THESIS

This is to certify that the

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ILLUMINATION OF FAT GLOBULE CLUSTERING
IN COWS' MILK

presented by

John R. Euber

has been accepted towards fulfillment
of the requirements for

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J. Robert Brunner
Major professor

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ILLUMINATION OF FAT GLOBULE CLUSTERING IN COWS' MILK

By

John R. Euber

A DISSERTATION

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ABSTRACT

ILLUMINATION OF FAT GLOBULE CLUSTERING
IN COWS' MILK

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Fat globule clustering is a prerequisite for normal creaming of milk. Fat globule clustering as characterized by the cream volume and cluster time was studied in raw milk, heated milk, and in model systems. The influence of various milk fractions, specific anti-sera, and simple carbohydrates were evaluated. Immunoglobulin M (IgM) was confirmed as the heat-labile component involved in fat globule clustering. Its participation was also demonstrated using raw milk. IgM was shown to function as a cryoagglutinin rather than as a cryoglobulin as previously indicated. Hapten inhibition studies demonstrated that the antigen is carbohydrate in nature. Skim milk membrane (SMM), isolated by ultracentrifugation or salt fractionation and gel filtration, was identified as the homogenization-labile component. Although IgM can agglutinate milk fat globules (MFG) to a limited extent, normal creaming requires both components. Approximately 7% of the IgM in normal milk participates in a single creaming. The lower portion of creamed milk (skim) failed to support creaming upon addition of washed MFG, but did so upon the

addition of SMM. The presence of SMM in the lower portion indicated that not all SMM is capable of participating in creaming. A theory of fat globule clustering consistent with the observed experimental results depicts IgM interacting in an antigen-antibody mode simultaneously with SMM and MFG through specific carbohydrate moieties.

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INTRODUCTION

The rapid rising of fat globules with a subsequent formation of a cream layer in normal cows' milk upon quiescent cold storage represents one of the fundamental physical properties of the milk system. This phenomenon is referred to as "creaming". Despite the number of investigations which have focused on this process, a clear-cut conception of the fundamental principles involved has not been established.

In the days of creamline milk, the consumer equated a deep cream layer with product quality. Early processors of dairy products used creaming as a means of obtaining a cream fraction from milk. With the introduction of efficient mechanical milk separators and because creaming is destroyed by heat treatment or homogenization - processes which virtually all commercial milk undergo - creaming is no longer of practical significance to the dairy industry. Nevertheless, elucidating the mechanism of creaming remains as an intriguing challenge.

Early studies demonstrated that fat globule clustering was a prerequisite for normal creaming. Immunoglobulin M (IgM) has been shown to promote fat globule clustering. Based on studies with IgM isolated from milk, the

immunoglobulin was designated as a cryoglobulin, i.e., undergoes cold-induced aggregation and precipitation. The cold-induced aggregation and the concomitant non-specific precipitation upon more than one fat globule, forming IgM "bridges", is proposed to result in clustering. This theory precludes an active role by other milk constituents and in particular the milk fat globule or its membrane. Although consistent with some of the observed phenomena, the theory fails to account for phenomena such as the Mertens and Samuelsson effects. In addition, the procedure employed in assessing the apparent cold-induced aggregation of purified IgM do not rule out a concentration and purity dependent phenomenon. This property is characteristic of purified immunoglobulins and in particular IgM and IgA. These factors prompted us to re-investigate the mechanism of fat globule clustering.

Research was directed toward determining the components involved in fat globule clustering and examining whether their role in creaming is passive or active. The nature of the interactions and their heat and homogenization lability was also examined. The ultimate goal of this study was to develop a model representative of fat globule clustering consistent with observed phenomena.

LITERATURE REVIEW

The Role of Fat Globule Clustering in Creaming

Babcock (1889a, 1889b) was the first to call attention to the clusters of fat globules which form shortly after milk is drawn from the cow and cooled. He postulated that the coagulation of fibrin, which he believed to be a normal constituent of milk, entangled the fat globules and other "solid and gelatinous" matter in milk and weighed them down enough to prevent them from rising. To minimize fibrin clotting or to maximize cream separation he suggested that milk be set at a cold temperature (ice water) immediately after milking. This theory was not generally accepted and was proven untenable when Hekma (1922) demonstrated that fibrin is not a normal constituent of milk.

Babcock and Russell (1896a, 1896b) and Woll *et al.* (1903) observed that clustering is an important factor influencing the consistency or viscosity of pasteurized milk and cream. Whereas raw milk or cream contained fat globule clusters, heated milk or cream failed to contain clusters. They did not indicate a correlation between fat globule clustering and creaming properties. However, their observations led to further investigations of fat globule clustering and the formulation of numerous theories to explain the phenomenon.

Henseval (1902) studied two types of milk, one showing a rapid and one showing a slow rising of cream. Both types had a normal composition but the milk which creamed rapidly had numerous large fat globule clusters while the fat globules in the slow creaming milk rarely showed any clustering.

In a study of the efficiency of creaming in the deep-setting and shallow-pan creaming methods, McKay and Larsen (1906) found a more rapid and complete creaming using the deep-setting method. This was attributed to the more favorable conditions provided for aggregation of the fat globules into "small bunches or masses."

Hammer (1916) attributed the loss of cream layer formation in milk having undergone agitation or pasteurization to the modification or size reduction of fat globule clusters.

Van der Burg (1921) called attention to the fact that individual fat globules should rise according to Stokes' law. The law which expresses the terminal velocity of small spheres submerged in a viscous fluid had been successfully applied to many systems. Rahn (1921), Van Dam and Sirks (1922), and Troy and Sharp (1928) confirmed that fat globules in milk do rise according to Stokes' law. The rate of rise of single globules was such that at least 50 hours would be required for most of the fat globules to rise from the bottom to the top of the creaming vessel. The rate of

rise of fat globule clusters agreed very well with that calculated by Stokes' law, and was rapid enough to account for the normal creaming time. These authors concluded that the fat globules in raw milk do not rise singly but that they aggregate into clusters which have considerably greater velocities of rise. Their work conclusively established that the clustering of fat globules plays the major role in explaining the creaming phenomenon.

A technique introduced by Dahlberg and Marquardt (1929), whereby thin films of milk are viewed with transmitted light, added indisputable evidence to the proof of the importance of fat globule cluster formation in gravity creaming.

Components Involved in Fat Globule Clustering

Early investigators realized that the percentage of fat in milk does not entirely determine its creaming ability (Hammer, 1916). The fact that milk with a high fat content occasionally produced a thin cream layer and the variability in creaming characteristics of milk from individual cows made it quite evident that factors other than the percentage of fat influence the creaming ability.

As discussed in the previous section, Babcock (1889a) incorrectly attributed the differences in creaming characteristics to a variable fibrin content.

Marcas (1903, 1904) attributed the poor performance of slow creaming milk to its elevated ash, phosphoric acid, and lime concentrations.

Van Dam et al. (1923) found that the addition of blood serum to milk which creamed poorly improved its creaming properties. If blood serum had been heated to 65 C or higher, little or no improvement was observed. The authors suggested that the clustering of fat globules in milk may be due to an agglutinin common to both milk and blood. Since milk is elaborated by the mammary gland from materials brought to it by the blood, these authors considered it conceivable that the blood serum from cows whose milk creamed well, when added to poor creaming milk, would influence the creaming more favorably than the blood serum from cows whose milk creamed poorly. Experiments failed to establish such a relationship. Brouwer (1924) later demonstrated that blood serum from steers was as beneficial in promoting creaming as that from cows.

In a continuation of these experiments, Hekma and Sirks (1923) examined the influence of various serum fractions on creaming. The substance responsible for the improvement in creaming was precipitated with sodium chloride or ammonium sulfate almost completely along with the globulin fraction. When the globulin fraction was dialyzed and added to milk, the creaming properties were almost identical to those of a control sample containing an equivalent volume of added

serum.

Brouwer (1924) fractionated the globulin fraction into euglobulin and pseudoglobulin fractions and demonstrated that euglobulin was the active component. This was supported by showing that blood serum of new born calves, which is devoid of euglobulin, failed to improve creaming properties.

Palmer et al. (1926) and Troy and Sharp (1928) demonstrated that the agglutinin was present in the whey fraction of milk as expected.

Orla-Jensen (1929) attributed the small amounts of colostrum required to restore creaming to heated milk to its high globulin concentration.

Rowland (1937) provided further support for the role of the globulin fraction in whey by showing that the reduction in creaming power is proportional to the percentage of total albumin and globulin denatured in heated milk.

Sharp and Krukovsky (1939) found that the agglutinin was concentrated in cream separated at low temperatures and was relatively absent in the corresponding skim milk. The opposite was true for milk separated at high temperatures. By first separating at a low temperature and then further separating the cream to a higher fat content at a higher temperature at which the fat is liquid, a cream plasma very active in agglutinating power was obtained. They concluded that the agglutinating substance normally present in milk is

adsorbed on the surface of solid fat globules but not on liquid fat globules.

Dunkley and Sommer (1944) found that the agglutinin nearly quantitatively precipitated from agglutinin-rich wheys when allowed to stand undisturbed for 12 hours at 5 C. Agglutinin-poor wheys gave very little or no precipitate. A euglobulin fraction prepared from the proteins in the precipitate produced practically the same creaming behavior as an equivalent amount of the precipitate indicating that the agglutinating power resides in the euglobulin fraction.

Samuelsson et al. (1954) also observed that the agglutinin responsible for normal creaming in milk precipatated at 2 C from rennet whey which had been heated to not greater than 60 C. The agglutinin formed opalescent solutions in warm whey or water and could be precipitated by adding sodium chloride or gum arabic. The agglutinin was shown to consist of two components, one inactivated by homogenization and the other by heating. In systems containing cream mixed with water, whey, or separated milk creaming would occur if one portion of the available agglutinin had been inactivated by homogenization and the other by heating, but if all the agglutinin had undergone either of these treatments, no creaming resulted. The activity of agglutinin was favored by factors contributing to its gradual precipitation, e.g., dilution, low-temperature aging in the range of 5 to 10 C, and mild heat treatment. Similar observations were reported

by Kenyon and Jenness (1958).

After concentrating the active clustering agent from milk in a cream plasma according to the procedure of Sharp and Krukovsky (1939), Gammack and Gupta (1970) subjected the aqueous phase to ultracentrifugation. They recovered 85% of the clustering activity in a small opalescent layer which sedimented above the casein pellet. A precipitate which formed when the opalescent layer was diluted with milk ultrafiltrate and held at 4 C contained most of the clustering activity. The precipitate could be redispersed on warming. Although the euglobulin fraction from colostrum also exhibited the cryoprecipitation behavior, precipitate from the opalescent layer was twenty times more active in terms of its protein content in promoting clustering and caused a more rapid formation of cream line than did euglobulin. When the precipitate was fractionated by gel filtration, the clustering activity was localized in a high particle-weight fraction, i.e., greater than $1x10^6$, which contained lipoprotein particles. The isolated lipoprotein particles themselves showed no clustering activity. Immunoglobulin G (IgG) isolated from colostral euglobulin had no clustering activity with or without added lipoprotein particles. A fraction containing immunoglobulin M (IgM) and A (IgA) isolated from colostral euglobulin showed some activity with a slow formation of cream line. On addition of lipoprotein particles, clustering activity was markedly

enhanced and cream line formation was rapid. These authors concluded that lipoprotein particles which exist in the aqueous phase augment the clustering activity of immune proteins such as those in euglobulin properties. In a set of related experiments, Franzen (1971) was not able to obtain similar results. This may have been due to different centrifugation conditions or the use of heated skim milk.

The observations of Gammack and Gupta support the earlier work of Hansson (1949) who noted that creaming in raw or low-temperature treated milk was enhanced by the addition of phospholipids isolated from brain matter. Cream rising was not improved in high-temperature treated milk.

Payens and Both (1970) and Franzen (1971) have also demonstrated that the IgM fraction is the euglobulin component active in restoring creaming to heated milk.

Stadhouders and Hup (1970) and Bottazzi et al. (1972) have suggested that there are 3 different types of agglutinins present in milk, binding either fat globules together, bacteria together, or bacteria to fat globules. Stadhouders and Hup demonstrated that the fraction which separates from euglobulin at low temperatures contains the immune proteins which agglutinate fat globules and those which attach the bacteria to fat globules.

Bottazzi and Premi (1977) contend that fat globules that remain in the skim phase during the agglutination process have a higher level of 5'-nucleotidase than the fat

globules which rise. They suggested fat globules that agglutinate most quickly are those with a low content of phospholipids.

In a study of factors associated with production of milk with a low creaming capacity, Bertoni et al. (1979) found milk with a high creaming capacity contained high levels of fat but low levels of phospholipids and cholesterol. They interpreted these results as indicating that fat globule membranes from milk with a high creaming capacity were less compact.

Bottazzi and Zacconi (1980) have recently isolated from milk a component active in aggregation of fat globules and bacteria using a new procedure. After concentrating the active clustering agent from milk in a cream plasma according to the procedure of Sharp and Krukovsky (1939), the aqueous phase was frozen. After thawing, a cryofloculate recovered by centrifugation was fractionated by gel filtration and ultracentrifugation. A fraction which greatly increased the degree and rate of cream rising was obtained. Virtually no cryoflocculate was observed in milk with a low creaming capacity.

Physical and Chemical Factors Influencing Creaming

Low Temperature

A low temperature has generally been recognized as a prerequisite of normal creaming. As discussed previously,

Babcock (1889a) incorrectly attributed this to the prevention of fibrin coagulation. Later investigators (Rahn, 1921; Van Dam and Sirks, 1922; Troy and Sharp, 1928) recognized the role of decreased temperatures in promoting cluster formation. Sharp and Krukovsky (1939) made the significant observation that agglutinin material was preferentially associated with fat globules in the cold and the skim phase in warm milk. Using labeled euglobulin, Payens et al. (1965) conclusively demonstrated that the amount adsorbed on fat globules is strongly temperature dependent. Reduced temperatures increased clustering of the fat globules and enhanced adsorption of euglobulin.

Dunkley and Sommer (1944) and Samuelsson et al. (1954) noted that factors which favor precipitation of the active component from whey also favor fat globule clustering. Rhee (1969) and Franzen (1971) associated fat globule clustering with temperatures low enough to promote euglobulin or IgM aggregation, respectively. Payens and Both (1970) suggested that the same functional groups are responsible for IgM cryoaggregation and fat globule cold agglutination.

High Temperature

Many of the early studies were initiated to examine the reduction in creaming capacity due to excessive heat (high temperature pasteurization). The reduced creaming power of heated milk was initially believed to be due to the precipitation of denatured lactalbumin onto fat globules resulting

in their failure to rise (Hunziker, 1921). Later investigators (Rahn, 1921; Van Dam and Sirks, 1922; Troy and Sharp, 1928) established experimentally that the reduction in creaming properties of milk caused by the use of high pasteurizing temperatures results from interference with subsequent cluster formation. Hekma and Sirks (1923) attributed the lack of clustering to the heat lability of the agglutinin responsible for creaming. Palmer and Anderson (1926) demonstrated that pasteurization primarily affected the milk plasma. Rowland (1937) supported this contention by showing that the reduction in creaming power was proportional to the percentage of the total albumin and globulin denatured. Based on electrophoretic studies with fat globules in samples of heated whole milk, heated skim milk plus raw cream, and raw skim milk plus heated cream, Dahle and Jack (1937) conclusively established that the electrokinetic potential of milk fat globules is not a major factor in the reduction of creaming by heat treatment. These studies were supported by experiments demonstrating the restoration of creaming to heated milk upon addition of euglobulin (Dunkley and Sommer, 1944) or IgM (Payens and Both, 1970; Franzen, 1971).

Whereas high-temperature treatment eliminates creaming capacity, Dunkley and Sommer (1944) demonstrated that it was necessary to warm (50 C) the fraction (skim or cream) containing active agglutinin for normal creaming to be observed in recombined samples. It was necessary to warm only that

portion which contained the agglutinin. Normal creaming was only restored in whole milk samples by warming prior to placement at reduced temperatures.

Homogenization

Hammer (1916) attributed the loss of creaming capacity in homogenized whole milk to the reduced size of fat globules which have less tendency to rise than the original globules. Doubt was shed on this theory when Mertens (1932) observed that milk recombined from normal cream and homogenized skim milk did not cream. Experiments conducted by Dunkley and Sommer (1944) confirmed these results. Furthermore, it was shown that milk recombined from unclumped homogenized cream and normal skim milk formed a cream layer. They attributed the loss of creaming capacity in homogenized milk to the denaturation of agglutinin. Samuelsson et al. (1954) demonstrated that the agglutinin consisted of a heat- and homogenization-labile component. Payens (1964) was unable to detect any differences in the physical properties and clustering ability of euglobulin isolated from colostrum prior to and following homogenization. In a subsequent study, Koops et al. (1966) observed normal creaming when washed cream was added to a homogenized model system containing milk dialyzate and euglobulin. However, when micellar casein or κ-casein was added to the system, creaming was eliminated despite the observation that labeled euglobulin

was adsorbed on the fat globule surface. The adsorbed euglobulin was accompanied by small amounts of casein, particularly κ-casein. The authors suggested that a euglobulin-κ-casein complex was formed during homogenization which was capable of adsorbing to the fat surface but unable to effect clustering, or that homogenization induced the adsorption of κ-casein on the fat surface, screening adsorption sites for euglobulin. Walstra (1980) homogenized rennet and acid whey (after pH adjustment to 6.6) and found creaming capacity was fully destroyed in both samples. The formation of a euglobulin-κ-casein complex was therefore doubted.

pH

In confirmation of previous work, Dunkley and Sommer (1944) found alkali added to milk increased the depth of the cream layer and resulted in a more rapid and complete creaming. Acid was found to have an opposite effect.

Added Salts

Dunkley and Sommer (1944) found the addition of sodium citrate and disodium phosphate to milk to have little effect on cream volume. Increasing concentrations of sodium chloride, calcium chloride, aluminum chloride, and ferric chloride caused marked reductions in the depth of the cream layer. The authors therefore suggested that the charge on the fat globule is not an important factor in determining

the creaming properties of milk.

Creaming in Milks Other than Cows'

The slow rate of creaming in buffaloes' milk has been attributed to the absence of cluster formation (Fahmi, 1951). Fahmi *et al*. (1956a, 1956b) supported this notion and reported that goats' and sheeps' milk are also devoid of fat globule clusters at room (22 C) or refrigerator (10 C) temperatures. Abo-Elnaga *et al*. (1966) reported clusters in buffaloes' milk held at 4 C for 3 hours but that they rarely contained more than 4 fat globules. They attributed the poor creaming of buffaloes' milk to low levels of agglutinin.

After a study of the factors influencing the creaming ability of carabaos' milk, Gonzales-Janolino (1968) concluded that carabaos' milk lacks the homogenization-labile component and while the heat-sensitive component is present, it is not in sufficient quantities for normal creaming. The poor creaming carabaos' milk was found to cream to an extent at least as exhaustive as that in cows' milk upon addition of an agglutinin concentrate from cows' milk.

Jenness and Parkash (1971) attributed the poor creaming of goats' milk at low temperatures to the lack of agglutinating euglobulins. Milks reconstituted from goat's cream and cows' skim milk creamed readily whereas those made by combining cows' cream and goats' skim creamed poorly.

The goats' milk fat globules failed to cream as exhaustively as those of cows' milk.

Whittlestone (1953) showed that creaming occurs in mixtures of sows' cream and cows' skim milk held at 37 C for 2-16 hours while sows' milk failed to exhibit cream formation under these conditions. Although the experiment was not performed at an optimal temperature, the author suggested that the agglutinin of cows' milk will cause the clustering of sows' milk fat globules. These experiments indicate sows' milk lacks a fat globule agglutinin.

Mechanisms of Fat Globule Clustering

The recognition of the significance of fat globule clustering in promoting cream layer formation led to numerous investigations and theories to explain the phenomenon. Early theories, which were critically reviewed by Dunkley and Sommer (1944), were based on (a) gravitation of fat globules, (b) electrokinetic potential of fat globules, (c) interfacial tension, (d) stickiness and state of hydration of the adsorbed membrane, and (e) fat clustering considered as an agglutination process. Based on experimentation designed to test these theories Dunkley and Sommer concluded that (a) the variable creaming properties of normal milk cannot be explained on the basis of differences in the rates of rise of individual fat globules, (b) the salts normally present in milk are sufficient to reduce the

surface potential on the fat globules and thus eliminate the charge variability of the fat globules, (c) the interfacial tension at the fat-serum interface does not promote creaming, and (d) evidence concerning the importance of hydration of the membrane on the fat globules was not sufficient to justify drawing a definite conclusion regarding the significance of this factor. Fat globule clustering was noted to share many similarities with bacterial agglutination. Both processes involve the aggregation of particles, require the presence of globulins, are prevented by heat denaturation, and require optimum salt concentrations. They used Marrack's (1938) theory of bacterial agglutination as a model and stated that if the mechanisms were similar, clustering would be promoted by (a) a partial dehydration of the adsorbed membrane on the fat globules due to specific polar adsorption of euglobulins, (b) aggregation of fat globules resulting from the adsorption of a single euglobulin molecule by two fat globules, and (c) maintenance of the surface potential of the fat globules below the critical level by the presence of salts. It was concluded that the clustering of fat globules in milk takes place by the same mechanism as that involved in the agglutination of bacteria.

Brunner (1974) has stated that it is unfortunate that this comparison has been made since it implies the operation of an antibody-antigen interaction. No evidence exists to suggest that the fat globule membrane contains antigenic

components to the euglobulin fraction. The results of Stadhouders and Hup (1970) indicating a lack of specificity in the euglobulin-fat globule complex but a specificity in the antibody-bacteria complex supports this suggestion. As the mechanism of bacterial agglutination was a matter of controversy in 1944 and data supporting fat clustering as an agglutination process was only based on comparison of limited aspects of the two phenomena, the use of the term agglutination for fat globule clustering can only be justified on the basis of convenience. Dunkley and Sommer's actual theory embracing a physical, as opposed to a specific, interaction between the euglobulin components and the fat globule surface has been generally accepted.

Jenness and Patton (1959) suggested that the adsorbed euglobulins serve to reduce the fat/plasma interfacial tension, thus permitting the globules to approach one another to form clusters. The fact that euglobulin-rich skim milk foams copiously was offered as evidence in support of the interfacial activity of euglobulin. Actually, cold separated skim milk has a lower surface tension than euglobulin-rich warm separated skim milk (Brunner, 1974). Furthermore, Jackson and Pallansch (1961) demonstrated that euglobulin is not as interfacially active as other milk proteins such as α-lactalbumin, and β-lactoglobulin, or the native interfacial milk fat globule membrane protein. It therefore seems improbable that the clustering of fat globules can be

explained solely on the basis of an increase in the interfacial activity resulting from the adsorption of euglobulin at the interfacial surface.

Payens (1964) suggested that the adsorbed euglobulin would cause agglutination by reinforcing the London-Van der Waals attraction between the fat globules. In view of the small amount of euglobulin experimentally determined to be adsorbed to the fat globules, this suggestion was retracted (Payens et al., 1965). Rather it was suggested that agglutination is brought about by the formation of euglobulin bridges between the fat globules, essentially in accordance with the model proposed previously by Dunkley and Sommer.

Kenyon et al. (1966) attributed the association of euglobulins with the fat globule surface to ionic interactions promoted by euglobulins having an isoelectric point relatively more positive than the fat globule surface. It was suggested that at low temperatures a more rigid system may develop forming lipoproteins held by weak ionic bonds with the basic proteins. Larger molecular weight proteins, such as euglobulins, would have an extended sphere of attraction thus more readily forming bridges with the ionic structure of phospholipids on adjacent fat globules. The Brucella Ring Test was postulated to involve immunoglobulins involved in fat globule clustering and having specific antibody activity (for Brucella cells). Adsorption to fat globules was proposed to occur in such a way that their

combining sites remain available for reaction and hence still capable of agglutinating bacteria.

The most recent and the generally accepted theory (Brunner, 1974; Mulder and Walstra, 1974) attributes agglutination to the precipitation of cryoglobulins (IgM) onto the fat globules, leading to clustering and subsequently to rapid creaming of the large clusters (Payens, 1968; Payens and Both, 1970). Since several observed phenomena are difficult to explain by the simple mechanism, Brunner (1974) and Mulder and Walstra (1974) have suggested that the clustering reaction is more complicated.

Facets of Fat Globule Clustering Warranting Further Investigation

Before a theory of fat globule clustering consistent with observed phenomena can be proposed, numerous questions regarding the nature of the components involved must be answered or further examined. Does all the IgM in milk undergo cryoprecipitation? Why does it associate only with the fat globules - is the reaction specific or non-specific? Do fat globules only play a passive role as has generally been assumed? What is the nature of the homogenization-labile component? No mechanism to date has prescribed a role to this component - how is it involved in fat globule clustering? How do the lipoprotein particles of Gammack and Gupta augment creaming?

The conditions required for fat globule clustering also warrant further investigation. Why is a low temperature prerequisite for creaming? Is only IgM effected? Do salts play an indirect or a direct role in the process? What component(s) involved in the process is effected as the pH is varied?

It is of interest that of the milks examined only the fat globules of cows' milk display extensive clustering. The other milks appear to contain the components required for creaming - euglobulins (IgM) and fat globules. What is unique about the IgM or fat globules in cows' milk? What is the biological significance with respect to immunoglobulin synthesis or milk secretion?

EXPERIMENTAL

Materials and Equipment

The milk used in this study was obtained from the Michigan State University Holstein dairy herd. Milk was collected in three or five gallon stainless steel cans from the milking parlor and separated as soon as possible at 40-45 C.

Rabbit anti-sera against bovine IgM, IgG, and IgA were purchased from Miles Laboratories, Inc. Rabbit anti-sera against bovine xanthine oxidase, bovine milk fat globule membrane, and navy bean (P. vulgaris L.) trypsin inhibitor were provided by Dr. J.R. Brunner.

Trypsin, type III, neuraminidase, type V, and rennet, type I, were purchased from Sigma Chemical Co. Pronase, grade B, was obtained from Calbiochem-Behring Corp. and mixed glycosidases from Miles Laboratories, Inc.

Bio-Gel A-0.5 m and A-5 m were obtained from Bio-Rad Laboratories; Sepharose 4B and Sephacryl S-200 from Pharmacia Fine Chemicals; and agarose (-m_r less than 0.2) from Miles Laboratories, Inc.

Chemicals used in this study along with their sources are listed in Table A1 of the Appendix. All chemicals were reagent grade unless otherwise indicated. Equipment used

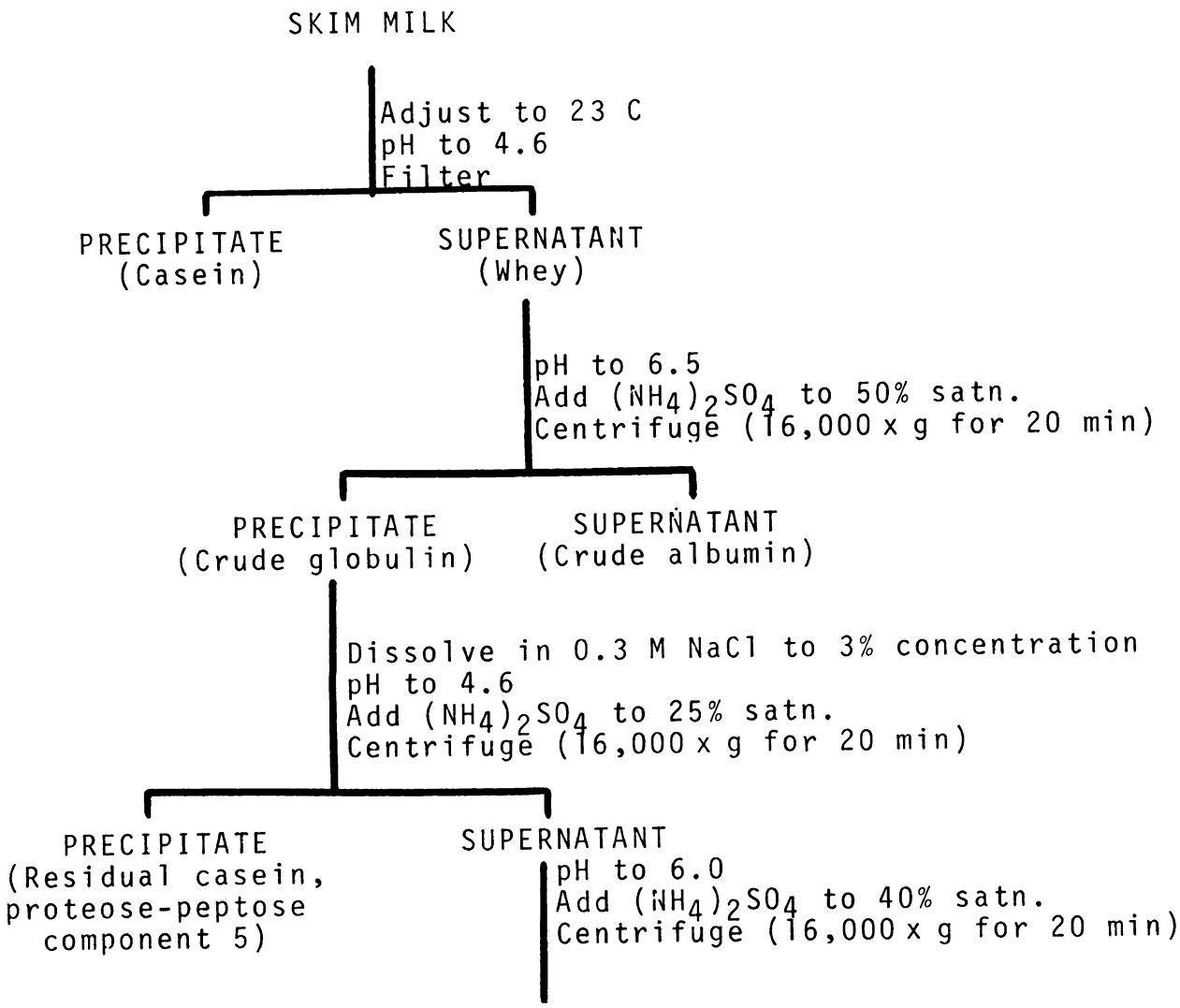
regularly during the course of this study is listed in Table A2 of the Appendix. Instrumentation specific for a certain experiment will be referred to in the appropriate section.

Preparative Procedures

Immunoglobulin M

Immunoglobulin M (IgM) was prepared by combining the ammonium sulfate precipitation method described by Smith (1946a, 1946b) and gel filtration on Bio-Gel A-5 m and A-0.5 m columns connected in tandem. Figure 1 outlines the isolation procedure of IgM from fresh skim milk. Whole milk was separated in a laboratory disk separator at 40-45 C. The resulting skim milk was cooled to room temperature and adjusted to pH 4.6 with 1 M HCl and allowed to stand 1 h. The precipitated casein was removed by filtration through 3 layers of cheese cloth. The acid whey was adjusted to pH 6.5 with 1 M NaOH and solid ammonium sulfate was added slowly to 50% saturation (313 g/l) to salt-out the crude lactoglobulin fraction. After standing overnight, most of the supernatant was siphoned off, and the precipitate was collected by centrifugation at 16,000 x g for 20 min at 20 C.

The precipitate was solubilized in a 0.3 M NaCl solution containing 0.02% (w/v) NaN_3 at approximately a 3% (w/v) protein concentration. The pH was adjusted to 4.6 with 1 M HCl and ammonium sulfate added to 25% saturation (144 g/l). After centrifuging at 16,000 x g for 20 min at 20 C, the


SKIM MILK
Adjust to 23 C
pH to 4.6
Filter
PRECIPITATE
(Casein)
SUPERNATANT
(Whey)
pH to 6.5
Add $(NH_4)_2SO_4$ to 50% satn.
Centrifuge (16,000 x g for 20 min)
PRECIPITATE
(Crude globulin)
SUPERNATANT
(Crude albumin)
Dissolve in 0.3 M NaCl to 3% concentration
pH to 4.6
Add $(NH_4)_2SO_4$ to 25% satn.
Centrifuge (16,000 x g for 20 min)
PRECIPITATE
(Residual casein,
proteose-peptose
component 5)
SUPERNATANT
pH to 6.0
Add $(NH_4)_2SO_4$ to 40% satn.
Centrifuge (16,000 x g for 20 min)

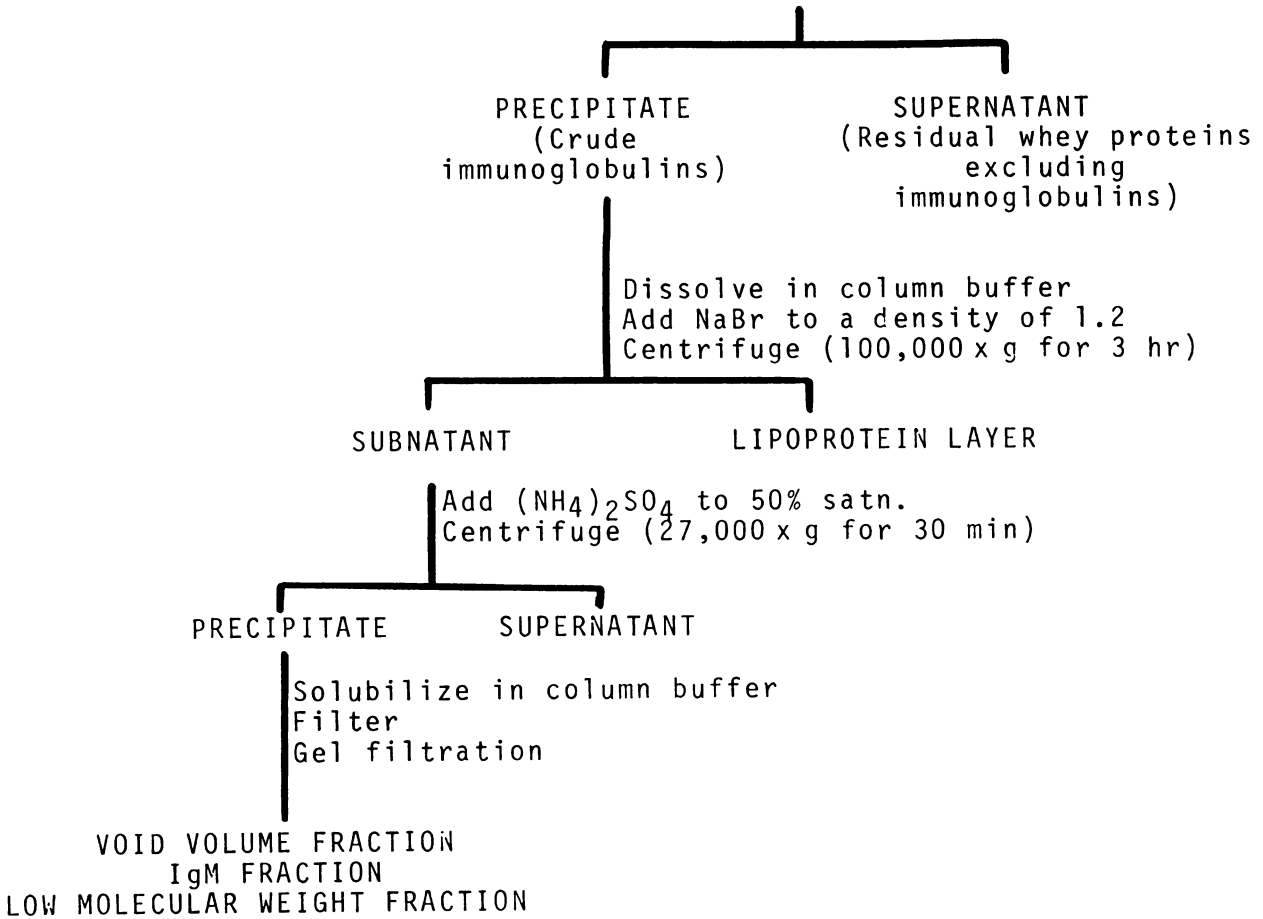


Figure 1. Schematic representation of the isolation procedure for obtaining IgM from skim milk.

supernatant was filtered through a single layer of cheese cloth. A crude immunoglobulin fraction was precipitated from this supernatant by adjusting the pH to 6.0 with 1 M NaOH and adding ammonium sulfate to 40% saturation (99 g/l).

The crude immunoglobulin fraction collected by centrifugation at 16,000 x g for 20 min at 20 C was dissolved in column buffer - 0.01 M Tris-HCl, pH 8.0, containing 0.30 M NaCl and 0.02% (w/v) NaN_3 - at approximately a 3% (w/v) protein concentration. In order to remove lipoproteins and residual lipid material, NaBr was added to a density of 1.2 (257 g/l) and the sample centrifuged at 100,000 x g for 3 h at 20 C. The subnatant was removed with a syringe and needle to which ammonium sulfate was added to 50% saturation. The crude immunoglobulin fraction was stored as such at 4 C until required.

Prior to gel filtration the precipitate was collected by centrifuging at 27,000 x g for 30 min at 20 C. The precipitate, representing approximately 2 l of whey, was dissolved in 8 ml of column buffer and filtered through a 5 µ Millipore filter. Gel filtration was performed in the ascending mode at room temperature using columns of Bio-Gel A-5 m (2.6 cm x 65 cm) and A-0.5 m (2.6 cm x 35 cm) connected in tandem. Pre-swollen agarose gels from Bio-Rad Laboratories were prepared and packed according to the manufacturer's recommended procedures. Columns and 3-way and 4-way valves were purchased from Pharmacia Fine Chemicals. The sample

applied to the A-5 m column at a rate of 0.20 ml/min was followed by 5 ml of a 10% sucrose solution prepared in column buffer and applied at the same rate. Elution was achieved at a rate of 0.35 ml/min. The column eluent was monitored at 254 nm with an ISCO Ultraviolet Analyzer, model UV-2. An ISCO, model 1100, fraction collector was used for collecting 11 min fractions. A Beckman Instruments DK-2A spectrophotometer was employed to read the absorbance of fractions at 280 nm. When required, IgM concentration was determined from absorbance at 280 nm and a specific absorption coefficient ($E^{1\%}_{280\ nm}$) of 12.6 (Mukkur and Froese, 1971).

Immunoglobulin M Fab Fragment

Immunoglobulin M Fab fragments were isolated according to a procedure adapted from Beale and Buttress (1972). Similar procedures have been shown to produce active Fab fragments (Stone and Metzger, 1968). A one-tenth volume of 2.5 M Tris-HCl, pH 8.0, buffer was added to IgM in column buffer at a concentration of 0.80 mg/ml. After flushing with nitrogen, dithiothreitol was added to a concentration of 5 mM. This was allowed to react for 1 h at room temperature and iodoacetamide, recrystallized 4 x from distilled water, was then added to a concentration of 35 mM. After reacting at room temperature for 15 min, the solution was dialyzed against several changes of column buffer for 18 h at 4 C. Iodoacetamide and all solutions containing iodoacetamide were maintained in the dark. The solution was

concentrated approximately 4-fold with a Millipore Corporation Immersible CX unit (nominal molecular weight cut-off 10,000 daltons).

After concentration and addition of $CaCl_2$ to a concentration of 0.01 M, the alkylated IgM subunits were digested with trypsin (protein:enzyme weight ratio, 50:1) for 3 h at 30 C. A 3-fold weight excess of soybean trypsin inhibitor (1 mg inhibits 1.5 mg trypsin) was added to stop digestion.

The Fab fragments were purified by gel filtration chromatography performed in the descending mode at room temperature using a column of Sephacryl S-200 (2.6 cm x 90 cm). Pre-swollen gel from Pharmacia Fine Chemicals was prepared and packed according to the manufacturer's recommended procedures. The column buffer was the same as used for IgM isolation - 0.01 M Tris-HCl, pH 8.0, containing 0.30 M NaCl and 0.02% (w/v) NaN_3. Before use, the column was calibrated (log MW vs K_d) using Blue Dextran, vitamin B_{12}, bovine IgG, bovine serum albumin, ovalbumin, and sperm whole myoglobin. A 10 ml sample containing 10% sucrose and representing approximately 30 mg of IgM, was applied to the chromatographic bed under the eluent. A flow rate of 0.45 ml/min was employed. The column eluent was monitored as for IgM isolation. A specific absorption coefficient ($E^{1\%}_{280\ nm}$) of 12.6 was employed for the Fab fragment.

Soluble and Insoluble Protein Fractions of the Milk Fat Globule Membrane

The procedure described by Herald and Brunner (1957) was used to isolate soluble and insoluble protein fractions from the milk fat globule membrane. This procedure was chosen since it yields a soluble glycoprotein fraction more representative of the total membrane fraction than other procedures commonly employed (Yamauchi et al., 1978). Approximately 8 gallons of fresh whole milk was separated at 40-45 C. The cream was collected and washed by adding 3 volumes of distilled water at 40-45 C followed by gentle mixing and reseparation. The cream was again collected and distilled water was added to bring the volume back to the original volume. This washing procedure was repeated 3 more times to ensure the complete removal of casein and whey components from the cream.

Following storage overnight at 4 C the washed cream was churned at room temperature. It required 40-60 min for the emulsion to break, after which churning was continued until the butter granules clumped to form large balls. The buttermilk was separated from the butter granules by straining through cheese cloth and an equal volume of saturated ammonium sulfate solution was added to the buttermilk to give a 50% saturated solution. This solution was allowed to sit overnight at 4 C in a graduated cylinder during which the salted-out membrane material formed a floating layer.

After removing the subnatant, the floating membrane material was centrifuged at 25,000 x g for 60 min to concentrate the membrane material into a packed, floating layer. The resulting concentrated total membrane preparation was delipidated as follows. Five ml of cold ethanol: diethyl ether (35:65, v/v) per gram of concentrated membrane material was added and the mixture stirred at 4 C for 60 min, followed by decantation and addition of an equal volume of fresh solvent. After stirring for an additional 60 min the membrane material was collected by centrifugation. Five ml of cold diethyl ether was added per gram of original membrane material. This mixture was stirred 60 min at 4 C. The diethyl ether was decanted and replaced with an equal volume of fresh diethyl ether. After stirring for 60 min the membrane material was collected by centrifugation. Fresh diethyl ether was added and the mixture stirred for 60 min at room temperature. The solvent was decanted and an equal volume of fresh diethyl ether added. After stirring for 60 min at room temperature the membrane material was collected by centrifugation. Residual solvent was removed under vacuum. The fat globule membrane proteins were then fractionated into a soluble and an insoluble fraction by extracting with 0.02 M NaCl and centrifuging the mixture for 30 min at 25,000 x g. This procedure was repeated 2 times. The soluble and insoluble fractions were dialyzed against distilled water for 48 h and lyophilized.

Skim Milk Membrane

Preparation of skim milk membrane via preparative ultracentrifugation. The procedure of Plantz et al. (1973) was employed. Fresh whole milk warmed to 37 C was centrifuged at 5,000 x g for 20 min at 30 C. The skim milk was removed with a syringe and needle inserted through the compacted cream layer. The membrane material in skim milk was obtained by centrifugation at 90,000 x g for 100 min at 20 C. After removing most of the whey, the fluff material located on the casein micelle pellet and tube bottom were suspended by gently shaking the tube and collected with a disposable pipette. Generally, 40 ml of skim milk membrane (SMM) solution was obtained from 500 ml of skim milk. If it was desired to obtain a SMM fraction with a lower whey protein content, 3 volumes of simulated milk ultrafiltrate (Jenness and Koops, 1962) was added and the mixture recentrifuged. Generally, 15 ml of SMM solution was obtained from 160 ml of solution.

Preparation of skim milk membrane via salt fractionation and gel filtration. A procedure adapted from Kitchen (1974) was employed. To skim milk, prepared as for preparation of SMM via ultracentrifugation, rennet was added at a rate of 0.8 g/l. After 30 min at 30 C, the curd was cut and allowed to sit an additional 30 min. The whey was then removed by filtration through cheese cloth. The whey was clarified by centrifugation at 16,000 x g for 20 min at 20 C.

Ammonium sulfate was then added to 50% saturation. After sitting overnight, most of the clear supernatant was siphoned off and the precipitate collected by centrifugation at 16,000 x g for 20 min at 20 C. The precipitate was solubilized in 0.5 M NaCl and dialysed against several changes of 0.5 M NaCl for 48 h at 4 C. After warming to 55 C for 30 min the solution was centrifuged at 15,000 x g for 20 min at 20 C.

The SMM was then purified by gel filtration chromatography performed in the descending mode at room temperature using a column of Sepharose 4B (2.6 cm x 35 cm). Pre-swollen agarose gel obtained from Pharmacia Fine Chemicals was prepared and packed according to manufacturer's recommended procedures. The column was equilibrated in 0.5 M NaCl. Ten ml of sample containing 10% sucrose and representing 150 ml of skim milk was applied to the chromatographic bed under the eluent. A flow rate of 0.35 ml/min was employed. The SMM was obtained in fractions which eluted at the void volume. Column eluent was monitored as for IgM isolation.

Milk Fat Globule Membrane Gangliosides

A ganglioside-containing fraction was isolated from milk fat globule membrane according to the procedure of Keenan (1974). The gangliosides were not purified using column or thin-layer chromatography. In addition to gangliosides, the aqueous fraction would contain neutral, mostly

fucose-containing, glycolipids, sulfatides, and some non-lipid contaminants. However, it should not contain sialoglycoproteins (Brunngraber et al., 1976).

Milk fat globule membrane was prepared from washed milk fat globules as described in the section on isolation of soluble and insoluble milk fat globule membrane proteins. The membrane material was collected by centrifugation at 90,000 x g for 100 min at 20 C. Lipids were extracted by stirring the membrane material with 20 volumes of chloroform:methanol (2:1, v/v) overnight at room temperature. Insoluble residues were recovered and stirred with 10 volumes (based on original sample volume) of chloroform:methanol (1:1, v/v) for 6 h at room temperature. Combined extracts were evaporated to near dryness with a rotary evaporator and redissolved in chloroform:methanol (2:1, v/v). Gangliosides were recovered by washing with 0.2 volumes of 0.88% KCl and then once with theoretical upper phase without KCl (chloroform:methanol:water::3:48:47, v/v/v). The combined upper phases were then dialysed against several changes of distilled water for 48 h at 4 C.

Casein Fractions

Preparation of whole casein. Whole casein was prepared from skim milk separated from whole milk at 40-45 C. After cooling to room temperature the pH was slowly adjusted to 4.6 with 1 M HCl while stirring. After sitting for 1 h, casein was collected by filtration through 3 layers of cheese

cloth. The casein was washed 2 times with a volume of distilled water equal to that of the original milk. It was then solubilized in an equal volume of distilled water by the slow addition of 1 M NaOH to pH 7.0 and again precipitated and washed 2 times with distilled water. After solubilization and dialysis against several changes of distilled water for 48 h at 4 C the casein was lyophilized.

Preparation of κ-casein. κ-Casein was prepared according to the procedure of Zittle and Custer (1963). Approximately 150 g of acid precipitated whole casein, prepared as for whole casein isolation, was dissolved in 500 ml of 6.6 M urea. This solution was acidified with 100 ml of 7 N H_2SO_4. After acidification, 1 l of distilled water was added. After standing for 2 h the precipitate, containing mainly α_s- and β-casein, was removed by centrifugation at 16,000 x g for 20 min at 20 C. The κ-casein was then precipitated by the addition of 132 g ammonium sulfate per liter of supernatant. The precipitate was collected by centrifugation at 16,000 x g for 20 min at 20 C, suspended in distilled water and dissolved by addition of 1 M NaOH to a final pH of 7.5. The solution was dialyzed against several changes of distilled water for 48 h at 4 C prior to lyophilization. The α_s- and β-casein-rich fraction was also solubilized, dialyzed, and lyophilized.

Preparation of caseinomacropeptide. Caseinomacropeptide was prepared according to a procedure adapted from

Brunner and Thompson (1959). Acid precipitated whole casein was prepared as for whole casein isolation. After solubilizing in distilled water by addition of 1 M NaOH to pH 7.0, one part of rennet was added per 100 parts of Na-caseinate present in solution at a 3% (w/v) concentration. After reacting for 60 min at 25 C the pH was adjusted to 4.6 with 1 M HCl to precipitate the casein. The precipitate was removed by centrifugation at 16,000 x g for 20 min at 20 C and trichloroacetic acid added to the supernatant to a final concentration of 2% (w/v). Insoluble protein was allowed to settle and the supernatant clarified by filtration. The filtrate was adjusted to pH 7.0 with 1 M NaOH and concentrated 2 fold by pervaporation. The solution was dialyzed against several changes of distilled water for 48 h at 4 C prior to lyophilization.

Chemical Methods

Lowry Determination of Protein

Protein was determined according to the Lowry modification of Markwell *et al*. (1978) using bovine serum albumin as a standard. Absorbance was read at 660 nm with a Bausch and Lomb Spectronic 21. The method employs solubilization of protein samples in sodium dodecyl sulfate and an increased copper tartrate concentration to eliminate the necessity of pretreating membrane or lipoprotein samples and minimized interferences from EDTA and sucrose.

Babcock Determination of Fat

The Babcock method was used to determine the fat content of whole milk. The procedure outlined by Atherton and Newlander (1977) was followed.

Electrophoretic Procedures

The following three electrophoretic procedures were performed using a tube-type apparatus manufactured by Buchler Instruments. Glass tubes (7.5 cm x 0.60 cm, inner diameter) which were acid-washed and Photo-Flo (0.5%, v/v, aqueous solution) rinsed were employed. Power was supplied by either a Bio-Rad Laboratories model 400 or a MRA Corporation model ml58 power supply. Gels were stained for protein with Coomassie Brilliant Blue R250 according to Weber and Osborn (1969) using a 2 h staining time and destained with a Bio-Rad Laboratories model 170 diffusion destainer.

Discontinuous Polyacrylamide Gel Electrophoresis with an Acid Buffer System

Polyacrylamide gel electrophoresis of immunoglobulins was performed using the acidic buffer system of Reisfeld *et al*. (1962). In this system, the basic immunoglobulins exhibit a reasonable migration distance and degree of resolution. A running gel with T = 4.10% and C = 2.60% and a stacking gel with T = 3.10% and C = 20.0% were employed. Chemically-induced polymerization was employed for the running and stacking gel. Methyl green prepared in stacking

gel buffer was added to samples as a marker dye. Electrophoresis was performed at a rate of 3 mA/tube.

Discontinuous Polyacrylamide Gel Electrophoresis with an Alkaline Urea-Containing Buffer System

Heavy and light polypeptide chains of IgM were electrophoretically resolved using the procedure of Reisfeld and Small (1966) with these modifications: (a) Heavy and light polypeptide chains were not separated prior to electrophoresis. (b) The discontinuous buffer system of Davis (1964) with running and stacking gels containing 8 M urea was employed. (c) A running gel with T = 4.10% and C = 2.60% and a stacking gel with T = 3.10% and C = 20.0% were employed. Urea solutions were deionized by stirring with mixed bed ion exchanger (equal volumes of Dowex 50W-X8 and Dowex AG1-X8, 20-50 mesh) of approximately one-tenth their volume for 12 h at 4 C. Specific conductivity did not exceed 8-12 μmho.

Immunoglobulin M (4 mg) was prepared for analysis by lyophilizing. After solubilizing in 1 ml 8 M urea, dithiothreitol was added to a concentration of 50 mM and the sample allowed to sit at room temperature overnight. To maintain the pH, 0.2 ml of pH 8.0 0.5 M Tris-HCl prepared in urea was added. Iodoacetamide, recrystallized 4 x from distilled water, was added in 0.5 M NaOH to a final concentration of 150 mM (0.3 ml). After reacting at room temperature for 15 min, the sample was dialyzed 24 h

against stacking gel buffer at 4 C. Iodoacetamide and all samples containing iodoacetamide were maintained in the dark. Electrophoresis was performed at a rate of 3 mA/tube using bromophenol blue as a marker dye.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Immunoglobulin M purity was evaluated and molecular weights of IgM heavy and light polypeptide chains determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis performed according to the general protocol as described by Weber and Osborn (1969). Protein samples were incubated at 100 C for 10 min and sucrose added to increase sample density prior to electrophoresis. Total acrylamide concentrations (T) of 5.50, 6.50, 7.50, 10.0, 12.0, and 14.0 were employed with a constant cross-linker concentration of 2.64%. A Pharmacia Fine Chemicals low molecular weight calibration kit was used to prepare standard curves. Asymptotic minimum molecular weights (Segrest and Jackson, 1972) and molecular weights based on retardation coefficients (MW vs K_r) were calculated (Rodbard and Chrambach, 1971).

Immunological Procedures

The following two immunological techniques were performed using a stabilizing medium of 1.0% agarose in 0.015 M veronal buffer, pH 8.6, containing 0.02% (w/v) NaN_3. A mold for casting the gel was prepared from 2 glass plates (8.3 cm x 10.0 cm) from Arthur H. Thomas, Co., polyethylene

tubing (inner diameter 1.14 mm, outer diameter 1.57 mm) from Clay Adams, and metal pinch clamps. One plate was coated with silicone vacuum grease for easy removal after the agarose had hardened and clamps removed. A gel of nominal thickness 1.5 mm was obtained. Anti-sera and sample wells of 3 mm diameter were cut with a sharp-edged stainless-steel tube. Agarose plugs were removed with a Pasteur pipette connected to a water aspirator. Wells were filled with either 7 µl of sample or anti-sera. Plates were allowed to develop in a closed, leveled chamber containing vessels of water and thymol crystals.

Following development the plates were pressed, washed in phosphate buffered saline for 24-48 h and stained with Coomassie Brilliant Blue R250 following the procedure of Weeke (1973).

Two-Dimensional Double Diffusion

A procedure adapted from Ouchterlony (1949) was used for two-dimensional double diffusion immunological identification of IgM. An electrophoretically homogeneous sample was subjected to two-dimensional double diffusion versus rabbit anti-bovine IgM (µ-chain specific) anti-sera. Prior to analysis the sample was dialyzed against 0.015 M veronal buffer, pH 8.6, for 8 h at room temperature. In preparing wells, a template with 6 antigen and 6 anti-sera wells placed on intersecting lines and with corresponding well-centers spaced from 0.50 to 1.40 cm apart was utilized. Diffusion

was allowed to proceed for 90-100 h.

Two-Dimensional Single Radial Diffusion

Two-dimensional single radial diffusion performed according to a modified procedure of Mancini et al. (1965) was employed for IgM quantitation in whey. Antibody concentrations of 0.12-0.60% (v/v) and IgM concentrations of 0.03-0.50 mg/ml were used. The concentration of IgM in standard solutions was based on absorbance at 280 nm and a specific absorption coefficient ($E^{1\%}_{280\ nm}$) of 12.6 (Mukkur and Froese, 1971). Prior to application, the IgM solution was dialyzed against 0.015 M veronal, pH 8.6, buffer for 8 h at room temperature. Whey samples were concentrated approximately 2 x by dialyzing samples against a 15-20% (w/v) polyvinylpyrrolidone solution prepared in 0.015 M veronal, pH 8.6, buffer at 4 C. Diffusion was allowed to proceed for 90-100 h. After staining, precipitate diameters were measured using a Nikon model 6 Shadowgraph Microcomparator equipped with an Ehrenreich Photo-Optical Industries Electromike. Plots of precipitate diameter versus log (concentration) yielded straight lines over limited concentration ranges.

Creaming-Studies Associated Procedures

Preparation of Samples

Milk. Creaming studies were generally performed using fresh whole milk. For some studies 1-2 day old milk stored at 4 C was employed. Prior to use all milk was placed in a

water bath at 55 C for 30 min with occasional gentle mixing. If skim milk was required, either cold- or warm-separated whole milk was centrifuged at 1,000 x g for 20 min at room temperature. Skim milk was removed with a syringe and needle inserted through the compacted cream layer. When required, washed milk fat globules were added to give a fat content of 3.2-3.6%. Substances added to milk were prepared in SMU or dialyzed against several changes of SMU for 6-8 h at room temperature.

Heated milk. Milk, either whole or skim, devoid of creaming capacity due to inactivation of the heat-labile factor was prepared by placing milk in a closed container in a water bath at 75 C for 30 min with occasional gentle mixing.

Homogenized milk. Skim milk devoid of creaming capacity due to inactivation of the homogenization-labile factor was prepared by homogenizing skim milk (40 C) at 2500 psi in a single stage model C-8 C.W. Logeman Co. homogenizer. If required, washed milk fat globules were added to give a fat content of 3.2-3.6%.

Washed milk fat globules. Washed milk fat globules were prepared from whole milk previously warmed to 55 C for 30 min. A cream layer was obtained by centrifuging the whole milk at 1,000 x g for 20 min at room temperature. After removing the skim layer, four volumes of distilled water at 40 C was mixed with the cream. The solution was

centrifuged again and the underlayer removed. This process was repeated 2 additional times.

Synthetic milk fat globules. Synthetic fat globules, or stable fat globules prepared by emulsification in an aqueous protein solution, were prepared using butter fat and either κ-casein, an α_s- and β-casein rich fraction, or a mixture of these 2 samples (α_s-, β-casein:κ-casein::15:85, w/w). Butter oil obtained from churned washed milk fat globules was washed twice by ultracentrifugal separation (1,000 x g) for 15 min at room temperature) using 1 M NaCl solution. Protein solutions at a 0.35% (w/v) concentration were prepared in SMU. A coarse emulsion was formed by mixing 4 g of butter oil (55 C) with 100 ml of protein solution (55 C) in a Waring blender for 45-60 sec. After sitting at 55 C for 10-15 min to reduce foam, the mixture was homogenized at approximately 500 psi with a C.W. Logeman Co. hand homogenizer. The fat globules were collected by centrifuging at 1,000 x g for 20 min at room temperature. They were then washed 2 times with 4 volumes of 55 C distilled water.

When observed microscopically (1000 x), the fat globule size distribution was predominantly in the 2-5 μ diameter range.

Quantitation of Creaming Capacity

Cream volume. Cream volume was determined by placing 37 C samples in 10 ml graduated cylinders into an 9-11 C

water bath. The water depth was sufficient to cover the sample. The milliliters of cream layer formed was read directly to the nearest 0.05 ml from the graduated cylinder scale with the aid of transmitted light. Cream volumes were generally read at 1, 2, 3, 4 and 24 h intervals. After 4 h the graduated cylinder was placed in a 4 C cold room. If a distinct cream line had not developed but clusters were visible throughout the cylinder, a cream volume of 10.0 was reported.

Cluster time. The cluster time, or the time in minutes for visible clusters to form, was also used as a measure of creaming capacity. A procedure similar to that employed by Dunkley and Sommer (1944) was used. Creaming cells with inside dimensions 76 mm x 20 mm x 1 mm were constructed from thin microscope slides. Two cells were mounted on a thick glass base plate. The cluster time was determined by placing 37 C samples in the creaming cells into a plexiglass water bath maintained at 9.5 - 10.5 C. The milk was continuously observed using transmitted light. The time at which clusters were first visible was reported as the cluster time.

Enzymic Treatment of Milk Fat Globules

Washed milk fat globules were treated with various enzymes in order to examine the role of membrane constituents in creaming. Twenty milliliters of washed milk fat globules was added to 100 ml of reaction buffer containing

enzyme to give a fat content of approximately 6%. Controls containing no enzyme were run in each case. For trypsin a reaction medium of 0.05 M Tris-HCl, pH 7.8, a reaction time of 20 min at 37 C, and a buffer enzyme concentration of 50 µg/ml were employed. For pronase the same reaction conditions were used except for a buffer enzyme concentration of 150 µg/ml. For mixed glycosidases (content and activities are given in the Appendix, Table A3) a reaction medium of 0.10 M Na-citrate, pH 4.6, a reaction time of 12 h at 37 C, and a buffer enzyme concentration of 1 mg/ml was employed. For neuraminidase the same reaction conditions were used except for a buffer pH of 6.0 and enzyme concentration of 100 µg/ml. For the latter 2 treatments, 1 drop of toluene was added to inhibit bacterial growth. For samples treated with mixed glycosidases and neuraminidase after trypsin treatment, appropriate buffers were employed using the washing procedure given below between treatments. For milk fat globules treated with mixed glycosidases and neuraminidase simultaneously, the Na-citrate, pH 6.0, buffer was employed using the enzyme concentrations listed above. The neuraminidase preparation exhibited no protease activity as measured with the Azocoll (Calbiochem-Behring Corp.) assay at pH 6.0 and 37 C. The mixed glycosidase preparation exhibited very low protease activity after a reaction time of 4.5 h at pH 4.6 and 37 C. A 3-fold weight excess of soybean trypsin inhibitor (1 mg inhibits 1.5 mg trypsin)

was added to trypsin treated milk fat globules to stop digestion. Milk fat globules were collected by centrifuging at 1,000 x g for 20 min at room temperature. They were then washed 3 times with 10 volumes of 40 C distilled water using the same conditions for separation. Milk fat globules were added back to skim milk to a fat content of 3.5% for creaming studies.

Spectrophotometric Evaluation of IgM Cold-Induced Aggregation

A procedure similar to that of Payens (1968) was employed. Immunoglobulin M purified as previously described was concentrated to approximately 1.2 mg/ml using a Millipore Corp. Immersible CX unit (nominal molecular weight cut-off 10,000 daltons). After concentration the solution was dialyzed against 3 changes of SMU for 6 h at room temperature. The sample absorbance was followed at 344 nm using a Gilford Instrument Laboratories, Inc. model 240 Spectrophotometer equipped with a model 2451A Automatic Cuvette Positioner while decreasing or increasing its temperature by means of circulating water at 0 C or 40 C. The temperature in a control cuvette was read intermittently. A final sample temperature of either 10 C or 37 C was achieved. The absorbance of an IgG and void volume fraction was also followed after dialysis against SMU.

RESULTS AND DISCUSSION

Isolation and Identification of Immunoglobulin M

Isolation

Immunoglobulin M (IgM) has been prescribed a role in milk fat globule (MFG) clustering due to its ability to restore creaming to heated milk (Franzen, 1971) or to bring about clustering of MFG in model systems (Gammack and Gupta, 1970; Payens and Both, 1970). To confirm these observations it was necessary to obtain an IgM fraction from cows' milk, colostrum, or blood serum. Salt fractionation and gel filtration were employed as outlined in Figure 1 to isolate IgM from milk.

Lipoproteins and residual lipid material were removed from the crude immunoglobulin fraction using ultracentrifugation, to avoid impairing column performance and to reduce the size of the void volume fraction. Table 1 details the relative purification and recovery of IgM (considering whey as 100%) for 3 steps in the isolation scheme. When the crude immunoglobulin fraction was subjected to gel filtration on columns of Bio-Gel A-5m and A-0.5m connected in tandem, 3 prominent peaks were obtained (Figure 2). The Bio-Gel A-0.5m column was employed to increase resolution

Table 1. The recovery and purification of IgM at selected steps in the isolation scheme.

Fraction	Volume (ml)	Protein Concentration (mg/ml)	IgM Concentration (mg/ml)	IgM Recovery (%)	Fold Purification
Whey	12420	18.4	0.077	100	-
Crude globulin	1500	8.18	0.44	69	5
Crude immunoglobulin	235	17.3	1.53	38	6
Crude immunoglobulin - lipoproteins	300	9.19	0.84	26	7

Figure 2. Gel filtration chromatogram of a crude immunoglobulin preparation. Bio-Gel A-5m (2.6 cm x 65 cm) and A-0.5m columns connected in tandem were employed. Approximately 500 mg of protein containing 40 mg of IgM was applied to the column.

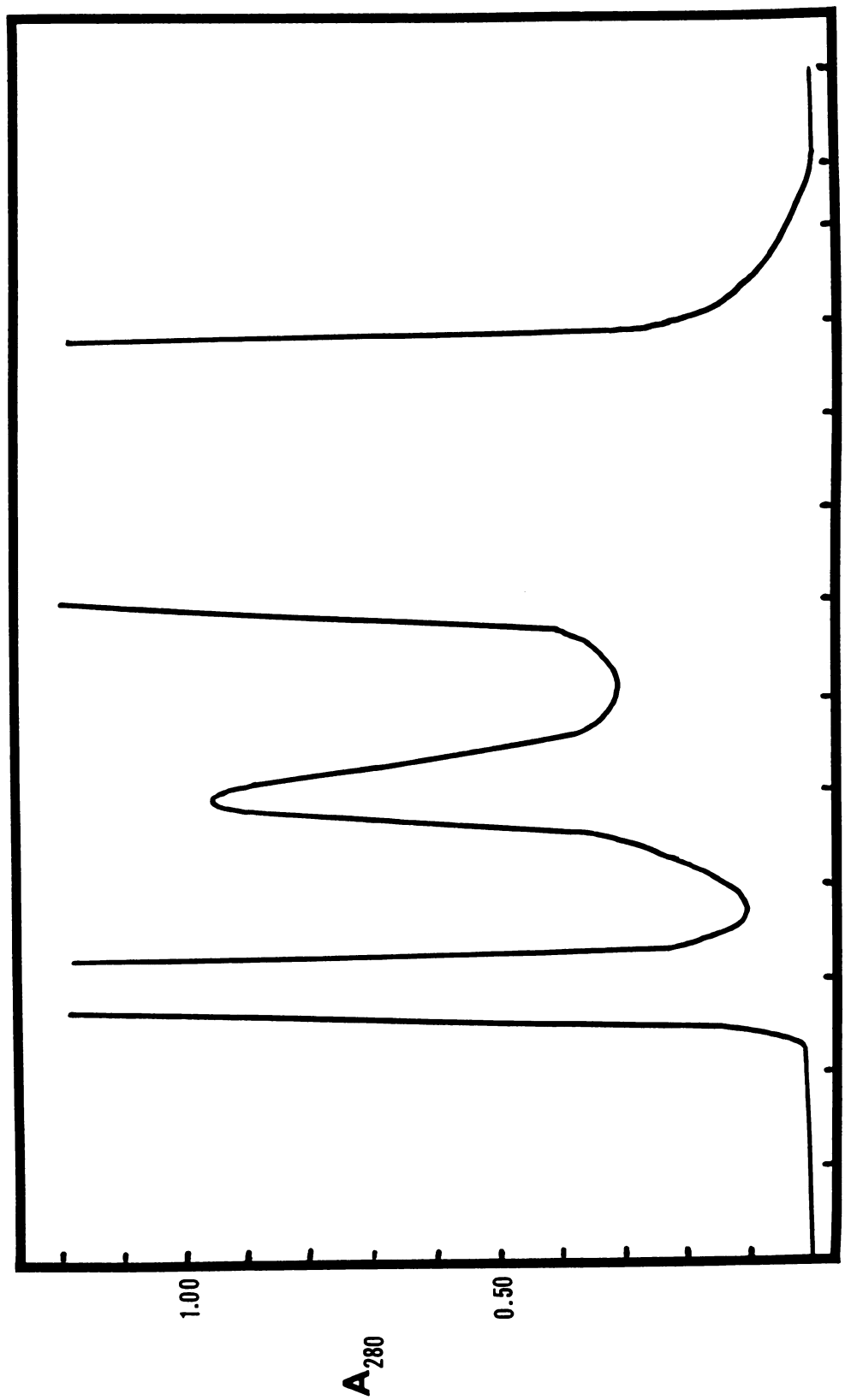


Figure 2

between the latter 2 peaks. The eluted fractions containing the first peak, corresponding to the void volume, was translucent. The second peak was identified as IgM and the last peak as primarily IgG using electrophoretic and immunological analysis (Results presented in the sections Electrophoretic Analyses and Two-Deminsional Double Diffusion). An IgM sample was obtained by pooling fractions 44-55. Sixty percent of the IgM applied to the column was recovered in this fraction. The remainder was distributed over the next 15 fractions which also contained IgG.

Re-chromatography of the IgM sample after concentration to 3.6 mg/ml yielded the chromatogram presented as Figure 3. The sample was essentially homogeneous except for a small void volume peak, probably consisting of aggregated IgM. The recovery of IgM was 65%. The significant loss of IgM is attributed to the removal by centrifugation (27,000 x g) for 30 min) and filtration of protein aggregates (turbidity) which formed during storage at 4 C and concentration. Protein aggregation is characteristic of purified IgM preparations (Metzger, 1970). As re-chromatography did not contribute to an increase in IgM purity, it was not included in the general isolation scheme.

Electrophoretic Analysis

Electrophoretic patterns of acid whey, crude globulin, crude immunoglobulin (after removal of lipoproteins), and

Figure 3. Gel filtration chromatogram of IgM. Bio-Gel A-5m (2.6 cm x 65 cm) and A-0.5m (2.6 cm x 65 cm) columns connected in tandem were employed. Approximately 20 mg of IgM was applied to the column.

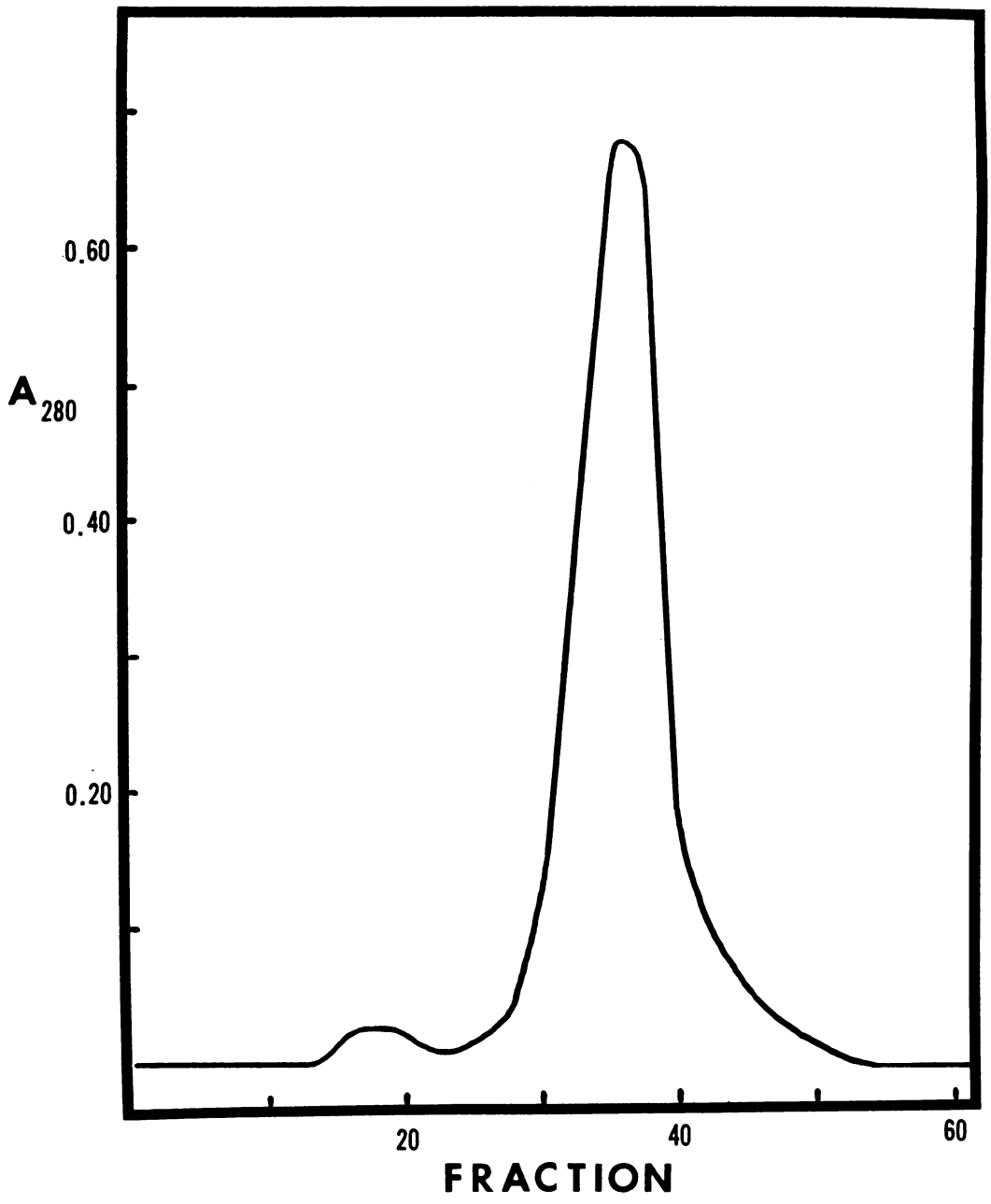


Figure 3

pooled fractions obtained from gel filtration of the crude immunoglobulin preparation (Figure 2) are presented in Figure 4 . The basic immunoglobulins separate into distinct zones with the acid buffer system employed. The immunoglobulins form diffuse zones near the stacking/running gel interface when alkaline buffer systems are used (Melachouris, 1969; Franzen, 1970). An enrichment of immunoglobulins is noted on progression from the whey to the crude globulin and crude immunoglobulin samples. Fractions 44-54, corresponding to the second peak in the gel filtration pattern, contained only IgM. Fractions 55-72 contained IgG in addition to IgM. Fractions 73-105 contained primarily IgG. The void volume fractions failed to yield protein bands which stain with Coomassie Brilliant Blue R250. IgA, which is commonly absent from cows' milk (Kumar and Mikolajcik, 1972), was not present in the samples.

When subjected to reduction and alkylation, electrophoretically homogeneous IgM yielded numerous components which were resolved by electrophoresis in an alkaline urea-containing buffer system (Figure 4, gel 8). Immunoglobulin heterogeneity or numerous heavy and light chains are expected in polyclonal antigenic responses (Kabat, 1976). The photographic technique employed here did not permit discernment of all protein bands which actually appear as numerous diffuse zones.

Figure 4. Electrophoretic patterns characterizing the isolation of IgM from milk. Samples applied to the gels are whey (1), crude globulins (2), crude immunoglobulins (3), and fractions obtained by gel chromatographic isolation of the crude immunoglobulin fraction (see Figure 2): fractions 44-54 (4), fractions 55-72 (5), fractions 63-78 (6), and fractions 88-105 (7). Gel 8 represents alkylated, 2-mercaptoethanol-reduced IgM. Discontinuous polyacrylamide gel electrophoresis with an acid buffer system was employed for gels 1-7 and an alkaline, urea-containing buffer for gel 8. Protein loads on the gels were: 90 μg (1), 40 μg (2-7), and 200 μg (8).

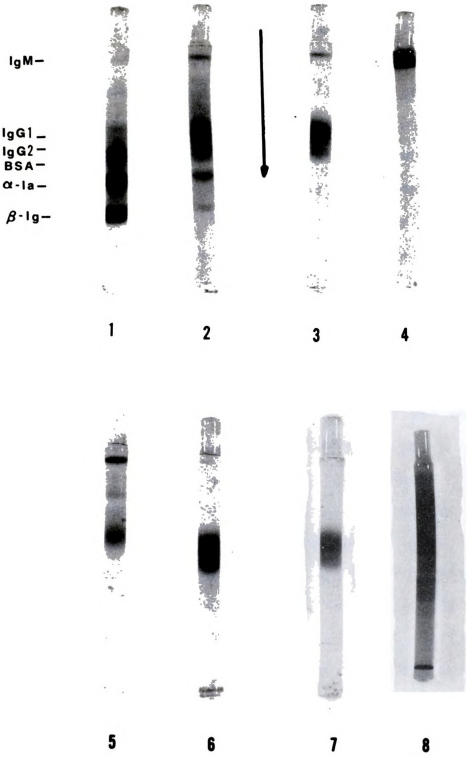


Figure 4

Sodium dodecyl sulfate polyacrylamide gel electropherograms of IgM are presented in Figure 5. As expected, 2 major protein zones representing heavy and light chains were obtained. A faint band having a mobility slightly greater than the heavy chain was evident at each gel concentration. Table 2 lists the apparent molecular weights of heavy and light chains for each gel concentration. The observed molecular weight for heavy chains varied with acrylamide concentration while for light chains was independent of acrylamide concentration. This is attributed to the relatively high carbohydrate content of heavy chains (12.0%) and low carbohydrate content of light chains (less than 0.5%) (Beale and Buttress, 1972; Segrest and Jackson, 1972). Asymptotic minimum molecular weights of 74,500 and 27,500 were estimated for the heavy and light chains, respectively. From a plot of molecular weight versus retardation coefficient (Figure 6), molecular weights of 70,000 and 25,000 were obtained for the heavy and light chains, respectively. Mukkur and Froese (1970), employing gel filtration, reported values of 76,000 and 22,500 and Beale and Buttress (1972), employing sedimentation equilibrium, reported values of 61,800 and 22,800 for heavy and light chain molecular weights, respectively.

Two-Dimensional Double Diffusion

The two-dimensional double diffusion pattern of pooled fractions 44-55 (Figure 2) developed against anti-IgM

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electropherograms of molecular weight standard proteins and IgM. Gel pairs 1, 2, 3, 4, 5, and 6 contained total acrylamide concentrations of 5.50, 6.50, 7.50, 10.0, 12.5 and 14.0%, respectively. IgM gels were charged with 25 μg of protein.

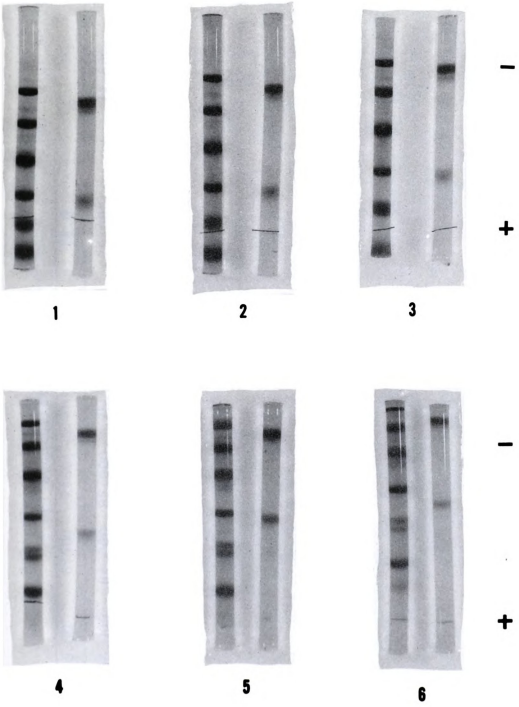


Figure 5

Table 2. Molecular weights of IgM heavy and light chains as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis as a function of gel concentration.

Protein	Molecular Weight as a Function of Gel Concentration[a] (%)						Asymptotic Minimum Molecular Weight
	5.50	6.50	7.50	10.0	12.5	14.0	
Heavy chain	86,000	84,000	82,000	75,000	74,000	75,000	74,500
Light chain	27,000	28,000	27,000	28,000	28,000	27,000	27,500

[a]Relative mobilities were the average of 3-5 measurements.

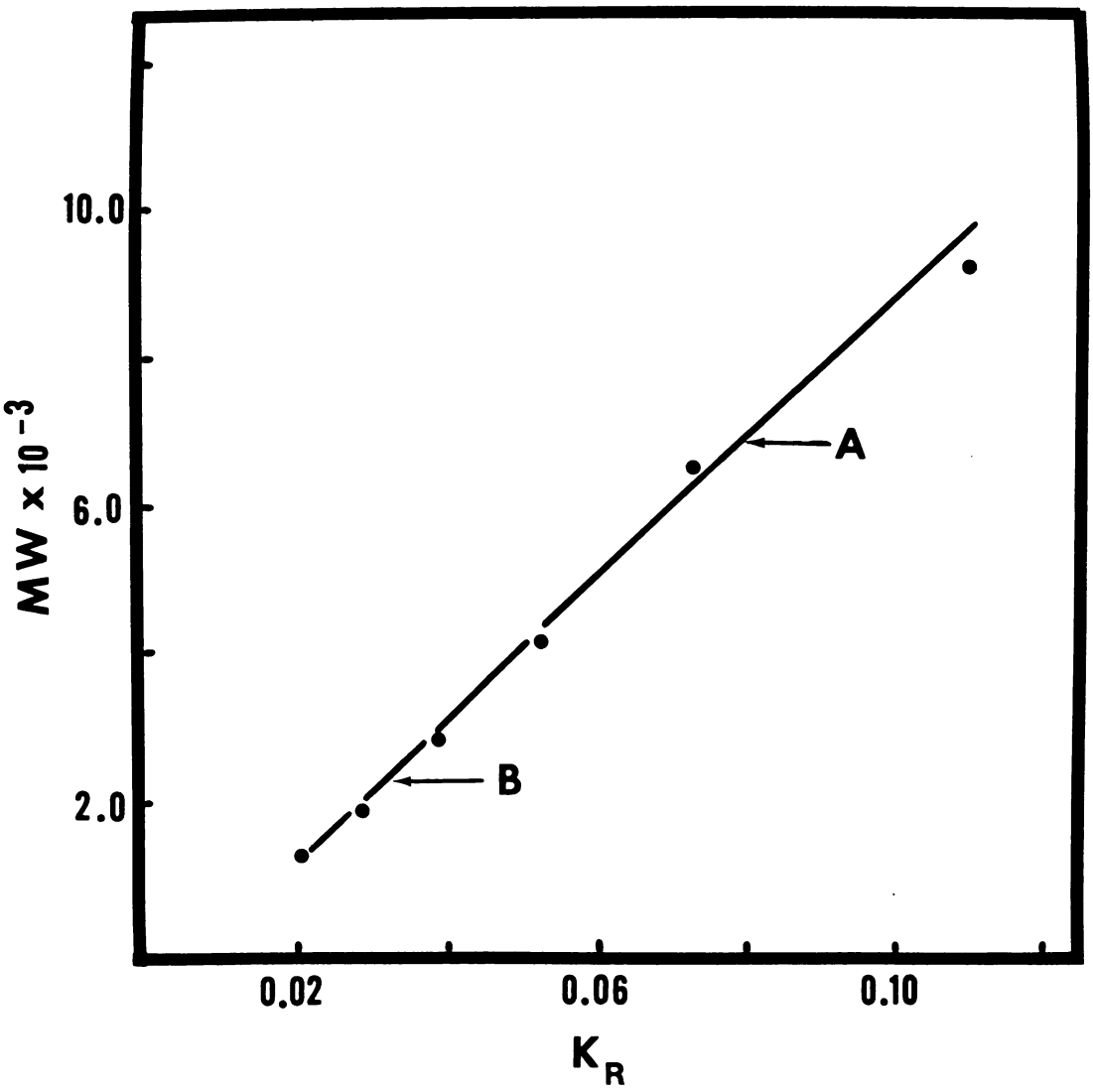


Figure 6. Plot of molecular weight versus retardation coefficient derived from sodium dodecyl sulfate polyacrylamide gel electrophoresis data (see Figure 5). Arrows indicate the retardation coefficients for IgM heavy (A) and light (B) chains.

anti-serum (μ-chain specific) is presented in Figure 7A. The precipitin reaction verifies that the designated sample is IgM. A faint reaction was obtained in the first set of wells versus anti-IgG anti-serum (γ-chain specific), see Figure 7B. The faint reaction demonstrates that an IgG contaminant is present in the sample - but at a very low concentration. Based on chromatographic, electrophoretic, and immunological results, fractions corresponding to 55-66 (Figure 2) were collected for experiments requiring IgM.

Confirmation of Immunoglobulin M as a Creaming-Active Component

Effect on Creaming of Heated Milk

Franzen (1971) demonstrated that IgM is the euglobulin component which restores creaming to heated milk. These results are confirmed by the data presented in Table 3. Whereas IgG and the void volume fraction failed to restore creaming to heated milk, IgM did restore creaming. Increased levels of IgM were found to restore increased levels of creaming capacity.

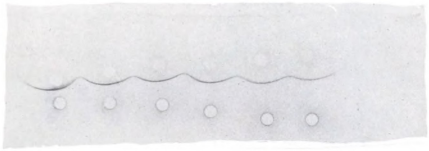
Identification of Immunoglobulin M as a Creaming-Active Component in Raw Milk

Removal from the reaction with specific anti-serum. To further examine or verify the role IgM plays in the creaming phenomenon, antibodies specific for IgM, IgG,

Figure 7. Two-dimensional double diffusion patterns of pooled gel filtration fractions 44-55 (see Figure 2) developed against (A) anti-IgM anti-serum (μ-chain specific), and (B) anti-IgG anti-serum (γ-chain specific).

A

IgM



anti-IgM

B

IgM

anti-IgG

Figure 7

Table 3. The effect of isolated IgG, void volume, and IgM fractions on creaming of heated milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Heated milk					
+IgG, 0.5 mg/ml	0.00	0.00	0.10	0.30	60 +
+IgG, 1.0 mg/ml	0.00	0.00	0.10	0.35	60 +
+IgG, 1.5 mg/ml	0.00	0.00	0.10	0.35	60 +
+Void volume	0.00	0.00	0.10	0.30	60 +
+IgM, 0.025 mg/ml	0.20	0.30	0.30	0.65	16.3
+IgM, 0.05 mg/ml	0.90	1.20	1.20	1.15	4.3
+IgM, 0.10 mg/ml	1.75	1.75	1.70	1.35	3.3
+IgM, 0.20 mg/ml	1.80	1.75	1.65	1.60	2.2
Raw milk	1.80	1.75	1.65	1.60	7.0

[a]One part of IgG, void volume, or IgM-SMU solution was added to 3 parts of heated milk. SMU was added to the heated and raw milk.

[b]Average of duplicate analyses.

and IgA heavy chains were added to raw milk. Indeed, if IgM is involved, the interaction with antibodies should remove it from the reaction, and decrease the extent of creaming. The results presented in Table 4 indicate that only in the case of added anti-IgM is the creaming effected, as measured by fat globule clustering time. At low levels of added anti-IgM, it is likely that soluble complexes involving only part of the IgM are formed due to an antigen excess. Only when the level is increased to amounts which bind significant levels of IgM is an effect seen.

Removal from the reaction with 2-mercaptoethanol. Mercaptoethanol (ME) treatment has been used as a means of differentiating between certain classes of immunoglobulins (Scott and Gershon, 1970; Kabat, 1976). Whereas ME-sensitive immunoglobulins lose their antibody combining activity, ME-resistant immunoglobulins suffer no or slightly diminished antibody combining activity. Although exceptions have been reported (Adler, 1965; Kim et al., 1966), ME-sensitive immunoglobulins are generally 19S-IgM, while ME-resistant immunoglobulins are usually 7S-IgG molecules. Since ME treatment destroys the antibody activity of bovine IgM molecules (Butler, 1969), its addition to milk may be expected to eliminate fat globule clustering and creaming. Data reported by Franzen (1971), presented in Table 5, demonstrate that creaming behavior of milk is eliminated upon the addition of ME.

Table 4. The effect of IgM, IgG, and IgA specific antisera on creaming of milk.

Sample	Cluster Time[a] (min)
Raw milk	6.7
+ 0.025 ml anti-IgM/2 ml	6.5
+ 0.05 ml anti-IgM/2 ml	6.5
+ 0.20 ml anti-IgM/2 ml	9.0
+ 0.30 ml anti-IgM/2 ml	21.3
+ 0.30 ml anti-IgG/2 ml	6.8
+ 0.30 ml anti-IgA/2 ml	6.7

[a]Average of duplicate analyses.

Table 5. The effect of 2-mercaptoethanol on creaming of milk.[a]

Sample	Cream Volume (ml/10 ml)		
	1 h	2 h	24 h
Raw milk	0.00	3.20	1.15
+ 0.5% 2-mercaptoethanol	0.00	3.50	1.20
+ 2.5% 2-mercaptoethanol	0.00	0.00	0.10

[a]Adapted from Franzen (1971).

At the lower level (0.5% ME), other milk proteins may have been preferentially reduced. However, at the higher level (2.5%), creaming was definitely eliminated. While indicating that IgM is involved in creaming, these results do not eliminate an indirect effect due to reduction of other proteins or components in milk. The failure of alkylated, reduced IgM to restore creaming to heated milk supports the notion that creaming is eliminated due to the effect on IgM (Franzen, 1971).

Effect of adding immunoglobulin M to raw milk. The addition of IgM to raw milk would be expected to augment creaming if it is involved in the phenomenon. The data of Table 6 support the contention that IgM is involved in creaming or fat globule clustering.

Immunoglobulin M Cryoaggregation

After it was established that IgM is involved in fat globule clustering, its cryoglobulin character, i.e., reversible low temperature-induced insolubility, was examined. The studies of Payens (1968) and Rhee (1968) with euglobulin and Payens and Both (1970) and Franzen (1971) with IgM implicated this characteristic of the protein as essential to fat globule clustering. The proteins were designated as cryoglobulins based on temperature-dependent turbidity measurements or sedimentation properties. The authors

Table 6. The effect of IgM on creaming of milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	1.75	1.55	1.50	1.55	7.1
+ IgM, 0.25 mg/ml	1.80	1.70	1.60	1.50	2.0

[a] Either one part of IgM-SMU solution or SMU was added to 4 parts of milk.

[b] Average of duplicate analyses.

failed to examine or address the evidence that immunoglobulins, and in particular IgA and IgM, polymerize or aggregate when purified (Butler, 1969; Metzger, 1970).

According to Zinneman (1978) human cryoglobulins may be classified into 3 categories based on their heterogeneity: (a) single monoclonal immunoglobulins, (b) mixed cryoglobulins, consisting of one monoclonal and one polyclonal immunoglobulin, and (c) mixed cryoglobulins, consisting of only polyclonal components. Mixed cryoglobulins are formed by two immunoglobulins, neither of which is a cryoglobulin in the single state. Data were presented to show that most cryoglobulins fall into the third category (186 of 411 cases) with the majority of these consisting of IgM and IgG (168 of 186 cases). It was suggested that IgM may combine with IgG bound to an unknown antigen. Brouet et al. (1974) reported similar results in a study of 86 cases of cryoglobulinemia - 50% of the cases were assigned to the third category with 85% of the cases involving IgM and IgG. Both authors found the remainder of the cases in group 3 to involve complexes containing IgM, IgG, and IgA. These studies demonstrated that a single immunoglobulin with the property of reversible cryoprecipitation is generally monoclonal. Based on experiments which demonstrate a role for IgM, and not IgG or IgA, in fat globule clustering and the electrophoretic pattern of alkylated, reduced IgM the cryoglobulins in milk fall into the third category.

The presence of cryoglobulins in serum is commonly associated with clinical symptoms characteristic of immunoproliferative and autoimmuno disorders. In approximately 10% of the cases, acute and severe symptoms necessitate emergency treatment with plasmapheresis and chemotherapy. More than half of the patients with high levels of single monoclonal cryoglobulins and approximately 15% of those with mixed polyclonal cryoglobulins are asymptomatic (Brouet et al., 1974). Cows fail to show symptoms of cryoglobulinemia.

It is of interest that Franzen (1971) found that the reduction of IgM inhibited creaming (Table 5) while Payens and Both (1970) reported that reducing agents did not inhibit IgM cryoaggregation. If the same functional groups are responsible for cryoaggregation of IgM and fat globule clustering, as suggested by Payens and Both, one would expect parallel results from the above experiments.

The following experiment was performed to examine the cryoglobulin character of isolated IgM. By studying conditions which influence cryoaggregation of IgM, factors which influence fat globule clustering should become apparent and better understood if the presently accepted theory is correct. A solution of IgM was concentrated to approximately 1.2 mg/ml and its turbidity monitored while temperature was varied from 10-37 C. The absorbance of IgG (1.1 mg/ml) and the void volume fraction (diluted

1:1 with SMU) was also monitored. Results are presented in Table 7. Absorbance of the IgM solution increased slightly when temperature was decreased, and decreased as temperature was increased. This was also observed for the void volume fraction. Absorbance of the IgG fraction did not change as the temperature was varied. During concentration of IgM at room temperature in preparation for this experiment, the absorbance at 280 nm increased approximately 2.6 fold while absorbance at 344 nm increased approximately 11 fold. The significant increase in absorbance at 344 nm which was observed during concentration and the slight increase recorded as a result of wide fluctuation in temperature indicate that a concentration and purity dependent aggregation may be the more significant factor promoting protein aggregation. As mentioned previously, this property is characteristic of IgM and IgA (Butler, 1969; Metzger, 1970). Based on the above discussion and experiment it was decided to further examine the role of IgM in creaming.

Quantitation of Immunoglobulin M Participating in Creaming

Experiments were performed to determine exactly how much of the IgM in milk participates in a single creaming. Milk was held quiescently overnight at 4 C and then

Table 7. The effect of temperature on the dispersion of IgM, IgG, and the void volume fraction.

Temperature (C)	Sample Absorbance at 344 nm		
	IgM	IgG	Void Volume
23.5	0.107	0.008	1.413
10.0	0.136	0.008	1.455
37.0	0.082	0.008	1.356
10.0	0.139	0.008	1.483
37.0	0.087	0.008	1.363

centrifuged for 10 min at 1000 x g. The skim milk layer, which will be referred to as gravity-separated skim milk, was examined for IgM. Approximately 93% of the IgM (0.0689 mg/ml) remained relative to a warm-separated control which contained 0.0743 mg/ml.

To determine if the amount of IgM associated with the milk fat globules (MFG) could be increased, known amounts of IgM were added to raw milk which was allowed to cream overnight. The amount of IgM remaining in the gravity-separated skim milk was determined as above. Results are presented in Table 8. The calculated IgM concentration is the amount in warm-separated skim milk (0.0513 mg/ml) plus the amount added to the original raw milk. Based on the ratio of calculated to experimental results or the % recovery, it was apparent that the added IgM, or some fraction of the added IgM, did not associate with the MFG. If the reaction merely involved aggregation of IgM and a non-specific precipitation onto the MFG, one would expect the added IgM or some fraction thereof to be associated with the MFG. These results are consistent with the hypothesis that there are a limited number of binding sites capable of reacting or interacting with IgM.

Table 8. Quantitation of IgM involved in creaming of milk supplemented with IgM.

IgM Concentration (mg/ml)		Ratio Calc./Exp.	% Recovery
Calculated	Experimental[a]		
0.0535	0.0539	0.993	121
0.0598	0.0623	0.960	130
0.0681	0.0691	0.980	106
0.0720	0.0752	0.957	116
0.0936	0.0938	0.998	101
0.115	0.115	1.00	100

[a]Average of duplicate analysis.

Examination of the Ability of Gravity-Separated Skim Milk to Support Creaming

Creaming of Recombined Milk-Washed Milk Fat Globules and Gravity-Separated Skim Milk

Washed MFG were added to gravity-separated skim milk to determine if the remaining IgM is capable of supporting creaming. As indicated in Table 9, the recombined milk failed to cream. This indicated that only a fraction of the IgM in milk is "active" or capable of promoting fat globule clustering, or that another component in the process is limiting and absent from gravity-separated skim milk. In order to examine these alternatives, the following experiments were performed.

Creaming of Recombined Milk - Washed Milk Fat Globules and Gravity-Separated Skim Milk from Raw Milk Supplemented with Immunoglobulin M

The following experiment was performed to determine if the IgM remaining in gravity-separated skim milk is "inactive". Raw milk was supplemented with IgM (0.25 mg/ml) and the resulting gravity-separated skim milk examined for its ability to support creaming upon addition of washed MFG. Since the added IgM will not be actively involved in creaming and will remain in the gravity-separated skim milk (Table 8), the gravity-separated skim milk would be expected to support creaming on the addition of washed MFG.

Table 9. Evaluation of gravity-separated skim milk to support creaming following the addition of washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	9.50	8.05	6.50	1.80	10.1
Gravity skim + MFG	0.10	0.10	0.10	0.50	60 +
Control[c]	8.00	6.55	4.00	1.60	11.0

[a]MFG were added to a final fat content of 3.5%.

[b]Average of duplicate analyses.

[c]Control consisted of raw milk which had creamed and been warmed to 55 C for 30 min with intermittent mixing.

Data presented in Table 10 indicate that the gravity-separated skim milk failed to support creaming, favoring the alternative hypothesis that gravity-separated skim milk fails to support creaming due to the absence of a limiting component other than IgM.

Effect of Immunoglobulin M Isolated from Gravity-Separated Skim Milk on Creaming of Heated Milk

To further examine the possibility that gravity-separated skim milk fails to support creaming due to the absence of another limiting component, the IgM remaining in gravity-separated skim milk was isolated according to the procedure outlined in Figure 1 and its ability to restore creaming to heated milk was examined. Results are presented in Table 11. "Inactive" refers to IgM isolated from gravity-separated skim milk which failed to support creaming and "total" refers to IgM isolated from warm-separated skim milk. Results are sufficiently similar to conclude that there is no significant difference in the ability of the two IgM fractions to restore creaming to heated milk, indicating further that another limiting component is absent from the gravity-separated skim milk. Since it is not the heat-labile component, one could speculate that it is the homogenization-labile component originally referred to by Samuelsson et al. (1954).

Table 10. Evaluation of gravity-separated skim milk from IgM-supplemented raw milk to support creaming following the addition of washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	1.75	1.55	1.50	1.55	7.1
+ IgM, 0.25 mg/ml	1.80	1.70	1.60	1.50	2.0
Gravity skim + MFG	0.00	0.00	0.20	0.95	60 +
Gravity skim (+IgM) + MFG	0.00	0.00	0.40	0.95	50.0

[a]Either one part of IgM-SMU solution or SMU was added to 3 parts of raw milk. MFG were added to a final fat content of 3.5%.

[b]Average of duplicate analyses.

Table 11. The effect of IgM isolated from warm-separated - "total" - or gravity-separated - "inactive" - skim milk on creaming of heated milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Heated milk	0.10	0.10	0.10	0.20	60 +
+ "Inactive" IgM, 0.05 mg/ml	0.35	0.55	0.65	0.85	14.3
+ "Inactive" IgM, 0.10 mg/ml	1.05	1.15	1.15	1.15	6.8
+ "Inactive" IgM, 0.20 mg/ml	1.50	1.45	1.35	1.10	4.9
+ "Total" IgM, 0.05 mg/ml	0.90	1.10	1.20	1.05	11.5
+ "Total" IgM, 0.10 mg/ml	1.45	1.45	1.45	1.10	6.5
+ "Total" IgM, 0.20 mg/ml	1.70	1.55	1.45	1.15	4.3
Raw milk	1.55	1.40	1.25	1.15	6.5

[a]Either one part of IgM-SMU solution or SMU was added to 4 parts of milk.

[b]Average of duplicate analyses.

Examination of the Role of the Antigen-Binding Properties of Immunoglobulin M in Creaming

Isolation of Fab Fragments

Tryptic digestion and gel filtration were employed to obtain Fab fragments from IgM. The gel filtration chromatogram of digested IgM-7S subunits is shown in Figure 8. Fractions 53-58 were collected as the Fab-containing fraction. The peak maximum corresponded to a molecular weight of 44,700. Sodium dodecyl sulfate polyacrylamide gel electropherograms (Figure 9) indicated the presence of low molecular weight contaminants. Species corresponding to intact heavy chains were not present. Light chain and Fd fragment molecular weights were 27,900 and 33,000, respectively.

The Effect of Fab Fragments on Creaming

If the IgM-MFG interaction is specific and involves the antigen-binding portion of the molecule, the addition of Fab fragments to milk should inhibit creaming by binding with reactive sites (antigens) without promoting cross-linking or clustering. When Fab fragments dispersed in SMU were added to raw milk (final concentration 0.10 mg/ml) the results shown in Table 12 were obtained. These data implicate a specific interaction in which the antigen-binding portion of the IgM molecule participates. An antigen-antibody interaction mode is suggested as opposed to an

Figure 8. Gel filtration chromatogram of alkylated, 2-mercaptoethanol-reduced, trypsin-treated IgM. A column of Sephacryl S-200 (2.6 cm x 90 cm) was employed.

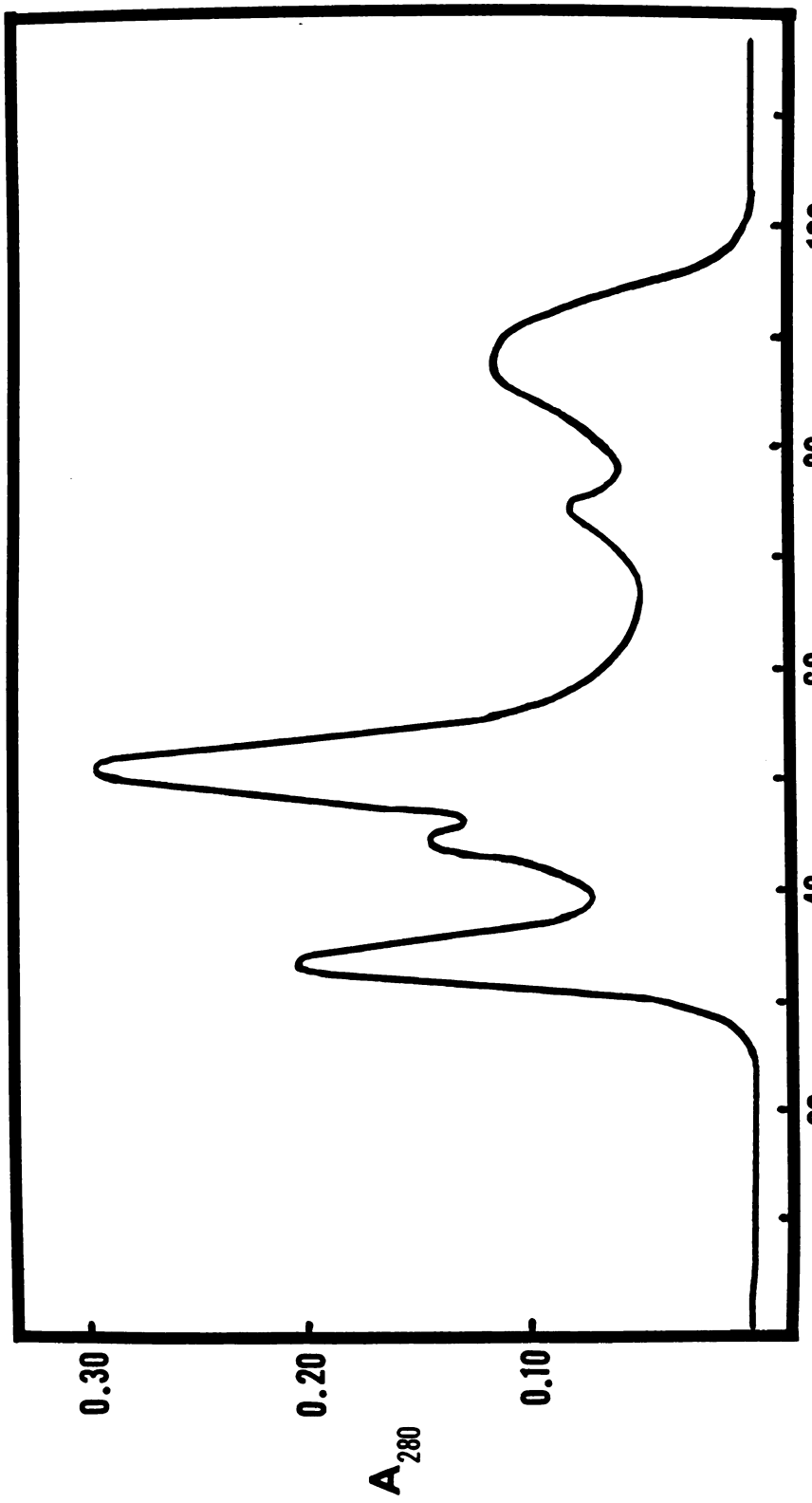


Figure 8

Figure 9. Sodium dodecyl sulfate polyacrylamide gel electropherograms of Fab fragments - pooled gel filtration fractions 55-58, see Figure 8 - (1 and 2) and IgM (3). Gels 1 and 3 contained 25 μg of protein and gel 2 contained 45 μg of protein.

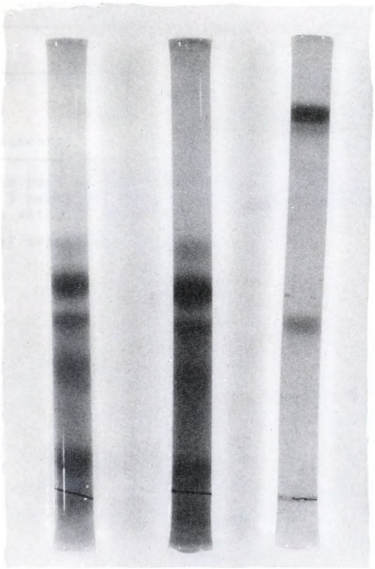


Figure 9

Table 12. The effect of IgM-derived Fab fragments on creaming of milk.

Sample[a]	Cluster Time[b] (min)
Raw milk	8.0
+Fab fragments	12.5

[a]Either one part of Fab fragment-SMU solution or SMU was added to 3 parts of milk. Final concentration of Fab fragments was 0.10 mg/ml.

[b]Average of triplicate analyses.

interaction involving the Fc portion and various effector functions.

Creaming in Simulated Milk Containing Synthetic Milk Fat Globules

If the reaction is specific, the MFG and in particular the milk fat globule membrane (MFGM) should play an active role in the fat globule clustering process, as opposed to a passive one in which the IgM merely precipitates onto its surface. To test this hypothesis, synthetic fat globules were prepared from butter oil and a stabilizing agent of either κ-casein, or an α_s-, β-casein-rich fraction, or a mixture of these two fractions. Synthetic fat globules and washed MFG were added separately to heated skim milk. IgM was added to one half of each sample and SMU to the other half which served as controls. Results in Table 13 show that all samples without IgM failed to form fat globule clusters or a cream layer. This was expected with the MFG and demonstrates that the synthetic fat globules do not undergo non-specific interaction or excessive gravitation. In the samples containing IgM, only the one with MFG formed normal fat globule clusters and a cream layer. This supports the concept that a specific interaction is involved in which both IgM and MFG play an active role.

Table 13. Creaming in reconstituted milk containing synthetic milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Heated skim					
+ MFG	0.00	0.00	0.00	0.20	60 +
+ α_s/β-FG	0.10	0.10	0.10	0.20	60 +
+ κ-FG	0.00	0.10	0.10	0.20	60 +
+ α_s/β/κ-FG	0.10	0.10	0.10	0.20	60 +
Heated skim + IgM					
+ MFG	0.50	0.80	0.95	1.20	18.0
+ α_s/β-FG	0.10	0.10	0.10	0.25	60 +
+ κ-FG	0.00	0.10	0.10	0.20	60 +
+ α_s/β/κ-FG	0.00	0.10	0.10	0.20	60 +

[a]Either one part of IgM-SMU solution or SMU and one part of fat globule solution were added to 4.5 parts of skim milk. MFG were added to a final fat content of 3.5%. α_s, β, and κ designate the appropriate casein fraction.

[b]Average of duplicate analyses.

Based on the ability of cows' milk euglobulins to cluster fat globules from goats' milk and Holland-Friesan and MRY cows' milk, Stadhouders and Hup (1970) concluded that the euglobulin-fat globule complex was not specific. Their conclusion fails to acknowledge the complexity, heterogeneity, and microheterogeneity of the carbohydrates on plasma membranes and hence milk fat globule membrane (MFGM) and the cross-reactivity of antibody molecules.

The Effect of Soluble Milk Fat Globule Membrane Proteins on Creaming

If an antigen-antibody reaction is involved, the agglutination reaction should be inhibited by the addition of soluble antigen - in this case soluble MFGM proteins. Soluble MFGM proteins were isolated according to the procedure of Herald and Brunner (1957). Their addition to raw milk did decrease cream volume and increase the time for visible clusters to be formed (Table 14). To verify if a specific effect was involved, ovalbumin was added to raw milk and found to be without effect. The results further support the notion that a specific interaction between IgM and the MFGM is involved in the cluster/creaming mechanism.

In two-dimensional double diffusion immuno-analyses of IgM versus soluble MFGM protein, precipitin lines failed to develop. This was attributed to either improper titers or

Table 14. The effect of soluble milk fat globule membrane (s-MFGM) proteins on creaming of milk.

Sample	Cream Volume[a] (ml/10 ml)				Cluster Time[a] (min)
	1 h	2 h	3 h	24 h	
Raw milk	1.50	1.40	1.50	1.40	8.5
+ s-MFGM, 2 mg/ml	1.10	1.15	1.30	1.40	12.8
+ s-MFGM, 6 mg/ml	0.70	1.00	1.05	1.10	15.7
+ s-MFGM, 10 mg/ml	0.20	0.85	0.90	1.10	20.7
+ Ovalbumin, 10 mg/ml	1.60	1.70	1.60	1.40	9.5

[a]Average of duplicate analyses.

the formation of soluble complexes. In instances where the antigen is bivalent or monovalent, precipitation may not occur due to the lack of formation of large aggregates or lattices.

The Effect of Milk Fat Globule Membrane Specific Anti-sera on Creaming of Heated Milk

To further verify that antibodies specific for MFGM antigens are involved, anti-serum against MFGM was prepared in rabbits and added to heated milk to examine its ability to restore creaming. Two different anti-serum preparations were employed. As a control, anti-serum against navy bean trypsin inhibitor (NBTI) was also prepared. The results in Table 15 show that only the anti-MFGM hyperimmune anti-sera restored normal creaming to heated milk. The limited clustering observed with anti-NBTI anti-serum after 31.5 min may be attributed to the presence of antibodies normally present in rabbit serum. These results support the belief that MFG clustering is the result of a specific interaction (antigen-antibody) between IgM and the MFGM.

In a study of the molecular mechanisms of milk secretion in which probe molecules were infused into the lactating gland of the goat via the teat canal, Patton et al. (1980) employed goat MFGM specific anti-serum. They observed that milk from the anti-serum-infused side of the udder showed

Table 15. The effect of anti-milk fat globule membrane (MFGM) and anti-navy bean trypsin inhibitor (NBTI) hyperimmune anti-sera on creaming of heated milk.

Sample	Cream Volume[a] (ml/10 ml)				Cluster Time[a] (min)
	1 h	2 h	3 h	24 h	
Heated milk	0.10	0.10	0.10	0.30	60 +
+ 0.05 ml anti-MFGM[b]/ml	10.10	1.60	1.60	1.45	11.5
+ 0.10 ml anti-MFGM[b]/ml					6.5
+ 0.05 ml anti-MFGM[c]/ml					12.7
+ 0.10 ml anti-MFGM[c]/ml					6.0
+ 0.10 ml anti-NBTI/ml	0.10	0.10	0.15	0.40	31.5
Raw milk	7.75	4.70	3.05	1.60	3.7

[a]Average of duplicate analyses.

[b]Anti-serum preparation 1.

[c]Anti-serum preparation 2.

extensive clustering of fat globules after storage for 12-24 h at 4 C. Milk from the non-infused side exhibited essentially no clustering. This observation is consistent with the suggestion of Jenness and Parkash (1971) that goats' milk creams poorly at low temperatures due to the lack of agglutinating euglobulins.

The Effect of Simple Sugars on Creaming

Since IgM is involved in fat globule clustering, and cows' milk generally creams throughout the lactation cycle, one may expect natural antibodies - or antibodies associated with histocompatability antigens or blood groups - to be involved. The isoagglutinins, which are predominantly IgM, occur with predictable regularity in all individuals without any apparent overt antigenic stimulation. They normally appear in serum 3 to 6 months after birth and remain throughout the life cycle. Their titer may vary throughout this period.

If natural antibodies are involved in the interaction, the antigen would be carbohydrate in nature. To test this possibility, hapten-inhibition studies, involving the addition of simple sugars to milk, were performed. The inhibition assay is essentially a competitive one in which the inhibitor competes with the native antigen on the fat globule surface for antibody molecules. The greater the

similarity between the added carbohydrate and the antigen, the greater the degree of cross-reactivity, and the greater the degree of creaming inhibition. When a number of simple sugars were tested, only glucosamine, galactosamine, and sialic acid were found to have an inhibitory effect (Table 16). In some milk samples sialic acid was found to completely inhibit creaming. The variability of response to the addition of sialic acid is a ramification of antibody heterogeneity. The complete and greater inhibition of creaming with sialic acid than with glucosamine or with galactosamine indicates sialic acid may be more closely related to the immunodominant group(s). The concentration of sugars required reflects the lack of identity with the MFGM antigen, but the notion of cross-reactivity does reflect the nature of the antigen.

That the antigen is carbohydrate in nature is supported by the observation that heat treatment does not destroy antigenic activity. Heated cream has been shown to cream as well as non-heated cream when added to raw skim milk (Dunkley and Sommer, 1944).

Fat Globule Clustering Interpreted in Light of Immunologic and Serologic Knowledge

It is interesting to speculate on the scenario drawn by the above experiments in light of information which has

Table 16. The effect of simple sugars on creaming of milk[a].

Sample	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	1.50	1.40	1.55	1.40	8.5
+ Sialic acid, 0.3 mg/ml	1.60	1.60	1.45	1.45	8.8
+ Sialic acid, 0.7 mg/ml	1.60	1.50	1.50	1.40	10.5
+ Sialic acid, 1.0 mg/ml	1.50	1.45	1.45	1.40	14.7
+ Sialic acid, 3.0 mg/ml	0.00	1.20	1.30	1.30	16.3
+ Glucosamine, 5.0 mg/ml	0.00	1.10	1.20	1.40	20.3
+ Galactosamine, 5.0 mg/ml	0.00	1.00	1.10	1.40	22.5

[a]The addition of lactose, glucose, mannose, galactose, xylose, fucose, N-acetylgalactosamine, and N-acetylglucosamine at a concentration of 10 mg/ml were without effect.

[b]Average of duplicate analyses.

been collected on the human and cattle immunologic and serologic systems. Cold agglutinins or antibodies more active (higher titer) in hemagglutination at lower temperatures are normally found in all fresh blood serum (Race and Sanger, 1968). Clinical symptoms are generally not present. Cold agglutinins are generally associated with the A, H, Le^a, P, I, Sp_1, M, and N antigens (Zmijewski and Fletcher, 1972). It is significant with respect to fat globule clustering that A and B antigen-associated antibodies have been detected in milk (Race and Sanger, 1968). The presence of antibodies associated with other antigens can therefore be expected. This could explain the specificity and temperature-dependence of the reaction between MFG and IgM.

The following information is presented in support of the conjecture that it is possible for the I-antigen and its associated IgM antibodies to be involved in MFG clustering. However in so doing, it should not be construed that the I-antigen is involved or that it is the only possible antigen involved. The I-antigen is widely distributed and considered a "public" antigen. It generally appears on the erythrocyte membrane in the first months of life, replacing the I-antigen observed at birth. Anti-I antibodies are of the IgM class and are essentially complete natural antibodies possessing an optimum activity at 4 C. After fixation on erythrocytes at 4 C they can be completely

eluted at 37 C. Because anti-I agglutinins occur in sera of individuals who also have the I-antigen, they can be considered autoantibodies.

I-active material (antigens) can be produced by the stepwise degradation of A and B substances. Thus, rather than being something separate, the I-antigens are internal to the A, B, H, Le^a, and Le^b determinants, and the I-gene exerts its influence prior to that of the ABH and Le genes (Race and Sanger, 1972; Goudemand and Delmas-Marsalet, 1975; Zmijewski and Fletcher, 1972).

Because blood group substances or antigens are present on nearly all tissues, it is expected that they are present on the apical section of the mammary gland secretory cell and hence on MFG. While possessing cell-type-specific antigens (Ceriani et al., 1977), numerous common complex carbohydrate species have been identified on the MFG (Glockner et al., 1976; Newman et al., 1976; Newman and Uhlenbruck, 1977; Farrar and Harrison, 1978). Complex carbohydrates corresponding to A, M, N, T_F, and T_n antigens have been identified. HLA-DR-like antigens have also been found on the MFG (Wiman et al., 1979). Due to the extensive microheterogeneity displayed by glycoproteins and glycolipids, the presence of carbohydrate moieties similar or identical with the I-antigen are possible.

Curtain (1969) found IgM cold agglutinins, mostly with anti-I specificity, in high incidence and titer in sera

from sheep, cattle, kangaroos, wallabies, wombats, and possums. Since all the cells tested from the various species possessed some I-antigen, the IgM species could be regarded as autoantibodies. Cattle erythrocytes had the lowest concentration of I-antigens, but their sera had the highest incidence and titers of cold agglutinins.

The presence of autoantibodies does not imply the presence of an autoimmune disorder. Since an antigen only induces an immune response when it is "foreign to circulation" or "foreign to antibody forming cells", anatomic location can prevent an antigen from being recognized as self. Tissue extracts, e.g., brain, kidney, testis, and crystalline lens of the eye, can induce an immune response when injected into animals of the same species from which they originated. This is especially true if the antigens occur intracellularly. The immunologic response to autologous, organ-specific antigens generally has no untoward consequences for the immunized animals (Milgrom, 1969; Kabat, 1976).

Wiener et al. (1956) suggested that cold hemagglutinins might be cross-reacting antibodies to antigenic determinants derived from organisms and substances in the environment, but requiring cooling to express their specificity towards the I-antigen. Curtain (1969) suggested that possibly the I-antigen, rather than anti-I, is inactive at 37 C. It is therefore not recognized as an autoantigen and

immunological tolerance to it and related configurations does not occur, enabling high titers of anti-I to be reached. If this postulation is correct, anti-I cold agglutinins are not autoantibodies, as the I-antigen would not exist at physiological temperature.

The Effect of Enzymic Treatment of Milk Fat Globules on Creaming

The above experiments indicate that the MFGM, and in particular its carbohydrate moieties, play an active role in fat globule clustering. One would therefore expect fat globule clustering to be effected if carbohydrate moieties are removed or modified enzymically. Isolation and fractionation of glycopeptides released with proteases could lead to the specific carbohydrate moiety or moieties involved through inhibition assays.

Results obtained using pronase, trypsin, neuraminidase, and mixed glycosidases are presented in Table 17. Treatment with mixed glycosidases, neuraminidase, or a mixture of these enzymes failed to significantly effect fat globule clustering. Neuraminidase treatment increased cluster time slightly while treatment with mixed glycosidases decreased cluster time slightly. Protease treatment significantly decreased cluster time. Trypsin treatment followed by mixed glycosidase treatment reduced cluster time to 0.5 min.

Table 17. The effect of enzymic treatment of milk fat globules on creaming of milk[a].

Milk Fat Globule Treatment[b]	Cream Volume[c] (ml/10 ml)				Cluster Time[c] (min)
	1 h	2 h	3 h	24 h	
Control	6.50	7.50	7.50	8.00	13.3
Trypsin	7.00	6.00	6.00	6.50	6.5
Pronase	3.75	3.75	4.50	9.00	6.0
Neuraminidase	6.00	6.00	6.50	6.20	16.0
Mixed glycosidases	6.00	6.40	6.00	6.35	10.8
Trypsin, neuraminidase	6.00	6.25	6.35	6.00	7.3
Trypsin, mixed glycosidases	7.15	6.75	6.60	6.50	0.5
Neuraminidase + mixed glycosidases	6.35	6.85	6.50	6.50	11.0

[a] In each case, milk fat globules added to heated skim milk failed to cream. Samples in which milk fat globules were treated with buffers not containing enzymes, produced results similar to the control.

[b] Milk fat globules were added to a final fat content of 3.5%.

[c] Average of duplicate analyses.

The failure of neuraminidase to increase cluster time to a greater extent than observed could be related to the enzyme's aglycon specificity or sialic acid accessibility. Tomich et al. (1976) demonstrated that gangliosides of the membrane are shielded from neuraminidase attack by membrane proteins. This could also be true for the mixed glycosidases. The slight decrease in cluster time may have been due to exposure of specific carbohydrate moieties. Proteases may have decreased cluster time through their effect on the primary (IgM binding to MFG) or secondary (MFG agglutination) phases of fat globule clustering (Discussed in the section Interpretation of the Effect of Environmental Factors on Creaming). Since all MFGM proteins, including several glycoproteins, are not equally susceptible to proteolysis (Mather and Keenan, 1975; Shimizu et al., 1979; Shimizu et al., 1980), cleavage of those which are susceptible may have exposed specific carbohydrate moieties. Gangliosides, which are located primarily on the environmental face, may also have been exposed (Tomich et al., 1976). Cleavage of sialoglycopeptides would effect the secondary phase of fat globule clustering by decreasing the zeta potential (Newman and Harrison, 1973; Harrison et al., 1975). This would facilitate agglutination by allowing MFG to approach one another in a closer relationship.

The Effect of Milk Fat Globule Membrane Gangliosides on Creaming

As alluded to in the above discussion, MFGM gangliosides contain complex carbohydrates which could participate in fat globule clustering. Erythrocyte blood group antigens are primarily associated with glycolipids (Kabat, 1976). To test the possibility that MFGM gangliosides participate in creaming, hapten inhibition assays were performed using a ganglioside fraction from MFGM (Table 18). The gangliosides failed to influence creaming when added to raw milk. This could be accounted for if they lacked the specific carbohydrate moieties involved in creaming or on the basis of their low effective concentration or inaccessibility to the reaction due to micellarization.

Confirmation of the Mertens and Samuelsson Effects

The experiments of Mertens (1932) and Samuelsson *et al.* (1954) contributed significantly to our understanding of the homogenization-induced destruction of creaming of milk. Mertens demonstrated that a size reduction of MFG was not sufficient to explain the phenomenon by showing that homogenized skim milk containing washed MFG also failed to cream. This experiment implicated a labile skim milk phase component. Samuelsson *et al.* showed that the agglutinin in

Table 18. The effect of milk fat globule membrane gangliosides on creaming of milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	10.0	10.0	9.25	5.55	5.0
+ gangliosides, 0.05 mM	10.0	10.0	9.50	5.50	5.0
+ gangliosides, 0.10 mM	10.0	10.0	9.50	6.15	5.2
+ gangliosides, 0.15 mM	10.0	10.0	9.50	5.75	5.0

[a]Either one part of ganglioside solution or distilled water was added to 4 parts of milk. Ganglioside concentrations are approximate.

[b]Average of duplicate analyses.

milk consisted of 2 components: heat- and homogenization-labile components. It was shown that both were necessary for normal creaming. The homogenization-labile component has not been identified. Payens (1964) was unable to detect any difference in the physical properties or clustering ability of euglobulin isolated from colostrum prior to and following homogenization. No proposed mechanism explaining fat globule clustering has prescribed a role to the homogenization-labile component.

Experimental results verifying the Mertens and Samuelsson effects are presented in Table 19. As expected, the samples containing only homogenized or heated skim milk failed to support creaming, while a mixture of both creamed to a limited extent on the addition of washed MFG. Creaming was less than that observed in the control sample. However, only one half of the usual concentrations of the heat- and homogenization-labile components were present.

Examination of the Role of κ-Casein in Homogenization-Induced Destruction of Creaming

Koops et al. (1966) demonstrated that when euglobulin was homogenized with κ-casein its ability to promote creaming was destroyed. They hypothesized that a euglobulin-κ-casein complex was formed, or that κ-casein was adsorbed to the MFG in such a manner as to prevent

Table 19. Confirmation of the Mertens and Samuelsson effects - creaming in heated skim milk, homogenized skim milk, or a mixture of heated skim milk and homogenized skim milk containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	1.90	1.55	1.50	1.35	7.5
Heated + MFG	0.00	0.00	0.20	0.25	60 +
Homogenized + MFG	0.00	0.00	0.10	0.10	60 +
Heated + homogenized + MFG	0.00	0.00	0.40	0.90	39.2

[a] Equal volumes of heated and homogenized skim milks were mixed. MFG were added to a final fat content of 3.5%.

[b] Average of duplicate analyses.

euglobulin bridging. Since κ-casein contains complex carbohydrate moieties (Whitney et al., 1976) which are similar to those found on the MFGM (Newman and Uhlenbruck, 1977), this theory was attractive. It was thought that homogenization may expose the carbohydrate moieties in such a manner as to allow reaction with IgM. However, experiments conducted with model systems of heated whole milk combined with one of the following: (a) homogenized κ-casein, (b) κ-casein, (c) homogenized IgM, (d) IgM, (e) homogenized (κ-casein + IgM), (f) κ-casein + IgM, (g) homogenized κ-casein + IgM, or (h) κ-casein + homogenized IgM, failed to support their observations. Substituting caseinomacropeptide or micellar casein for κ-casein or increasing homogenization pressure to 2500 psi failed to change the results obtained. Walstra (1980) has shown that homogenization of whey, either rennet or acid, inhibits the ability of the whey to support creaming and sheds doubt upon an IgM-κ-casein complex being formed during homogenization. The author did not propose an alternative mechanism of inactivation. Although whey was not a very good creaming medium (Discussed in the section Creaming in Model Systems - Simulated Whey Model Systems), these results were reproduced in this study (Table 20).

Table 20. Creaming in homogenized and non-homogenized acid and rennet wheys containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Acid whey + MFG	0.10	0.10	0.15	0.50	60 +
Homogenized acid whey + MFG	0.10	0.10	0.10	0.20	60 +
Rennet whey + MFG	0.10	0.15	0.20	0.60	60 +
Homogenized rennet whey + MFG	0.10	0.10	0.10	0.20	60 +

[a]MFG were added to a final fat content of 3.5%.

[b]Average of duplicate analyses.

Examination of the Participation of Skim Milk Membrane in Creaming

Because the reaction between IgM and the MFG was shown to be specific, IgM could react with carbohydrate-containing species other than MFG in milk. Two possible components are milk oligosaccharides - the soluble complex carbohydrates found in milk - and skim milk membrane (SMM) - much of which has an origin similar to the MFGM (Plantz et al., 1973; Kitchen, 1974; Patton and Keenan, 1975). Due to the low level of milk oligosaccharides in cows' milk (Blanc, 1979), their possible participation was not examined. The possible participation of SMM was examined.

Effect on Creaming of Homogenized Skim Milk Containing Washed Milk Fat Globules

The addition of SMM, isolated by ultracentrifugation or salt fractionation and gel filtration, to homogenized skim milk containing washed MFG produced the results reported in Table 21. The results clearly show that while IgM failed to restore creaming, SMM did restore creaming to homogenized skim milk containing washed MFG. A very low cluster time, very large clusters, and a deep cream layer were obtained. The results indicate that SMM is the homogenization-labile component. The different cluster times for the two SMM preparations may be due to (a) differences in quantity of SMM added to the milks (due to

Table 21. The effect of skim milk membrane (SMM) isolated by ultracentrifugation or salt fractionation and gel filtration on creaming of homogenized skim milk containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Homogenized skim + MFG	0.00	0.00	0.20	0.55	60 +
+ IgM, 0.10 mg/ml	0.00	0.00	0.30	0.75	60 +
+ SMM[c]	9.00	8.50	8.35	7.05	2.8
Homogenized skim + MFG	0.10	0.15	0.20	0.50	60 +
+ SMM[d]	10.0	10.0	1.30	1.65	13.1

[a]Samples were prepared by mixing 18.5 ml skim milk, 5 ml IgM-SMU solution, 4 ml SMM solution, and 2.5 ml washed MFG (3.5% final fat content). SMU was substituted for IgM and SMM when required.

[b]Average of duplicate analyses.

[c]SMM prepared by ultracentrifugation.

[d]SMM prepared by salt fractionation and gel filtration.

approximations and losses in preparation), (b) differences in IgM content of the milks employed, or (c) the relative distribution of components isolated by the two procedures.

Effect on Creaming of Heated Skim Milk Containing Washed Milk Fat Globules

While IgM did restore creaming to heated milk, SMM failed to do so (Table 22). This further demonstrates that SMM is distinct in function from the heat-labile IgM component.

Effect on Creaming of Gravity-Separated Skim Milk Containing Washed Milk Fat Globules

Based on the failure to support creaming despite the presence of active IgM, it was concluded that the failure of gravity-separated skim milk to support creaming was due to the absence of another limiting component. To determine if the limiting component is SMM, it was added to gravity-separated skim milk prior to creaming experiments. The results in Table 23 show that the addition of SMM did restore normal creaming to the gravity-separated skim milk. As expected, IgM failed to do so. These results further indicate that SMM is the limiting component in determining how many times a given skim milk will cream and probably also in the amount of IgM incorporated into the cream layer.

Table 22. The effect of skim milk membrane (SMM) isolated by ultracentrifugation on creaming of heated skim milk containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Heated skim + MFG	0.10	0.10	0.10	0.25	60 +
+ IgM, 0.08 mg/ml	1.50	1.60	1.80	1.20	14.1
+ SMM	0.10	0.10	0.10	0.30	60 +

[a]Samples were prepared by mixing 18.5 ml skim milk, 5 ml IgM-SMU solution, 4 ml SMM solution, and 2.5 ml washed MFG (3.5% final fat content). SMU was substituted for IgM and SMM when required.

[b]Average of duplicate analyses.

Table 23. The effect of skim milk membrane (SMM) isolated by ultracentrifugation or salt fractionation and gel filtration on creaming of gravity-separated skim milk containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Gravity skim + MFG	0.00	0.20	0.30	0.65	60 +
+ IgM, 0.10 mg/ml	0.00	0.35	0.60	1.00	28.5
+ SMM[c]	1.90	1.85	1.80	1.40	8.1
Gravity skim + MFG	0.10	0.40	0.50	0.70	31.7
+ SMM[d]	10.00	10.00	1.50	1.25	6.2

[a]Samples were prepared by mixing 18.5 ml skim milk, 5 ml IgM-SMU solution, 4 ml SMM solution, and 2.5 ml washed MFG (3.5% final fat content). SMU was substituted for IgM and SMM when required.

[b]Average of duplicate analyses.

[c]SMM prepared by ultracentrifugation.

[d]SMM prepared by salt fractionation and gel filtration.

Creaming in Mixtures of Gravity-Separated, Homogenized, and Heated Skim Milks Containing Washed Milk Fat Globules

To further examine or verify the presence or absence of IgM and SMM in various skim milk preparations, selected skim milks were mixed and examined for creaming ability (Table 24). As previously shown, gravity skim - devoid of or lacking functional SMM, but still containing active IgM - failed to cream normally upon the addition of washed MFG. A mixture of gravity-separated skim milk and homogenized skim milk - both of which are devoid of or lacking functional SMM, but containing active IgM - also failed to cream. When gravity-separated skim milk and heated skim milk - which is devoid of active IgM - are mixed, significantly improved results were obtained. Creaming was not returned to normal levels, but only one half of the usual concentrations of the heat- and homogenization-labile components were present. These results support the contention that gravity-separated and homogenized skim milks failed to support creaming due to the absence of the homogenization-labile SMM, whereas heated skim milk failed to support creaming due to the absence of the heat-labile IgM.

Effect on Creaming of Raw Milk

When SMM was added to raw milk the results presented in Table 24 were obtained. The cream layer depth was increased and cluster time decreased. This observation

Table 24. Creaming in mixtures of gravity-separated, homogenized, and heated skim milks containing washed milk fat globules (MFG).

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Gravity skim + MFG	0.00	0.10	0.20	0.65	60 +
+ Heated skim	0.45	0.75	0.85	1.40	20.4
+ Homogenized skim	0.00	0.15	0.25	0.70	60 +

[a]Washed MFG were added to a final fat content of 3.5%. Equal volumes of gravity and heated or homogenized skim milks were mixed.

[b]Average of duplicate analyses.

Table 25. The effect of skim milk membrane (SMM) isolated by ultracentrifugation on creaming of milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Raw milk	9.50	7.30	6.05	2.00	6.5
+ SMM, 5 ml/75 ml	10.0	10.0	9.00	7.00	6.8
+ SMM, 10 ml/75 ml	10.0	10.0	9.50	8.15	5.4
+ SMM, 15 ml/75 ml	10.0	10.0	9.80	9.30	4.2

[a]Whey prepared by ultracentrifugation was added to the control and other samples as required (15 ml total addition to each sample).

[b]Average of duplicate analyses.

further demonstrates that SMM does play an active role in the fat globule clustering process.

Effect on Creaming of Cold Milk

Cold-agitated milk fails to re-cream as exhaustively as milk pre-warmed prior to placement at reduced temperatures (Dunkley and Sommer, 1944). Mulder and Walstra (1974) suggested that cold skim fails to support creaming due to the formation of a few large IgM cryoaggregates with reduced ability to flocculate fat globules. An analogous situation can be postulated for cold milk which has previously creamed prior to agitation. However, since IgM has been shown to function as a cryoagglutinin as opposed to a cryoglobulin, this phenomenon was re-examined. IgM and SMM were added to homogeneous cold-agitated milk which was then allowed to re-cream (Table 26). The sample to which SMM was added creamed significantly better than the cold milk or cold milk supplemented with IgM. The pre-warmed milk creamed as expected. A possible reason for the poor re-creaming of cold milk and its improvement upon the addition of SMM will be discussed in the section, A Model Representative of Milk Fat Globule Clustering.

Isolation of Skim Milk Membrane from Warm- and Cold-Separated Skim Milks

Based on the above experiments, cold-separated skim milk should contain less SMM than warm-separated skim milk.

Table 26. The effect of skim milk membrane (SMM) isolated by ultracentrifugation on creaming of cold milk.

Sample	Cream Volume[a] (ml/10 ml)				Cluster Time[a] (min)
	1 h	2 h	3 h	24 h	
Cold milk[b,c]	1.50	1.50	1.30	1.20	15.0
+ IgM, 0.10 mg/ml[c]	1.50	1.50	1.35	1.25	15.2
+ SMM	2.30	2.00	1.80	1.55	6.5
Warm milk[d]	10.0	9.05	8.00	2.25	4.0

[a]Average of duplicate analyses.

[b]Raw milk was allowed to cream at 4 C overnight and then mixed by transferring between 2 containers. Two milliliters of either IgM-SMU solution or SMM solution at 37 C was then added to 18 ml of cold milk.

[c]Loosely packed cream layer and diffuse creamline. The skim milk was opaque indicating poor creaming.

[d]An aliquot of cold milk was warmed to 55 C for 30 min prior to analysis.

Relative quantities of SMM can be determined from the area of the void volume peaks in the Sepharose 4B chromatograms of warm and cold-separated skim milk (5,000 x g for 20 min) as presented in Figure 10. The cold-separated skim milk contained less SMM than the warm-separated skim milk. An area ratio of 0.62 was obtained. The fact that the SMM remaining in the cold-separated skim milk would not support creaming indicates that not all SMM is capable of participating or is required in creaming. The corollary experiment was performed to ascertain if the SMM content returned to the original level when cold milk is warmed prior to separation. An area ratio, relative to warm-separated skim milk, of 0.80 was obtained. Mechanical entrapment or incomplete elution from the MFG may have resulted in the lower than expected value. However, the trends support previously presented results.

Isolation of Skim Milk Membrane from Non-Homogenized and Homogenized Skim Milks and Model Systems

To acquire an insight into the inactivation of SMM by homogenization, SMM was isolated by gel filtration from homogenized (2500 psi) and non-homogenized skim milks and model systems - consisting of whole casein and SMM isolated from skim milk using gel filtration. A void volume peak area ratio (homogenized/non-homogenized) of 3.7 was obtained for the skim milk, whereas for the model system a

Figure 10. Gel filtration chromatograms of (A) warm-separated skim milk (2.67 ml sample) and (B) cold-separated skim milk (2.60 ml sample). A column of Sepharose 4B (2.6 cm x 35 cm) was employed.

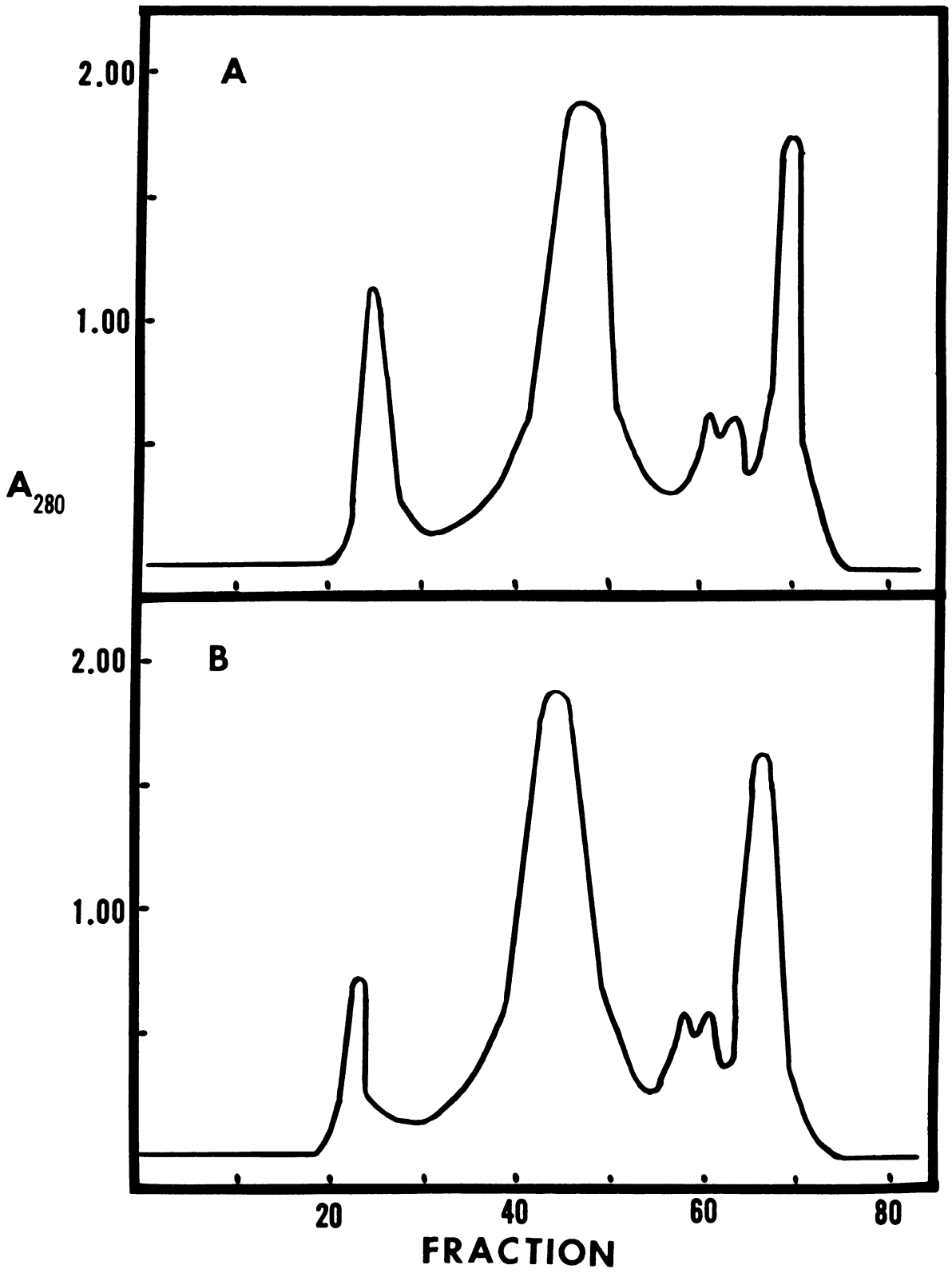


Figure 10

value of 1.2 was obtained. The increased turbidity of the skim milk void volume fraction significantly increased absorbance at 280 nm. The non-homogenized skim milk void volume fraction contained 86% as much protein as the homogenized sample. The non-homogenized SMM/casein sample void volume fraction contained 91% as much protein as the homogenized sample.

These few results do not allow a mechanism of SMM inactivation to be proposed. However, they do indicate that the material isolated as SMM does change upon homogenization. One can speculate that SMM is inactivated by (a) emulsification of lipoidal membrane particles by proteins in milk or (b) by a reduction in size of membrane particles. In the first case carbohydrate reaction sites may be shielded by adsorbed proteins, while in the second size reduction of particles may lead to a decrease in the number of reactive sites/particle and hence a reduced ability to act as bridging-agents and possibly even to acting as inhibitors of creaming (Discussed in the section, A Model Representative of Milk Fat Globule Clustering).

Effect of Replacing Skim Milk Membrane with Milk Fat Globule Membrane

Because SMM and MFGM have origins in common (Plantz et al., 1973; Kitchen, 1974; Patton and Keenan, 1975) it was suspected that MFGM obtained by churning washed MFG may

function in restoring creaming capacity to gravity-separated and homogenized skim milks in the same fashion as SMM. However, when added at various levels to whole milk, homogenized skim milk, or gravity-separated skim milk it had no effect. This behavior may have been a result of differences in reaction site (specific carbohydrate moieties) density or lack of physical availability of reactive sites due to different states of aggregation of vesiculation.

Creaming in Model Systems

Simulated Whey Model Systems

The results in Table 27 demonstrate that acid, rennet, and centrifuge whey poorly support creaming. The addition of SMM and to a lesser extent IgM improved their creaming capacity. In general, centrifuge whey was a better medium than rennet whey which was better than acid whey. When subjected to gel filtration, void volume peak area ratios (whey/skim milk) were: 0.0 for centrifuge whey, 0.23 for rennet whey, and 0.11 for acid whey. These ratios indicate that the wheys lack functional SMM as was anticipated for centrifuge whey. The low pH employed to precipitate casein probably results in SMM precipitation in acid whey (Brunner, 1974) and physical entrapment of the SMM in the casein clot may account for its decrease in rennet whey. Due to the

Table 27. Creaming in simulated whey model systems.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Acid whey + MFG	0.10	0.10	0.15	0.50	60 +
+ IgM	0.10	0.10	0.20	0.65	37.0
+ SMM	0.80	0.70	0.60	0.90	22.5
+ IgM + SMM	0.85	0.90	0.90	1.10	23.0
Rennet whey + MFG	0.10	0.15	0.20	0.60	60 +
+ IgM	0.10	0.20	0.20	0.80	31.3
+ SMM	1.00	0.70	0.85	0.80	21.3
+ IgM + SMM	1.10	1.00	0.95	1.20	18.0
Centrifuge whey + MFG	0.20	0.40	0.50	0.60	60 +
+ IgM	0.30	0.70	0.75	0.85	25.2
+ SMM	10.0	7.00	4.20	1.35	11.0
+ IgM + SMM	1.70	1.30	1.25	1.40	11.5

[a]Whey samples were adjusted to pH 6.6. Samples were prepared by mixing 21.0 ml whey, 2.5 ml IgM-SMU solution (0.10 mg/ml final concentration), 4 ml skim milk membrane (SMM) solution, and 2.5 ml washed milk fat globules (MFG) (3.5% final fat content).

[b]Average of duplicate analyses.

low SMM contents, MFG clustering in whey is primarily IgM mediated (Discussed in the section Creaming in Model Systems - Simulated Milk Ultrafiltrate Model Systems). The ranking of their capacity to support creaming is anti-parallel to their ionic salt content. Since the addition of salt to milk has been shown to decrease creaming (Dunkley and Sommer, 1944), this is probably a contributing factor. Dialysis of the various wheys versus SMU may equalize their capacity to support creaming.

Simulated Skim Milk Model Systems

The importance of SMM in creaming is further demonstrated by the results of Table 28. Simulated milk prepared from centrifuge whey, casein micelles, and washed MFG creamed better (characteristics more like the raw milk control) in the presence of than in the absence of SMM. The sample to which SMM was not added contained some SMM attributable to that co-pelleted with the casein micelles. However, the results indicate the importance of SMM in the "native" milk system with respect to fat globule clustering.

Simulated Milk Ultrafiltrate Model Systems

In order to ascertain the concerted and individual contributions of SMM and IgM to creaming, washed MFG were mixed with SMM, IgM, or a mixture of these two components in SMU and creaming capacity evaluated (Table 29). The results indicate that only the sample containing SMM and

Table 28. Creaming in a simulated skim milk model system.

Sample	Cream Volume[a] (ml/10 ml)				Cluster Time[a] (min)
	1 h	2 h	3 h	24 h	
Raw milk	9.80	9.50	9.50	7.80	5.8
Centrifuge whey + casein + MFG[b]	1.40	1.35	1.20	1.05	7.0
Centrifuge whey + casein + SMM + MFG[c]	9.30	9.50	8.30	4.85	5.5

[a]Average of duplicate analyses.

[b]Casein micelles prepared by ultracentrifugation were added to centrifuge whey after removal of the skim milk membrane (SMM) fluff layer. Washed milk fat globules (MFG) were added to a final fat content of 3.5%.

[c]The entire tube contents were mixed after centrifugation under conditions to prepare SMM.

Table 29. Creaming in a simulated milk ultrafiltrate model system.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
SMU + MFG	0.10	0.10	0.10	0.30	60 +
+ IgM[c]	0.25	0.40	0.50	1.15	13.3
+ SMM[c]	0.20	0.40	0.45	0.50	19.3
+ IgM + SMM	1.90	1.70	1.65	1.30	3.5

[a]Samples were prepared by mixing 17.5 ml SMU, 6 ml IgM-SMU solution (0.10 mg/ml final concentration), 4 ml skim milk membrane (SMM) solution, and 2.5 ml washed milk fat globules (MFG) (3.5% final fat content).

[b]Average of duplicate analyses.

[c]Very diffuse creamline and small clusters.

IgM displayed normal creaming. The sample with SMM creamed to a limited extent - probably due to the small amount of IgM present in the whey carrier. The sample of IgM creamed to a limited extent - indicating IgM has the ability to cluster MFG to a limited extent in the absence of SMM.

The results of this experiment are similar to those obtained by Gammack and Gupta (1970). The authors noted that lipoprotein particles isolated from milk augmented creaming. Although the particles were not recognized as SMM, they were probably SMM or some fraction thereof.

Interaction of Milk Fat Globules with Skim Milk Membrane

To determine if SMM interacts with MFG without promoting clustering, heated whole milk was washed 4 times with washed MFG at 4 C. After quiescent storage at 4 C for 6 h, the heated milk was centrifuged (1000 x g for 20 min) and the MFG-containing layer removed. Fresh washed MFG were added to the skim layer and the process repeated three times. Heated milk served as a control. Results are presented in Table 30. The addition of IgM alone produced sufficiently similar results in the heated milk and MFG-washed heated milk to conclude that SMM did not interact with MFG.

Table 30. The effect of IgM and skim milk membrane (SMM) on creaming of heated milk and milk fat globule (MFG)-washed heated milk.

Sample[a]	Cream Volume[b] (ml/10 ml)				Cluster Time[b] (min)
	1 h	2 h	3 h	24 h	
Heated milk	0.10	0.10	0.10	0.20	60 +
+ IgM	1.40	1.50	1.45	1.45	9.8
+ IgM + SMM	2.00	1.80	1.70	1.65	7.0
Heated MFG-washed milk	0.10	0.10	0.10	0.20	60 +
+ IgM	0.20	0.70	1.05	1.45	9.9
+ IgM + SMM	1.90	1.95	1.80	1.45	7.0

[a]Samples were prepared by mixing 20.5 ml of milk, 3 ml of IgM-SMU solution (0.10 mg/ml final concentration), 3 ml of SMM or centrifuge whey, and 3.5 ml of washed MFG (3.5% final fat content).

[b]Average of duplicate analyses.

Interaction of Immunoglobulin M with Skim Milk Membrane

The precipitation of the heat- and homogenization-labile components from whey stored at 4 C (Samuelsson et al., 1954) is taken as evidence of an IgM-SMM interaction. Perhaps this behavior can be considered as a typical antigen-antibody agglutination or precipitation reaction. The absence of a precipitate in the whey of poor-creaming milk (Dunkley and Sommer, 1944; Bottazzi and Zacconi, 1980) could be attributed to an antigen or antibody (pro-zone effect) excess.

Interpretation of the Effect of Environmental Factors on Creaming

Dunkley and Sommer (1944) demonstrated that salts are required for MFG clustering, but excessive levels result in inhibition of cluster formation. The ionic strength of milk is higher than optimum as evidenced by the fact that dilution (up to 50%) favors the formation of a deep cream layer and rapid formation of MFG clusters. Polyvalent cations added to raw milk decreased creaming to a greater extent than monovalent cations. Sodium citrate and disodium phosphate had very little influence on creaming. Creaming capacity of milk was reduced as the pH was varied from the original value of 6.6. These observations were supported

by the results of Rhee (1969).

It was expected that sodium citrate and disodium phosphate might decrease the creaming properties by increasing the zeta potential of the fat globules and that $CaCl_2$, $AlCl_3$ and $FeCl_3$ might improve creaming by decreasing the potential. An improvement in creaming was also expected as the pH was lowered, due to a decrease in zeta potential. Based on electrophoretic mobility measurements of fat globules and the above results, the author concluded that the charge on the MFG is not an important factor in the creaming of milk.

In examining the effect of environmental factors, i.e., ionic strength, pH, and dielectric constant, on fat globule clustering it is convenient to visualize the process as occurring in two steps: the first, corresponding to the uptake of IgM by MFG or SMM and, the second, the aggregation of the MFG by the formation of inter-MFG (or SMM) protein bonds (Pollack et al., 1964; Pollack, 1965; Zmijewski and Fletcher, 1972). The two stages probably do not occur in isolated steps once the first stage has progressed sufficiently for the second to begin. A fat globule cluster will result only if the MFG can approach each other closely enough for the intervening distance to be spanned by the IgM or IgM-SMM complex. This distance will vary with the effective length of the IgM molecule or the IgM-SMM complex and the position of the antigen receptor site on the MFG.

The increase in the effective length mediated by SMM may contribute to its augmentation of MFG clustering.

Whether or not aggregation occurs is dependent on the sum of the individual forces which drive the MFG together and those which drive them apart. In the absence of gravitational and centrifugal forces, the major aggregating force is through interfacial tension. With every surface there is associated a definite amount of energy (γ ergs/ cm^2). When clustering occurs, two surfaces each of area A cm^2 are lost and the amount of energy contained by these two surfaces ($2A\gamma$ ergs) becomes free. The MFG surface tension acts to produce clustering by reducing the free energy to a minimum (equilibrium). If the surface is extensively hydrated, the energy lost is not significant, but there is no reason to assume that the MFG surface is extensively hydrated or that the surface is influenced to a marked extent by the binding of IgM (Dunkley and Sommer, 1944; Payens, 1964; Payens et al., 1965). The electrostatic force of repulsion, which is dependent on the zeta potential, is the determining factor in maintaining two MFG a given distance apart. The zeta potential is proportional to the surface charge density and the thickness of the double layer. It is therefore influenced by changes in the suspending medium, i.e., ionic activity and dielectric constant, which may alter the double layer without a concomitant change in the surface charge. An increase in the ionic strength or

dielectric constant brings about a decrease in the double layer thickness and zeta potential. The term "critical zeta potential" has been used to define the voltage above which agglutination cannot occur. For human erythrocytes, the critical zeta potential is about 18-20 mV for saline agglutinins and 8-9 mV for incomplete Rh antibodies. For bovine, dog, and mouse erythrocytes which are seldom agglutinated, the critical zeta potential would be lower.

Factors influencing the first stage of clustering or the uptake of IgM by MFG or SMM are the ionic strength, dielectric constant, pH, and temperature of the suspending medium. There is generally an optimum salt concentration for antibody binding above which binding is decreased. As pH is varied from the optimum value of 6.7, antibody binding is reduced. The optimum dielectric constant depends on the ionic strength of the medium but a decreased value will reduce antibody binding. Antibody binding is favored by reduced temperatures.

From the above discussion, it is obvious that the influence of changes in pH and ionic strength are such that the first and second stages of MFG clustering are inversely affected. A decrease in pH or an increase in ionic strength is detrimental to the first stage but favors the second. The effect of factors on the two stages of fat globule clustering and cream layer formation are difficult to separate. Optimum fat globule clustering and creaming

occurs at conditions sub-optimal for the two individual stages which comprise the whole. This led Dunkley and Sommer (1944) to conclude that the charge on the MFG is not important in fat globule clustering. The decreased creaming capacity of milk on salt addition and decreased pH are probably due to the reduced IgM-MFG interaction, while the requirement of salts for clustering is probably associated with the zeta potential. A decreased zeta potential on removal of sialoglycopeptides probably contributed to the decreased clustering times observed with protease-treated MFG.

A Model Representative of Fat Globule Clustering

The most recent and generally accepted theory of fat globule clustering (Brunner, 1974; Mulder and Walstra, 1974) attributes agglutination to the precipitation of cryoglobulins (IgM) onto the fat globules, leading to clustering and rapid creaming of the large clusters (Payens, 1968; Payens and Both, 1970). The model can be pictorially presented as in Figure 11. Important features are: (a) the precipitation of aggregated IgM onto MFG, and (b) aggregates of IgM which precipitate in cold whey. The mechanism does account for the clustering of fat globules. The temperature prerequisite is attributed to the nature of

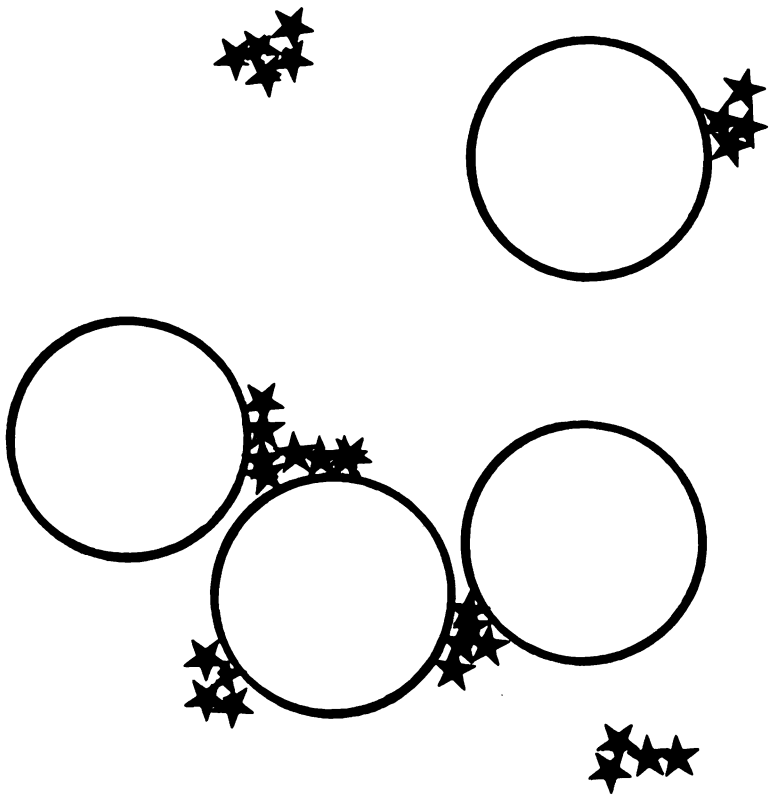


Figure 11. A model representing an interpretation of milk fat globule clustering as presented in the contemporary literature. Circles and stars represent milk fat globules and skim milk membrane, respectively.

the cryoglobulins. The effect of heat would be to denature the proteins which would no longer function as cryoglobulins. The proposed mechanism does not explain why aggregated IgM associates with MFG, as opposed to casein micelles. It also can not account for the Mertens and Samuelsson effects. One of the major problems appears to be that it fails to prescribe a role to the homogenization-labile component.

A model representative of fat globule clustering based on the experiments and discourse of this dissertation is presented in Figure 12. Significant features of the model are: (a) three components are involved - IgM, SMM, and MFG interacting through specific carbohydate moieties, (b) IgM interacts with MFG and SMM, not SMM with MFG, (c) IgM can cluster MFG to a limited extent, (d) SMM acts as a focal point or cross-linking agent which reduces the distance which MFG must approach to allow culstering, (e) IgM is free in solution, not cryoaggregated, and (f) IgM can react with SMM free in solution - yielding the precipitate in cold whey containing the heat- and homogenization-labile components.

As an application of the proposed model, Figure 13 contains a pictorial presentation of events proposed to occur in cold-agitated milk and cold-agitated milk supplemented with SMM. In the absence of added SMM, the free IgM in solution competes with IgM attached to MFG and

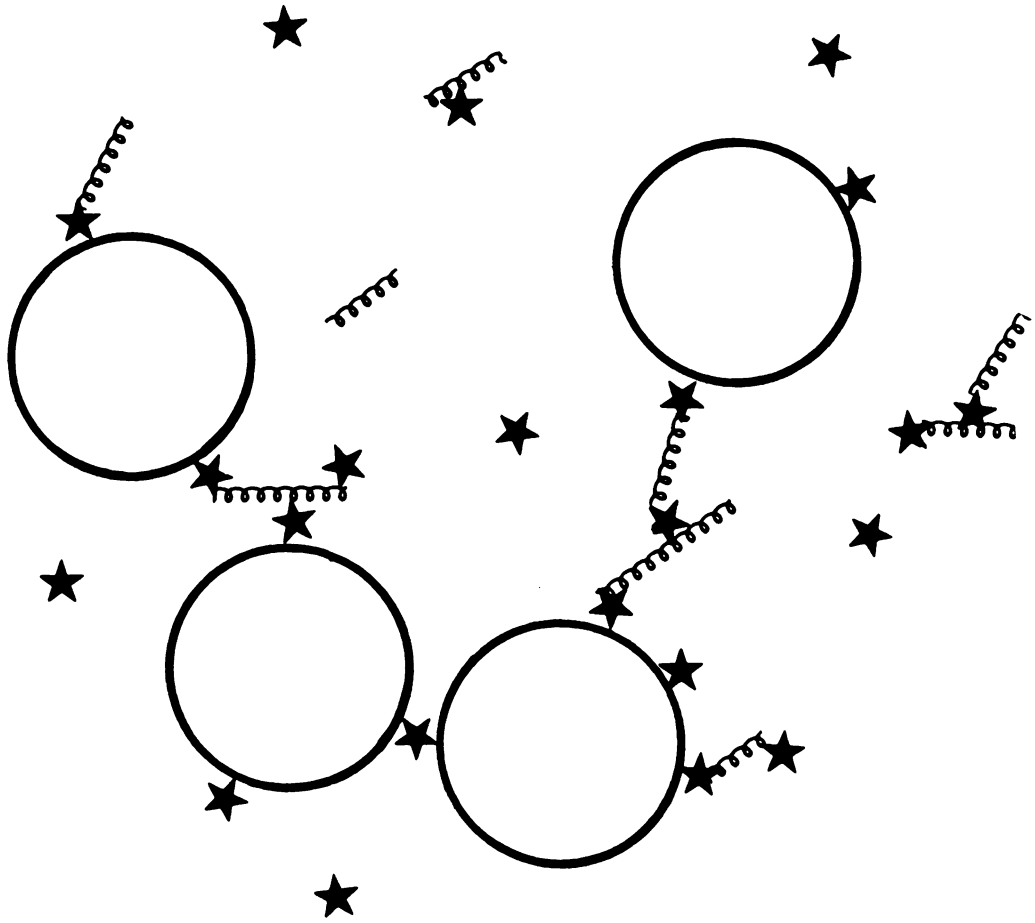


Figure 12. A proposed model of milk fat globule clustering based on the experiments and discourse of this dissertation. Circles, stars, and spiral segments represent milk fat globules, IgM, and skim milk membrane, respectively.

Figure 13. A pictorial presentation of events occurring in cold-agitated milk in the absence and presence of supplemental skim milk membrane. Circles, stars, and spiral segments represent milk fat globules, IgM, and skim milk membrane, respectively.

associated SMM for the free carbohydrate moieties on SMM and MFG. This results in fewer "bridges" and hence reduced clustering and poorer creaming. In the presence of added SMM, a situation similar to that in warm milk is obtained and numerous "bridges" are formed.

CONCLUSIONS

IgM was confirmed as the heat-labile component involved in fat globule clustering. Its participation was also demonstrated using raw milk. IgM was shown to function as a cryoagglutinin rather than as a cryoglobulin as previously indicated. The antigen is proposed to be carbohydrate in nature. SMM was identified as the homogenization-labile component. Although IgM can agglutinate MFG to a limited extent, normal creaming requires both components. Approximately 7% of the IgM in normal milk participates in a single creaming. The lower portion of the creamed milk (skim) fails to support creaming upon addition of washed MFG due to the absence of functional SMM. The presence of SMM in the lower portion indicates not all SMM is capable of participating in creaming. A theory of fat globule clustering consistent with the observed experimental results depicts IgM interacting in an antigen-antibody mode simultaneously with SMM and MFG through specific carbohydrate moieties.

RECOMMENDATIONS

Questions raised by this project and warranting further investigation are: (a) What is the structure of the MFG/SMM antigen(s) involved in fat globule clustering?, (b) What specific SMM component (origin) is involved?, (c) What is the mechanism of homogenization-inactivation of SMM?, (d) Exactly how does SMM augment creaming - is it merely through its effect on the distance which MFG must approach to allow clustering?, (e) Is all of the IgM in milk capable of participating in creaming?, (f) What does the temperature-dependence of the reaction depend upon - the nature of the antigen, antibody, or reaction conditions?, (g) How does salt addition and pH change effect the two separate stages of MFG clustering?, and (h) What is the structure of the SMM-IgM-MFG complex?

APPENDICES

Table A1. Chemicals used in this study and their sources.

Chemical	Company
Potassium chloride	Allied Chemical
Ammonium persulfate	J.T. Baker Chemical Co.
Potassium carbonate	
Acrylamide	Bio-Rad Laboratories, Inc.
Bisacrylamide	
Sodium dodecyl sulfate	
N-acetylgalactosamine	Calbiochem-Behring Corp.
N-acetylglucosamine	
Azocoll	
Dithiothreitol	
Fucose	
Galactose	
Glucose	
Lactose	
Mannose	
Xylose	
High vacuum grease	Dow Corning Corp.
Photo-Flo 200	Eastman Kodak
N,N,N',N'-Tetramethyl-ethylenediamine	
Ammonium sulfate	Fisher Scientific Co.
Bromophenol blue	
Calcium chloride	
Magnesium citrate	
Potassium citrate	
Potassium phosphate, monobasic	
Potassium sulfate	
Sodium citrate	
Folin-Ciocalteu phenol reagent	Harleco
Acetic acid	Mallinckrodt
Barbital	
Chloroform	
Citric acid	
Copper sulfate	
Diethyl ether	
Hydrochloric acid	
2-Mercaptoethanol	
Methanol	
Sodium bromide	
Sodium carbonate	
Sodium chloride	
Sodium hydroxide	
Sodium phosphate, monobasic	
Sodium phosphate, dibasic	
Sodium tartrate	
Sucrose	

Table A1. (continued)

Chemical	Company
Sulfuric acid	
Trichloroacetic acid	
Calcium carbonate	MCB Manufacturing Chemists
Magnesium carbonate	
Methyl green	
Thymol	
Polyvinylpyrrolidine	Oxford Laboratories
Iodoacetamide	Pfaltz and Bauer, Inc.
Blue dextran	Pharmacia Fine Chemicals
Low molecular weight SDS calibration kit	
N-acetylneuraminic acid	Sigma Chemical Co.
Bovine serum albumin	
Coomassie Brilliant Blue R250	
Dowex AG1-X8, 20-50 mesh	
Dowex 50W-X8, 20-50 mesh	
Galactosamine-HCl	
Glucosamine-HCl	
Myoglobin	
Ovalbumin	
Sodium azide	
Soybean trypsin inhibitor	
Tris (trishydroxymethylaminomethane)	
Glycine	U.S. Biochemical Corp.

Table A2. Equipment routinely used in this study.

Equipment	Company
Analytical balance, type 2463	Satorius Balances
Top-loading balance, type 3716	Satorius Balances
Top-loading balance, type K7T	Mettler Instrument Corp.
Camera, MP-3 Land Camera	Polaroid Corp.
Preparative centrifuge, model U type 277 head	International Equipment Co.
Preparative refrigerated centrifuge, model RC2-B, type SS-34 and GSA rotors	Sorvall Instruments
Preparative refrigerated ultracentrifuge, model L-2-65, type 21, 30, and 65 rotors	Beckman Instruments, Inc.
Conductivity meter	Industrial Instruments, Inc.
Visking dialysis tubing	Union Carbide Corp.
Disk milk separator, type LWA 205	Westfalia Separator
Hand-operated homogenizer	C.W. Logeman, Co.
Mechanical homogenizer, model C-8	C.W. Logeman, Co.
Lyophilizer	Laboratory-constructed, Dr. J.R. Brunner
Convection oven, model OV510	Blue M Electric Co.
Vacuum oven, no. 95050	Central Scientific Co.
pH meter, model 245	Instrumentation Laboratories Inc.
pH meter, model 12	Corning Scientific Instruments
Rotary shaker	Eberbach
Water bath, no. C6566	Precision Scientific Co.
Water bath, model RW-150	New Brunswick Scientific Co.

Table A3. Activities of glycosidases from Turbo cornutus as supplied by Miles Laboratories, Inc.

Glycosidase	Activity (units)*	Substrate**
α-Mannosidase	94	PNPG
β-Mannosidase	106	PNPG
α-Glucosidase	3.0	PNPG
β-Glucosidase	7.6	PNPG
α-Galactosidase	40	PNPG
β-Galactosidase	110	PNPG
α-L-Fucosidase	60	PG
α-D-Fucosidase	-	PNPG
β-Xylosidase	9.8	PNPG
α-N-Acetylglucosaminidase	2.2	PG
β-N-Acetylglucosaminidase	460	PNPT
α-N-Acetylgalactosaminidase	30	PG
β-N-Acetylgalactosaminidase	44	PG
β-L-Arabinosidase	-	PG
β-D-Arabinosidase	-	PG
Neuraminidase	-	SL

*One unit will release 1.0 μmole of p-nitrophenyl or phenyl per minute from glycoside at pH 4.0 at 37 C.

**PNPG: p-nitrophenyl glycoside, PG: phenyl glycoside, SL: sialyllactose.

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