48	11.00	70.10	70.80	79.84	8.64	9.50	7.41
67.00	68.00	71.28	16.79	15.16	11.00	68.04	72.48	81.28	8.64	9.22	7.04
68.02	66.20	69.25	16.70	15.84	11.45	69.02	69.00	80.25	9.43	9.00	5.85
67.42	67.20	69.25	17.89	16.00	11.45	69.02	69.00	80.25	8.56	8.66	6.07

^aExpressed as percentage of added iron.

Table 11. Analysis of variance of the distribution of added iron in acid casein, acid whey, rennin casein, and rennin whey,^a

Source	D.F.	Mean Square			
		Acid casein	Acid whey	Rennin casein	Rennin whey
Total	35				
Processing	2	20.34**	97.74**	262.15**	45.53**
Replicates	5	0.68	0.80	3.49	0.28
Processing x Replicates	10	1.02	1.11	5.71	0.47
Residual Error	18	0.64	0.42	1.38	0.14

^aCalculations were based on the percentage of added iron in milk.

**Highly significant at the 0.01 level of probability.

Table 12. Effect of processing on the distribution of added iron in proteose-peptone, whey proteins, and fat-globule membrane.^a

Proteose-peptone			Whey proteins			Fat-globule membrane		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
4.56	4.38	4.22	4.04	2.88	6.97	0.20	0.18	0.42
4.56	4.70	4.22	3.86	2.74	6.70	0.19	0.18	0.45
4.29	4.06	4.75	4.37	3.14	6.57	0.20	0.20	0.42
4.69	4.13	4.88	4.02	3.00	6.92	0.23	0.20	0.45
4.28	5.24	5.16	3.85	2.73	7.07	0.20	0.20	0.42
4.46	5.00	4.98	3.74	2.94	6.81	0.24	0.18	0.40
4.84	4.86	5.14	3.67	3.03	6.69	0.18	0.16	0.44
4.51	4.94	4.77	3.92	3.10	6.92	0.22	0.20	0.40
4.05	4.00	4.89	3.95	2.59	6.54	0.22	0.18	0.41
4.29	4.14	5.35	4.14	2.76	6.86	0.20	0.20	0.45
4.50	4.05	5.21	3.91	2.50	7.17	0.23	0.17	0.46
4.87	4.99	5.02	3.91	2.62	6.94	0.21	0.20	0.44

^aExpressed as percentage of added iron.

from processing are shown in the analysis of variance (Table 13). Unlike casein and proteose-peptone, whey proteins were affected by heat treatment (Table 3, page 36). Whey proteins isolated from pasteurized milk contained approximately 1% less added iron than that from raw milk. This may be due to the fact that casein is not a heat denaturable protein, as are the whey proteins (57). Whey proteins from homogenized milk contained about twice as much added iron as that from raw and pasteurized milk. This may be due to the fact that casein is partially denatured by homogenization, and is thus precipitated with whey proteins or remain with the proteose-peptone fraction. This finding is in keeping with the results reported by Iwaida and Tsugo (55) who found a reduction in casein nitrogen due to homogenization. Fox et al. (40) found milk fat reacted with casein micelles when homogenized,

Only 0.43% of the added iron was found in the fat-globule membrane preparation even though approximately 10% of the added iron was present in thrice washed cream from homogenized milk. The amount of added iron in fat-globule membrane isolated from homogenized milk was about double that from raw and pasteurized milk. This may be due to the fact that when the surface of the fat phase is greatly extended by high-pressure homogenization, a situation is created in which new lipid-protein complexes are formed which again bind additional iron. Proteins other than the normal membrane are involved in the lipid-protein interactions. Jackson and Brunner (56) demonstrated that the membrane material in homogenized milk included casein and whey proteins. The proportions in which casein and lipid materials may combine was quite variable, and the

Table 13, Analysis of variance of the distribution of added iron in proteose-peptone, whey proteins, and fat-globule membrane,

Source	D.F.	Mean Square		
		Proteose-peptone	Whey proteins	Fat-globule membrane
Total	35			
Processing	2	0.54**	51.46**	0.2155**
Replicates	5	0.25	0.03	0.0003
Processing x Replicates	10	0.21	0.05	0.0002
Residual Error	18	0.06	0.03	0.0004

^aCalculations were based on the percentage of added iron.

**Highly significant at the 0.01 level of probability.

complex may be less dense than the serum or more dense depending on the conditions of homogenization and the composition of the sample. Fox et al. (40) considered that the complex was formed because of interaction between the fat surface and hydrophobic regions on the casein made momentarily accessible by the turbulent conditions at the homogenizing valve.

According to Koops and Tarassuk (66), heating of milk (80 C for 15 min and 90 C for 15 sec) caused the transfer of phospholipids which was present in iron-containing fat-globule membrane to the plasma to the extent of about 20%. Koops and Tarassuk (66) further stated that agitating or heating of the milk resulted in a migration of membrane phospholipids into the skimmilk portion. Loewenstein and Gould (71) indicated that phospholipids and protein were lost in the same ratio, which suggested that lipoproteins were displaced from the fat-globule surface as a unit and not as free phospholipids. Also, they showed that in re-made milk the proteins of heat-treated skim milk (82 C for 15 min) were adsorbed at the fat/plasma interfacial surface in lower concentrations than the proteins from skimmilk heated to 40 C and cooled immediately.

The data on the iron distribution in total protein and TCA-filtrate are presented in Table 14. Analysis of variance (Table 15) revealed that processing caused a significant difference in iron distribution in these fractions. However, comparison of mean values (Table 3, page 36) showed that pasteurization did not affect the distribution of the added iron. Total proteins isolated from homogenized milk contained about 3% more added iron than that from raw and pasteurized milk, apparently due to the fact that protein in homogenized milk binds more iron than protein

Table 14. Effect of processing on the distribution of added iron in total protein and TCA-filtrate.^a

Total protein			TCA-filtrate		
Raw milk	Pasteurized milk	Homogenized milk	Raw milk	Pasteurized milk	Homogenized milk
80.11	78.05	83.13	5.47	5.25	4.44
79.55	79.08	82.36	5.73	5.34	4.38
79.80	78.71	82.74	4.85	4.84	4.14
80.15	80.12	81.14	5.11	5.06	4.22
81.60	80.99	83.63	5.32	5.00	4.23
80.00	78.11	84.36	5.08	5.00	4.23
79.00	80.20	84.15	5.34	5.45	3.86
77.65	81.00	83.24	5.56	4.94	3.67
80.00	80.54	82.40	5.58	4.78	4.10
78.43	80.84	82.80	5.41	4.90	3.94
78.15	81.71	83.79	5.12	5.46	4.36
80.00	82.10	85.37	4.88	5.66	4.36

^aExpressed as percentage of added iron.

Table 15, Analysis of variance of the distribution of added iron in total protein and TCA-filtrate.^a

Source	D.F.	Mean Square	
		Total protein	TCA-filtrate
Total	35		
Processing	2	48.10**	4.50**
Replicates	5	2.00	0.13
Processing x Replicates	10	2.11	0.13
Residual Error	18	0.84	0.04

^aCalculations were based on the percentage of added iron in milk.

**Highly significant at the 0.01 level of probability.

in nonhomogenized milk. This is borne out by the fact that the TCA-filtrate from homogenized milk contained less iron than that from non-homogenized milk (Table 3).

Due to the dilution factor in the determination of iron in beta-lactoglobulin, food grade ferric pyrophosphate (0.8333 g/l milk) was added to raw milk to yield 100 ppm of iron concentration. The effect of pasteurization on the binding of iron with this particular protein is shown in Table 16. The data show that the ratio of iron to protein in the beta-lactoglobulin fraction of raw milk to be somewhat greater than in the beta-lactoglobulin from pasteurized milk. Beta-lactoglobulin from raw milk contained approximately 0.85% of iron and that from pasteurized milk had approximately 0.49% iron. Using 36,000 as the dimer molecular weight of beta-lactoglobulin these data show about six atoms of iron and three atoms of iron are associated with each beta-lactoglobulin dimer in raw and pasteurized milk, respectively.

Groves (44) discussed research showing that heat treatment of beta-lactoglobulin causes a conversion of the dimer, about 36,000 dalton molecular weight, to a monomer of about half that size. If the protein molecule was broken down in the present investigation, the iron-binding capacity per molecule may have changed.

Larson and Jenness (70) found increasing activity of the sulfhydryl group as they heated bovine beta-lactoglobulin. Stauff and Uhlein (95) concluded from light-scattering studies that the dimer dissociates to the monomer on heating and that aggregation occurs with increasing time.

Table 16. Concentration of iron in beta-lactoglobulin preparations from raw and pasteurized milk

Replicates	Microgram iron per milligram protein	
	Raw milk	Pasteurized milk
I	9.2	3.1
II	8.2	4.4
III	9.8	5.9
IV	7.4	4.7
V	8.1	6.5
Average $\pm$ std. dev.	8.54 $\pm$ 0.95	4.92 $\pm$ 1.23

George et al. (41) also demonstrated by light-scattering measurement that bovine beta-lactoglobulin B shows increasing dissociation to the monomer at a given pH in the region 6-9 as the temperature is increased from 20 to 45 C.

It has been found that heating of skimmilk concentrate to 79 C for 25 sec had no effect on the whey protein nitrogen. Above 85 C whey protein values decreased with increasing temperature (27). These heat treatments are more drastic than those used in this study.

III. BINDING OF IRON WITH MILK PROTEINS

In 1960, iron-binding proteins were isolated from human milk and were called red protein and lactotransferrin (44). The other iron-binding protein found in milk but absent in blood was called lactoferrin.

The iron-binding protein was present in milk in two forms - the iron-complexed red protein and the iron-free protein. The red protein of cow milk was distributed between the casein, whey, and, apparently, the fat fractions of milk. This distribution was probably a consequence of its ability to form protein complexes which, in some instances, were strong enough to change its chromatographic behavior (73).

In considering the binding of metal ions to proteins, two broad types of binding may be distinguished. In the first the ions were bound in what may be called a nonspecific manner. The binding site depends on the pH of the solution. The extent of binding increases with increasing free metal ion concentration without always approaching a well-defined limiting value. In the second the limiting stoichiometry was approached

at comparatively low metal ion concentrations, for example the binding of two ferric ions per mole of lactoferrin (109). The data on the binding of iron with milk proteins are shown in Table 17. An analysis of variance on the binding of iron with milk proteins in raw milk shown in Table 18 clearly indicates that different milk proteins have different binding capacities for added iron. Mean values calculated as microgram iron per gram protein are presented in Table 19. Among five milk proteins investigated, fat-globule membrane protein showed the greatest binding capacity while whey proteins bound the least amount of added iron. Fat-globule membrane protein bound about 10 times more added iron than whey proteins. A different binding capacity was found between acid-precipitated casein and rennin-coagulated casein. However, in raw milk, there was no significant difference in binding capacity between acid-precipitated casein and proteose-peptone. The order of binding capacity was as follows: fat-globule membrane protein, rennin-coagulated casein, proteose-peptone, acid-precipitated casein, and whey proteins.

Aulakh and Stine (5) from their binding studies with copper ion found that the casein micelle had a greater binding capacity than fat-globule membrane protein. Koops (65) showed that affinity of the proteins for copper at pH 6.8 decreased in the following order: fat-globule membrane protein, sodium caseinate, beta-lactalbumin and beta-lactoglobulin. According to Fless and Klotz (34), the affinity of the proteins for copper at pH 6.5 was found to decrease in the following sequence: alpha-casein, beta-casein, bovine serum albumin, beta-lactoglobulin and lysozyme.

Table 17. Binding of added iron with milk proteins. ^a

Raw milk					Pasteurized milk					Homogenized milk				
Acid casein	Rennin casein	Whey proteins	Proteose-peptone	Fat-globule membrane protein	Acid casein	Rennin casein	Whey proteins	Proteose-peptone	Fat-globule membrane protein	Acid casein	Rennin casein	Whey proteins	Proteose-peptone	Fat-globule membrane protein
254.73	312.05	47.28	279.76	451.80	252.38	283.67	37.26	261.29	477.90	295.97	406.38	87.75	255.36	369.82
268.48	290.48	51.56	271.48	460.37	260.54	302.38	37.26	268.30	470.84	315.97	395.40	91.81	249.11	361.37
262.75	307.48	46.36	264.50	455.45	248.89	292.44	34.95	265.69	467.75	280.14	395.42	88.69	247.92	371.34
261.05	284.69	46.36	270.40	459.38	228.89	294.98	38.13	269.45	470.63	274.30	401.66	85.12	240.66	374.56
266.98	295.60	48.65	268.87	477.10	250.15	287.26	34.34	263.50	477.60	301.40	390.35	89.47	269.02	367.61
280.88	312.78	50.97	261.34	469.24	237.47	310.12	36.93	262.64	465.88	295.11	413.16	89.56	274.45	371.49
280.77	318.49	46.64	272.29	470.00	224.36	312.38	35.58	256.29	454.60	312.45	393.61	90.66	254.68	378.06
270.82	300.36	46.64	254.08	459.75	218.54	293.66	35.58	264.11	450.24	291.39	410.30	92.10	254.68	369.33
269.44	310.70	50.44	264.86	462.00	215.64	306.48	37.56	253.32	460.54	320.47	407.04	87.17	263.73	374.03
284.72	320.60	48.28	254.08	460.29	227.16	298.69	38.34	250.47	452.77	307.24	418.18	87.17	274.11	370.66
262.35	308.65	47.00	270.49	459.32	234.64	302.48	34.87	253.22	453.22	310.65	398.34	89.21	256.13	359.54
275.29	314.53	47.22	276.86	448.39	226.70	312.45	36.70	258.44	453.22	318.57	410.37	89.21	250.44	348.75

^aExpressed as micrograms iron per gram protein.

Table 18. Analysis of variance showing the binding of added iron with milk proteins.

Source	D.F.	Mean Square		
		Raw milk	Pasteurized milk	Pasteurized-homogenized milk
Total	59			
Samples	4	277113,51**	264696.88**	181177.55**
Replicates	5	67.85	83,59	100.80
Samples x Replicates	20	77,51	71.74	101.31
Residual Error	30	61.23	45.11	49.61

^aCalculations were based on microgram iron per gram protein.

**Highly significant at the 0.01 level of probability.

Table 19. Binding of added iron with milk proteins in raw, pasteurized, and pasteurized-homogenized milk.^{a, b}

	Raw milk	Pasteurized milk	Pasteurized-homogenized milk
Casein by acid	259.85 ^b	245.45 ^b	301.97 ^c
Casein by rennin	306.37 ^c	299.75 ^d	403.35 ^e
Whey proteins	48.16 ^a	36.46 ^a	88.91 ^a
Proteose-peptone	267.42 ^b	260.56 ^c	257.52 ^b
Fat-globule membrane protein	461.09 ^d	462.68 ^e	367.05 ^d

^a Means of 12 observations.

^b Expressed as micrograms iron per gram protein.

Note: Means in a column followed by the same letter are not significantly different at the 0.01 level of probability.

Analysis of variance on the binding of milk proteins with added iron in pasteurized milk is shown in Table 18 (page 60). Table 19 (page 61) shows the comparison of mean values based on microgram iron per gram protein between different milk proteins. As in raw milk membrane material, pasteurized milk membrane protein bound the greatest amount of added iron while whey proteins showed the least added iron-binding capacity. Fat-globule membrane protein bound over ten times more added iron than whey proteins. Though the order of iron binding was the same for the various proteins, pasteurization changed the capacity of binding iron with whey proteins. Proteose-peptone bound significantly higher amounts of added iron than acid-precipitated casein.

Analysis of variance on the binding capacity of homogenized milk presented in Table 18 shows different binding capacities by different milk proteins. In Table 19 the mean value was compared with each protein. In pasteurized-homogenized milk, casein coagulated by rennin showed the greatest binding capacity. The fat-globule membrane protein bound less added iron than rennin-coagulated casein. Even though the binding capacity of whey proteins was doubled due to homogenization, they bound the least amount of added iron among the proteins tested. The order of binding capacity in homogenized milk was as follows: rennin-coagulated casein, fat-globule membrane protein, acid-precipitated casein, proteose-peptone and whey proteins,

CHAPTER V

SUMMARY

This investigation was to determine the effect of pasteurization and homogenization on the binding of added iron to milk proteins. The iron-treated milk was pasteurized at 62.8 C for 30 min. The pasteurized milk was homogenized through two stage commercial homogenizer by employing 2,500 p.s.i. on the first stage and 500 p.s.i. on the second stage.

Based on the results of this study, the following conclusions were reached:

1. About 86% of added iron was recovered in the skim milk portion of raw and pasteurized milk and approximately 84% from the pasteurized-homogenized milk.
2. In the skim milk fraction most of the added iron was bound to protein fractions. Among the proteins in skim milk the casein fraction bound most of the added iron, Rennin-coagulated casein bound slightly higher amounts of added iron than acid-precipitated casein.
3. Pasteurization changed the binding capacity of whey proteins only, Whey proteins from raw milk bound more added iron than that from pasteurized milk and beta-lactoglobulin from raw milk contained more iron than that from pasteurized milk.

4. Fat-globule membrane protein from raw and pasteurized milk bound about 0,20% of the added iron and that from homogenized milk contained about 0,40%.
5. Proteins from homogenized milk bound more added iron than proteins from nonhomogenized milk.
6. The order of binding capacity of milk proteins (microgram Fe/g protein) from raw and pasteurized milk was as follows: fat-globule membrane protein, rennin-coagulated casein, proteose-peptone, acid-precipitated casein, and whey proteins.
7. The order of binding capacity in homogenized milk (microgram Fe/g protein) was as follows: rennin-coagulated casein, fat-globule membrane protein, acid-precipitated casein, proteose-peptone, and whey proteins.



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