ISOLATION AND CHARACTERIZATION OF BOVINE LACTEAL IMMUNOGLOBULINS

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
ROGER W. FRANZEN, JR.
1971

This is to certify that the

thesis entitled

ISOLATION AND CHARACTERIZATION
OF BOVINE LACTEAL IMMUNOGLOBULINS

presented by

ROGER W. FRANZEN, JR.

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Food Science & Human Nutrition

J. Robert Brunner
Major professor

Date 11/10/71

O-7639

ABSTRACT

ISOLATION AND CHARACTERIZATION OF BOVINE
LACTEAL IMMUNOGLOBULINS

By

Roger W. Franzen, Jr.

The immunoglobulins present in bovine milk are heterogeneous, high molecular weight proteins possessing antibody activity. Three antigenically distinct classes of bovine immunoglobulins have been described. They are designated IgM, IgA, and IgG, the latter being divided into two subclasses, IgG1 and IgG2. These proteins comprise between 1.4 and 2.8% of the skim milk proteins.

The purpose of this investigation was to determine which immunoglobulin was responsible for clustering milk fat globules and to better elucidate its mode of action in the creaming phenomenon. The immunoglobulins were isolated from whey by ammonium sulfate precipitation, then fractionated by preparative ultracentrifugation and Sepharose 6B column chromatography into enriched molecular size classes. The identification of components within fractions was accomplished with polyacrylamide disc gel electrophoresis and immunoelectrophoresis, as well as other physical and chemical procedures.

The 100S pellet was observed to be very opaque, and contained approximately 29% lipid and 12% carbohydrate. A 15S species of IgG1 appeared to be loosely associated with the major 190S component when this fraction was centrifuged in 0.15 M NaCl at 20°C. A similar

association was observed when this fraction was centrifuged at 4°C in a simulated milk salt buffer; however, the major component sedimented as a 150S species.

The eluate corresponding to the void peak of Sepharose 6B column chromatography, fraction 1, also exhibited some opacity. It contained approximately 73% lipid and 14% carbohydrate and was observed to be heterogeneous in sedimentation velocity determinations. A majority of the components in this fraction sedimented in the 20-70S range. A lipoprotein-like boundary gradient was observed when this fraction was centrifuged in 20°C 0.15 M NaCl. In 4°C simulated milk salt buffer, this lipoprotein gradient was not apparent.

Fraction 2 contained approximately 8.5% lipid and 10% carbohydrate and comprised principally IgM when analyzed by acid polyacrylamide disc gel electrophoresis and immunoelectrophoresis. The sedimentation velocity patterns of this fraction in 20°C 0.15 M NaCl showed a 20S major component which aggregated to a 25S component in 4°C simulated milk salt buffer.

Fraction 3 was lipid free and contained 2.9% carbohydrate. Immunoelectrophoresis indicated that this fraction consisted of IgG; however, a minor quantity of IgA was also present. The sedimentation velocity studies revealed that a 7.1S species predominated in 0.15 M NaCl at 20°C, which aggregated to an 8.7S species in 4°C simulated milk salt buffer.

Serine was found to be the most abundant amino acid in all fractions. The fractions were all mildly acidic and contained more hydrophobic than hydrophilic residues.

A qualitative lipid analysis indicated that cholesterol and sphingomyelins make up a majority of the lipids present in the fractions.

When a heat-inactivated, recombined creaming system was used to evaluate the creaming ability of the unheated immunoglobulin fractions, only fraction 2 exhibited a normal creaming function. This fraction contained mainly IgM. When reduced to monomers with 2-mercaptoethanol, its creaming ability was destroyed. The cryoaggregation of IgM from a 20S to a 25S molecule in the presence of simulated milk salt buffer was postulated as being of fundamental importance to mechanisms explaining the creaming phenomenon.

ISOLATION AND CHARACTERIZATION OF BOVINE LACTEAL IMMUNOGLOBULINS

By

Roger W. Franzen, Jr.

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1971

DEDICATION

This manuscript is dedicated to the memory
of my father,

Roger W. Franzen, Sr.

April 30, 1920-April 8, 1969

ACKNOWLEDGMENTS

The author wishes to express his profound appreciation to Dr. J. R. Brunner for his most valuable advice and never-ending patience, understanding and encouragement throughout the course of this study.

Grateful appreciation is also expressed to the other members of his guidance committee, Dr. L. R. Dugan, Dr. H. A. Lillevik, Dr. P. Markakis, and Dr. A. M. Pearson, for their helpful suggestions.

Special thanks are also extended to Dr. J. E. Butler (USDA) for providing his excellent disc gel electrophoretic technique as well as anti-sera which were invaluable to this study. The author is also indebted to Miss Ursula Koch for providing technical assistance with the amino acid and analytical ultracentrifugation analyses used in this study.

Grateful acknowledgment is also due Dr. B. S. Schweigert and Dr. G. A. Leveille for providing the research facilities and to the Food and Drug Administration, Grant No. FD00210-04 BAC for providing the funds necessary for this research.

Finally, the author wishes to express most grateful thanks to his wife, Linda, for her steadfast love and devotion, as well as her valuable assistance throughout his graduate program and in the preparation of this manuscript.

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INTRODUCTION

The immunoglobulins present in bovine milk are described as heterogeneous, high molecular weight proteins possessing antibody activity. They are usually isolated from the whey portion of milk as a minor fraction and are also present in blood serum and other milk secretions. Three antigenically distinct classes of bovine immunoglobulins have been described. They are designated IgM, IgA, and IgG, the latter being divided into two subclasses, IgG1 and IgG2.

In the earlier dairy science literature, the immune proteins were isolated in the "lactoglobulin" fraction which is classically defined as that portion of the whey proteins precipitated by saturation with magnesium sulfate, or by half-saturation with ammonium sulfate. Upon exhaustive dialysis this fraction separates into two components; a precipitate called euglobulin and a protein remaining in solution called pseudoglobulin. The euglobulin has recently been shown to contain IgA, IgM, IgG2 and electrophoretically slower IgG1 globulins, while the pseudoglobulin consists mainly of IgG1 and secretory IgA.

Previous studies have indicated that the euglobulin fraction is very active in promoting the fat globules in milk to cluster, which is preliminary to the creaming phenomenon observed in milk. However, this fraction, as described previously, is made up of different types of immunoglobulins all of which are heterogeneous and exhibit different physicochemical properties. A limited amount of information is available

on which immunoglobulin is responsible for creaming and even less is available concerning how it functions in the creaming phenomenon.

This study was undertaken to identify which of the bovine immunoglobulins is responsible for the clustering and creaming of milk fat globules. The immunoglobulins were isolated by classical methods and were then fractionated and enriched, based on molecular size, into their respective classes. The identification of components within fractions was accomplished with polyacrylamide disc gel electrophoresis and immunoelectrophoresis, as well as other physical and chemical procedures.

Sedimentation velocity studies were performed on the isolated fractions and the information obtained was considered pertinent to mechanisms explaining the creaming phenomenon.

Creaming studies were performed in natural as well as model systems to better understand the role played by immunoglobulins in the creaming phenomenon.

REVIEW OF LITERATURE

Immunoglobulins

The term immunoglobulins when used in reference to milk proteins describes a group of heterogeneous, large molecular weight proteins, sharing common physicochemical properties and antigenic determinants. The third revision of the Nomenclature of the Proteins of Cows Milk (Rose *et al.*, 1970) recommends the adoption of this term to update the earlier dairy science literature and to be consistent with the nomenclature of more extensively studied species in accord with the World Health Organization Report (1964). The term immunoglobulin therefore would replace terms like "immune lactoglobulin", "gamma globulin", "euglobulin", "pseudoglobulin", and "T-globulin", terminology used in earlier dairy science literature.

Butler (1969) states that three antigenically distinct classes of bovine immunoglobulins have been described. They are designated IgM, IgA and IgG, the latter being divided into two subclasses, IgG1 and IgG2. All have been found to be present in serum as well as the lacteal secretions. Murphy *et al.* (1964) employed physicochemical as well as immunochemical methods to demonstrate that the three immunoglobulins were present in milk during lactation, dry secretion and colostrum synthesis.

Butler (1969) states that immunoglobulins are found in the serum and other body fluids of animals and possess γ- or slow β-electrophoretic

mobility, and that these include all molecules with antibody activity, as well as other chemically related normal or pathological proteins. He further reports that all immunoglobulins appear to be either monomers or polymers of a four-chain molecule consisting of two light polypeptide chains (L-chains:20,000 molecular weight) and two heavy polypeptide chains (H-chains) with molecular weights varying from 50,000 to 70,000 daltons for the different immunoglobulin classes.

Carpenter (1965) states that the principal immunoglobulin of normal serum is a 7S molecule with a molecular weight of approximately 160,000. He also reports that most animal species contain a small amount of macroglobulin in the 17S to 20S class, with a molecular weight of 900,000 to 1,000,000. Traces of 28S to 44S components are also found. The macroglobulins appear to be polymers held together by disulfide bridges, but they are not simple polymers of 7S globulin. They differ notably in carbohydrate content; approximately ten per cent for macroglobulins and about 2.5% for the 7S immunoglobulins.

Physicochemical Properties of the Three Bovine Immunoglobulin Classes[a]

IgM is a macroglobulin with a sedimentation constant of 19S that contains approximately 12% carbohydrate. It is reported to represent between 0.1 to 0.2% of the protein present in skim milk and has a molecular weight approaching 1,000,000. This protein is eluted in the void volume peak of Sephadex G-200 when the protein fraction from whey, insoluble in 33% ammonium sulfate, is used as the starting material. When the same starting material is fractionated on acid-disc electrophoresis at

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[a]From Rose *et al.* (1970) when specific reference not indicated.

pH 4.3, the IgM present does not enter the separating gel but forms a dense band at the stacking gel-separating gel interface. IgM is thought to be a pentamer of the basic four chain immunoglobulin molecule which loses its antibody activity and is reduced to a monomer in the presence of 2-mercaptoethanol.

Murphy *et al.* (1964) suggest that the length of the immunoelectrophoretic arc for IgM indicates the presence of a continuum of molecules differing in net charge in a manner similar to the spectrum of 7S gamma globulin molecules. Murphy *et al.* (1965) found bovine serum IgM to have properties quite similar to those of the analogous protein in human serum. This was determined by comparing results from gel filtration, immunoelectrophoresis, anion exchange chromatography, ultracentrifugation and reduction with mercaptoethanol.

The antigenically distinct immunoglobulin IgA is reported to represent 0.05 to 0.10% of the protein present in bovine skim milk. It has a molecular weight in the range of 300,000 to 500,000 and often exists as a dimer of the basic four polypeptide immunoglobulin structure. The carbohydrate content of this molecule is between 8 and 9%. Jenness *et al.* (1965) report that it is sensitive to 2-mercaptoethanol and sediments as a 12S molecule.

Lacteal IgA is eluted between the IgM and IgG peaks during Sephadex G-200 fractionation of whey. Groves and Gordon (1967) reported the isolation of glycoprotein-a from cows milk (48,000 daltons) and was later confirmed by Butler *et al.* (1968) to be present in a free form and also bound to lacteal IgA. The bound form is probably analogous to the "secretory IgA", described for other species. Porter (1969) found that IgA in sow colostrum was present in many polymeric forms

since it appeared in gel filtration fractions over a wide molecular weight range. He states that the secretory IgA in humans has a molecular weight of 390,000 compared with 170,000 for serum IgA. He also postulated that in the sow the high molecular weight form might be synthesized in the mammary gland while the lower molecular weight components were transferred directly from the serum.

Of the immunoglobulins present in bovine milk, IgG is by far the most abundant class. IgG1 represents one to two per cent while IgG2 comprises approximately 0.2 to 0.5% of the skim milk protein. These molecules have a sedimentation coefficient of approximately 7S. Smith *et al.* (1946) and Nolan and Smith (1962) reported a carbohydrate content of between 2 and 4%. Murphy *et al.* (1965) separated IgG into two subclasses by chromatographic elution positions, electrophoretic migration rates and biological activities (complement fixation). They characterized these immunoglobulins by anion exchange chromatography, immunoelectrophoresis, zone electrophoresis, ultracentrifugation and analysis of the products of papain digestion. They concluded that their properties were similar to those of analogous components in human serum.

The more basic IgG molecules are the IgG2 subclass of immunoglobulins which have a mean $S_{20,w}$ coefficient of 6.6. These molecules migrate most rapidly toward the cathodic electrode during electrophoresis at pH 4.3 in polyacrylamide disc gels. The IgG2 immunoglobulins are not retained on DEAE-Sephadex in low ionic strength environment at pH 8.0 and thus are eluted in the breakthrough peak (Murphy *et al.*, 1965).

The subclass IgG1 consists of the less basic molecules which are eluted at higher ionic strength than the IgG2 fraction on DEAE-Sephadex. They also appear more heterogeneous on immunoelectrophoresis than the IgG2 subclass. IgG1 has a mean $S_{20,w}$ of 6.3.

Rose *et al.* (1970) report that the two subclasses of IgG can be correlated with the early preparations of Smith (1948) in the following manner. Smith's pseudoglobulin fractions contain mostly IgG1 and secretory IgA, while his euglobulin fraction consists of IgG2-like globulins, slower IgG1 globulins, IgA and IgM.

Isolation of Immunoglobulins

Carpenter (1965) discusses the three classical methods of fractionation that have been employed for many years in the isolation of serum proteins. These methods utilize the solubility characteristics of the serum proteins in the (1) presence of neutral salts such as sodium, ammonium and magnesium sulfate; (2) in the presence or absence of electrolyte; and (3) in the presence of cold alcohol at various hydrogen ion concentrations.

Crowther and Raistrick (1916) precipitated milk globulins with magnesium sulfate and further separated these proteins into water insoluble euglobulin and water soluble pseudoglobulin by means of exhaustive dialysis.

Howe (1921, 1922) employed sodium sulfate to fractionate milk proteins in an analogous procedure to that used for blood protein fractionation. He found that euglobulin was precipitated at up to 14.2% salt at 34°C and that pseudoglobulin I and II were precipitated at between 14.2-18.4% and 18.4-21.5% salt, respectively.

Smith (1946) stated that classical methods such as those of Crowther and Raistrick (1916), using repeated precipitation with half-saturated ammonium sulfate or saturated magnesium sulfate, yielded preparations which show complex electrophoretic patterns. He described a method for preparing electrophoretically homogeneous immunoglobulin by a stepwise

fractionation with ammonium sulfate. The crude globulin was precipitated from pH 6.5 whey by the addition of solid ammonium sulfate to 0.5 saturation. This fraction was redissolved at about 3% protein concentration, the pH was adjusted to 4.6, and ammonium sulfate added to 0.25 saturation. After removing the precipitate by centrifugation, the globulins were filtered and the immune proteins were precipitated from the filtrate by adjusting it to 0.4 saturation with ammonium sulfate at pH 6.0. This fraction which contained 80% of the immune proteins, was reworked by dissolving in water at 1°C, adjusting to pH 4.5, and removing the insoluble residue by filtration. The filtrate was brought to 0.3 saturation with solid ammonium sulfate. The supernatant was adjusted to pH 6.0 and 0.4 saturation with ammonium sulfate and the precipitate collected. Both immune globulin fractions were dialyzed separately against distilled water at 2°C and resolved into water-soluble "pseudoglobulins" and water-insoluble "euglobulin" fractions. The last precipitate was electrophoretically homogeneous in moving boundary electrophoresis.

Creaming

When normal, freshly drawn cows milk is chilled under quiescent conditions, the fat phase of the milk rises at a rapid rate and packs into a relatively loose lipid-rich layer. This phenomenon is known as creaming and usually occurs within one hour after optimum conditions have been attained. Troy and Sharp (1928) estimated the rate of rise of fat globules from Stoke's law, concluding that if the fat globules rose individually approximately fifty hours would be required before a cream layer was attained. These workers also measured the rate at which clusters of fat globules rose and concluded that clustering accounted for the rapid formation of the cream layer. The mechanism that is

involved in the clustering of fat globules is still poorly delineated; thus it was with this fact in mind that the present study was undertaken.

In 1899 Babcock observed that the fat globules in freshly drawn cows milk tended to aggregate and form clusters. His "coagulated fibrin" theory explaining this occurrence was later shown to have been an artifact. However, his recognition of the significance of fat globule clustering led to further investigations and the formulation of numerous theories to explain the phenomenon.

Among these early theories, which were extensively reviewed by Dunkley and Sommer (1944) were: (a) gravitation of fat globules; (b) electrokinetic potential of fat globules; (c) fat-serum interfacial tension; (d) stickiness and state of hydration of the adsorbed membrane; and (e) fat clustering considered as an agglutination process. Dunkley and Sommer concluded, from experimentation designed to test the above theories, 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) that 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) that the interfacial tension at the fat-serum interface does not promote creaming; (d) that evidence concerning the importance of hydration of the adsorbed membrane on the fat globules was not sufficient to justify drawing a definite conclusion regarding the significance of this factor. However, these workers isolated a protein from cold-separated cream which had euglobulin characteristics and promoted creaming. Sharp and Krukovsky (1939) also isolated a similar protein and considered the clustering of fat globules as an agglutination process. 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) also compared fat globule clustering with bacterial agglutination, citing their many similarities. Both processes involved 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 affected by a specific polar adsorption of the euglobulin; (b) an aggregation of fat globules resulting from the adsorption of a single euglobulin molecule, jointly by two fat globules; (c) maintenance of the surface potential of the fat globules below the critical level by the presence of salts.

Samuelsson *et al.* (1954) observed that the agglutinin responsible for normal creaming in milk was separated at 2°C as a yellowish powder from rennet whey which had been heated to 60°C. The agglutinin formed opalescent solutions in warm whey or water and consisted of two components, one inactivated by homogenization and the other inactivated by heating in water, whey or milk to 70-75°C for 15 seconds. 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.

Kenyon and Jenness (1958) substantiated the agglutinin inactivation work of Samuelsson *et al.* (1954) and also reported that the addition of 15 µg of colostral euglobulin per 100 ml of solution restored the

creaming ability of heated skim milk but not of homogenized skim milk.

Payens *et al.* (1965) performed creaming experiments using ^{131}I-labeled euglobulin from bovine colostrum. They were able to show that euglobulin is definitely adsorbed to the fat globule membrane, and that various amounts of β-lactoglobulin and casein are also adsorbed. Their studies at various temperatures showed that adsorption is temperature dependent with a considerable decrease at 45°C and a much greater adsorption at 5° and 10°C. They also demonstrated that at surface concentrations of about 2 mg of euglobulin per gram of fat, clustering time became constant. These results led them to the postulation that agglutination is brought about by the formation of euglobulin bridges between the fat globules with euglobulin peptide chains projecting into the plasma phase.

Koops *et al.* (1966) reported that low homogenization pressures, i.e., 10 kg/cm^2, significantly decreased the creaming ability of milk reconstituted from homogenized skim milk and unhomogenized cream (separated at 45°C). They suggested that denaturation of the euglobulin at these low pressures would be very unlikely. Instead, the results of these experiments indicated that casein micelles, particularly k-casein, were the inhibitory agents of clustering and creaming. They found that euglobulin was still adsorbed to the fat globule surface after homogenization and theorized that (a) homogenization caused euglobulin and k-casein to complex and that this complex was capable of adsorbing onto the fat surface but unable to effect clustering; or that (b) homogenization induces the adsorption of k-casein which would screen off the clustering sites of the adsorbed euglobulin.

Stadhouders and Hup (1970) found that Smith's (1946) euglobulin F isolated from colostrum not only contained the active agglutinin responsible for clustering and creaming but also an agglutinin for many bacterial species as well as one to attach these bacteria to fat globules. They reported that all three agglutinins were different. The cryoglobulin fraction, separated after aggregation at low temperature, was found to be a minor fraction (≈8%) which appeared to contain the antibodies responsible for agglutinating the fat globules as well as those which attach the bacteria to the fat globules. The antibodies which agglutinate the bacteria were found to remain completely in the non-cryoglobulin fraction.

Gammack and Gupta (1970) concentrated the active clustering agent from milk by separating the cream at low temperatures and desorbing the adsorbed material from the fat globules into a small volume of aqueous phase. After ultracentrifugation of this aqueous phase, 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. On warming, it redispersed. Further, the euglobulin fraction from colostrum also exhibited the cryoprecipitation behavior, but the precipitate from the milk preparation 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 aqueous phase obtained from milk was fractionated by gel filtration on agarose, the clustering activity was localized in a high particle weight fraction, i.e., 1×10^6, which contained lipoprotein particles. However, the isolated

lipoprotein particles themselves showed no clustering activity. Then, they separated colostral euglobulin by gel filtration on Sephadex G-150 at 50°C and found that IgG had no clustering activity with or without added lipoprotein particles. The fraction containing IgM and IgA showed some activity with a slow formation of cream line, but, on addition of lipoprotein particles, clustering activity was markedly enhanced and cream line formation was rapid. They further state that while protein molecules such as those in euglobulin preparations from colostrum promote clustering of fat globules these are not the most effective clustering agents in milk. Lipoprotein particles which exist in the aqueous phase augment the clustering effect of certain immune protein fractions such as those present in euglobulin preparations. They concluded that certain immune proteins as well as lipoprotein particles have an affinity for each other as well as for sites on the fat globule surface to which they adsorb as a preliminary step to cluster formation.

EXPERIMENTAL

Apparatus and Equipment

The milk used in this study was collected in five- or ten-gallon stainless steel cans and separated with a DeLaval disc-type separator (Model 518). Stainless steel, plastic or Pyrex containers were used in performing all experiments. A Beckman Model 115 or an Instrumentation Laboratory Model 245 pH meter, equipped with glass electrodes, was used to measure pH values. For weighings, a top-loading, direct-reading Mettler Type K-7 or a Sartorius series 2400 balance were used.

Low-speed centrifugations were performed with an International clinical centrifuge or a Sorvall super-speed centrifuge with a type SS-1 rotor. Intermediate-speed centrifugations were performed with a Sorvall RC2-B refrigerated centrifuge using type GSA rotor. For high-speed centrifugation, a Beckman Model L-65 refrigerated preparative ultracentrifuge, equipped with type 30 fixed-angle rotor was used.

Sephadex laboratory columns, Type K25/45, equipped with up-flow adaptors and cooling jackets, were used for packing the supporting materials. The eluates from the columns were monitored at 254 nm with a recording ultraviolet analyzer manufactured by Instrumentation Specialties Company, Inc.

Protein solutions were dried from the frozen state on a laboratory-constructed lyophilizer. Lyophilized protein samples as well as some chemicals were dried in a vacuum obtained with a Cenco Hyvac 2 vacuum pump and stored over P_2O_5.

Laboratory-constructed Plexiglass electrophoretic cells were used for polyacrylamide disc gel electrophoresis as well as immunoelectrophoresis. Power was supplied by a Heathkit variable voltage regulated power supply Model IP-32. A Polaroid Land Camera (Model MP-3) was used to photograph the electrophoretograms and immunoelectrophoretograms.

A Perkin-Elmer, Model 38-A Tiselius electrophoresis apparatus, using circulating refrigerated water to maintain the bath temperature at 2°C, was used for the free-boundary electrophoretic analyses. Buffer resistances were determined with an Industrial Instruments, Model RC, conductivity bridge.

Amino acid analyses were performed with a Beckman/Spinco Model 120C amino acid analyzer. A Beckman DK-2A ratio recording spectrophotometer, equipped with quartz cells with a 1 cm light path, was used for all spectrophotometric determinations.

A Beckman/Spinco Model E analytical ultracentrifuge equipped with a RTIC temperature control unit and a phase plate as a schlieren diaphragm was used for sedimentation-velocity studies. A capillary-type, single-sector synthetic boundary cell was used for sedimentation studies. In all determinations, 12 mm filled Epon centerpieces with quartz windows were used. An An-D Duralumin rotor was used for centrifuging and a General Electric AH-6 Mercury lamp served as the light source. Kodak metallographic glass plates were used for recording the schlieren patterns and a Nikon Model 6 Shadowgraph microcomparator was used for measuring the plates.

Chemicals and Materials

The principal chemicals used in this research and their suppliers are given below:

Tris-hydroxymethyl aminomethane (Sigma 121 and Trisma Base) was used as a primary standard and Sigma 7-9 was used to prepare buffers. Both were obtained from Sigma Chemical Co. as was iodoacetamide and neuraminidase (from *Cl. perfringens*). Diethyl-barbituric acid and sodium diethylbarbituric acid for veronal buffer, and monobasic potassium phosphate, potassium chloride, potassium sulfate, potassium citrate, sodium citrate, anhydrous calcium chloride, magnesium citrate hydrate, potassium carbonate, and potassium hydroxide for simulated milk salt solution were purchased from Fisher Scientific Company. Reagent grade ammonium sulfate was also purchased from Fisher Scientific Co. 2-thiobarbituric acid, N,N,N',N'-tetramethylenediamine, acrylamide, N,N'-methylenebisacrylamide and sodium azide were obtained from Eastman Organic Chemicals.

Cyanogum 41 used in polyacrylamide disc gel electrophoresis was purchased from E. C. Apparatus Company. Ammonium persulfate was obtained from the Baker Chemical Company and 2-mercaptoethanol was acquired from Fisher Scientific Co.

Special Agar-Noble used in immunoelectrophoresis was obtained from Difco Laboratories.

Orcinol was obtained from the Matheson Company, and phenol was acquired from Mallinckrodt and redistilled prior to use. Acetylacetone was purchased from Fisher Scientific Company. The Sepharose-6B used in this study was obtained from Pharmacia Fine Chemicals, as was the Blue Dextran 2000.

Mannose was acquired from Fisher Scientific Co. and galactose was obtained from Pfanstiehl Laboratories, Inc. (Germany). Glucosamine hydrochloride, galactosamine, and fucose were purchased from Nutritional Biochemicals Corp. N-acetyl neuraminic acid and tryptophan were acquired

from Calbiochem. The Buffalo Black NBR and Thiazine Red R were obtained from Allied Chemical Corp.

The other organic and inorganic chemicals used in this research were of reagent grade.

Preparative Procedures

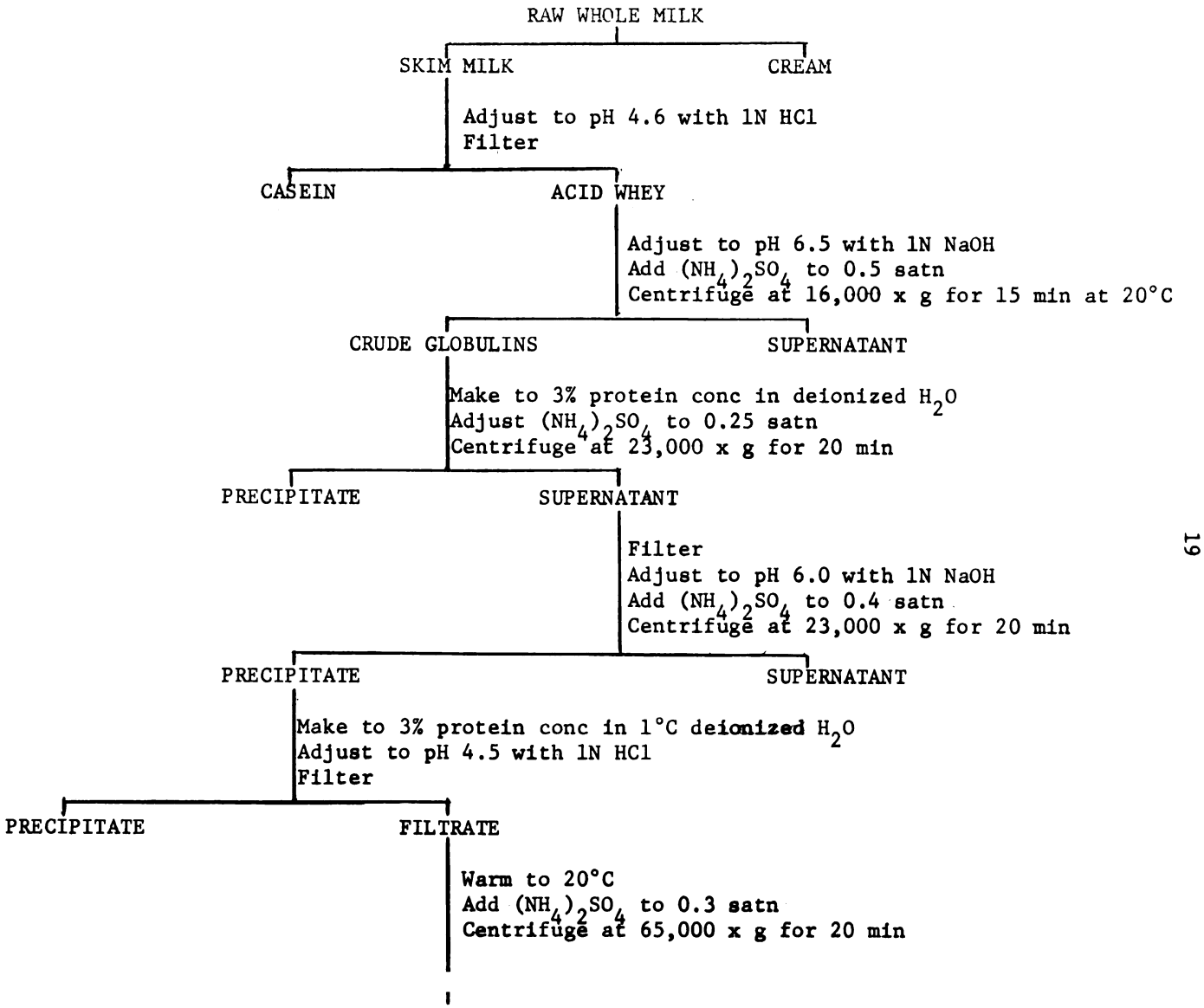
The milk used in this study was obtained from the Michigan State University dairy herd which consisted of Holstein cows. All milk was collected immediately after milking and before the milk reached the cooling bulk tank. The whole milk was heated to 45°C before separating in a laboratory separator, and the fresh skim milk was used as the starting material in this study.

Preparation of immunoglobulins.--The immunoglobulin fractions were prepared by combining the ammonium sulfate precipitation method described by Smith (1946), with minor modification, followed by preparative ultracentrifugation and column chromatographic technique using Sepharose 6B. The isolation procedure for the immunoglobulin fractions employed in this study is presented diagrammatically in Figure 1. Details of this procedure are described in the following sections.

Precipitation of immunoglobulins with ammonium sulfate.--After the cream was separated at 45°C, the resulting skim milk was cooled to 25°C and adjusted to pH 4.6 with 1 N HCl and allowed to stand for three hours. The precipitated casein was removed by filtration through multi-layered cheese cloth. The acid whey was adjusted to pH 6.5 with 1N NaOH and solid ammonium sulfate (313 g/l) was added slowly to 0.5 saturation while stirring, to salt-out the crude globulin fraction.

After standing overnight, the majority of the supernatant was siphoned off, and the precipitate was collected by centrifugation at 16,000 x g for 15 min at 20°C in the Sorvall GSA rotor. All further centrifugations were accomplished at 20°C in the Sorvall GSA rotor.

The precipitate was redissolved to about 3% protein concentration in deionized water, the pH adjusted to 4.6 and ammonium sulfate added to 0.25 saturation (144 g/l). After centrifugation at 23,000 x g for 20 min to remove the suspended precipitate, the supernatant was filtered through Whatman No. 42 filter paper. The filtrate was then adjusted to pH 6.0 with 1 N NaOH and the immunoglobulins precipitated by adding ammonium sulfate to 0.4 saturation (99 g/l). The precipitate was collected by centrifugation at 23,000 x g for 20 min and redissolved to approximately 3% protein concentration in 1°C deionized water. It was then adjusted to pH 4.5 with 1 N HCl and refiltered through Whatman No. 42 filter paper. The filtrate was warmed to 20°C and ammonium sulfate was added to 0.3 saturation (176 g/l). The precipitate was removed by centrifugation at 65,000 x g for 20 min, corresponding to Smith's fraction E. The supernatant was adjusted to pH 6.0 with 1 N NaOH and was brought to 0.4 saturation with ammonium sulfate (67 g/l). The precipitate was collected by centrifugation at 23,000 x g for 30 min at 20°C in GSA and was dissolved in deionized water to 3% protein concentration. The pH was determined to ensure a pH of 6.0 and ammonium sulfate was added to 0.4 saturation (243 g/l). The resulting precipitate corresponds to Smith's fraction F and was stored at 4°C under 0.4 saturated ammonium sulfate until further use. It was designated as "crude immunoglobulin preparation" throughout this study.


RAW WHOLE MILK
SKIM MILK
CREAM
Adjust to pH 4.6 with 1N HCl
Filter
CASEIN
ACID WHEY
Adjust to pH 6.5 with 1N NaOH
Add $(NH_4)_2SO_4$ to 0.5 satn
Centrifuge at 16,000 x g for 15 min at 20°C
CRUDE GLOBULINS
SUPERNATANT
Make to 3% protein conc in deionized H_2O
Adjust $(NH_4)_2SO_4$ to 0.25 satn
Centrifuge at 23,000 x g for 20 min
PRECIPITATE
SUPERNATANT
Filter
Adjust to pH 6.0 with 1N NaOH
Add $(NH_4)_2SO_4$ to 0.4 satn
Centrifuge at 23,000 x g for 20 min
PRECIPITATE
SUPERNATANT
Make to 3% protein conc in 1°C deionized H_2O
Adjust to pH 4.5 with 1N HCl
Filter
PRECIPITATE
FILTRATE
Warm to 20°C
Add $(NH_4)_2SO_4$ to 0.3 satn
Centrifuge at 65,000 x g for 20 min

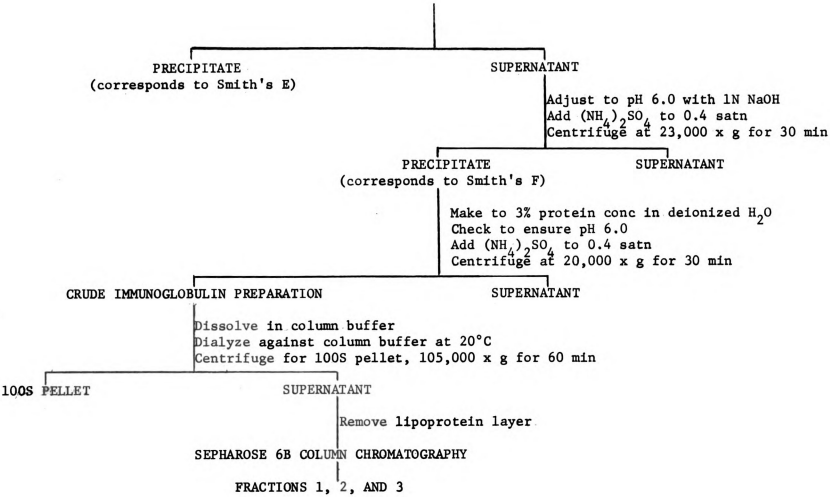


Figure 1. Schematic for the fractionation of immunoglobulins from cow's milk.

Separation of immunoglobulin fractions by gel filtration.--The principle of gel filtration, employing Sepharose 6B, was used for separating the immunoglobulin fractions from the crude immunoglobulin preparation. Sepharose 6B has a protein molecular weight exclusion limit of approximately 4×10^6. The column support was washed with deionized water three times to eliminate fine particles, followed by equilibration with pH 7.2 Tris-NaCl column buffer before packing the column.

The column buffer contained 0.1 M Tris, 1 M NaCl, 0.02% sodium azide and was adjusted to pH 7.2 with 1 N HCl. Sephadex chromatographic columns (2.5 x 45 cm), equipped with up-flow adaptors, were partially filled (2 in) with column buffer. A relatively dilute suspension of Sepharose 6B was added with constant stirring until the suspension had exceeded the length of the column and partially filled the column extender attached to the column prior to pouring. The outlet height was adjusted so that the beads were never under a head pressure of greater than 30 cm. When a one centimeter layer of beads settled to the bottom of the column, the outlet was opened and the column was allowed to pack under a head of approximately 27 cm. When a bed height of 35 cm was attained, the outlet was closed and the extender removed. The top upflow adaptor was secured in place, and the column was connected to a three-way glass stopcock which facilitated sample application in the up-flow direction. The head was maintained at approximately 25 cm, using a buffer vessel with a Mariott tube. The column was allowed to equilibrate with the buffer by passing through at least two column volumes. The gel volume was approximately 170 ml, and the column void volume was found to be 63 ml when a 1% solution of Blue Dextran 2000 in column buffer was eluted through the column. The flow rate was approximately 34 ml/hr.

The crude immunoglobulin preparation stored under 0.4 saturated ammonium sulfate was removed from the cold storage and stirred. Aliquots of this suspension were centrifuged in a Sorvall bench top centrifuge at 20,000 x g for 30 min. The supernatant was decanted, and the precipitate was dissolved in column buffer followed by dialysis against column buffer for 24 hr at room temperature to ensure good equilibration.

Twenty ml aliquots of the protein solution were then transferred to 30 ml polycarbonate tubes and centrifuged at 105,000 x g for one hour at 20°C in a Spinco Model L ultracentrifuge using a Type 30 rotor. These conditions were calculated to yield approximately a 100S pellet. After centrifugation, the flocculant lipoprotein layer in the upper part of the tube was discarded and the middle fraction of the tube was removed with a syringe. The 100S pellet was dissolved in column buffer and stored at 4°C for further analysis. The middle fraction of the tube was fractionated on the Sepharose 6B column. Usually 5 to 7 ml of this sample was applied to the column.

Preparation of cream and skim milk for creaming experiments.--Warm whole milk was collected from the Michigan State University dairy herd and separated in a laboratory cream separator at 40°C. Approximately two liters of the skim milk and 0.3 liters of the cream were heated to 70°C for 20 min on a steam bath to inactivate the creaming factor. Raw cream and raw skim were used in some experiments. Cream which was washed three times with a two-fold volume of simulated milk salt buffer described by Jenness and Koops (1962) was used in a model system containing this buffer as the aqueous phase. The composition of this buffer is presented in the Appendix.

Chemical Methods

Nitrogen

Nitrogen analyses were performed in duplicate. A micro-Kjeldahl apparatus with round bottom flasks and ball and socket ground glass joints was employed. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated H_2SO_4. Ten to 15 mg of dried protein were digested in 4 ml of the digestion mixture over a gas flame for one hour. After cooling, one ml of 30% H_2O_2 was added to each flask and digestion was continued for one additional hour. Each digestion flask was cooled and rinsed with 10 ml of deionized water. The digestion mixture was neutralized with 25 ml of 40% sodium hydroxide after the digestion flask was connected to the distillation apparatus. The released ammonia was steam distilled into 15 ml of 4% boric acid which contained three drops of indicator consisting of 400 mg bromocresol green and 40 mg methyl red in 100 ml of 95% ethanol. The distillation was completed when a total volume of approximately 75 ml was collected in the receiving flask. The ammonia-borate complex was titrated with 0.0172 N HCl which had been standardized with trishydroxymethyl aminomethane (Sigma 121) serving as a primary standard. The average recovery for a tryptophan standard was 99.4%.

Hexose

Hexose analyses were determined by the colorimetric method reported by Dubois *et al.* (1956). A weighed amount of dried protein (1-3 mg/ml) was dissolved to a known volume with 0.1 M NaCl. A one milliliter aliquot was taken from this mixture and delivered to a 15 x 150 mm test tube. One milliliter of 5% phenol in water was added and mixed. Reagent

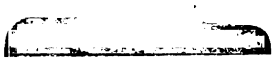
grade phenol was redistilled before preparation of the 5% solution. Next, 5 ml of concentrated sulfuric acid was added with a fast-delivery five ml pipette with a portion of the tip removed. The acid stream was directed against the liquid surface to ensure proper mixing and thus good reproducibility. The tubes were allowed to stand 10 min and placed in a water bath at 25°C for 20 min. Transmission (%) was read at 490 nm with a Beckman DK-2A spectrophotometer.

A blank was prepared by omitting the protein from the reaction mixture. A standard curve was constructed covering the range of 0 to 100 µg of mannose-galactose (0.8:1.2 w/w) dissolved in one milliliter of water.

Hexosamine

The method described by Johansen *et al.* (1960) was used to determine the hexosamine content of the fractions. Approximately 3 mg samples of protein were placed directly into 5 ml ampoules. After adding one milliliter of 4 N HCl the samples were frozen in a dry ice-ethanol bath, evacuated, refrozen and sealed under vacuum. The samples were hydrolyzed for six hours at 100°C in a hot air oven. After cooling, the hydrolyzed samples were transferred to distillation flasks. The empty ampoules were rinsed with one milliliter of 4 N NaOH, followed by two one milliliter rinsings with deionized water.

The acetylacetone reagent was prepared by dissolving one milliliter of acetylacetone in 25 ml of 1 M Na_2CO_3 solution plus 20 ml of water. The pH was adjusted to 9.8 and the volume was made up to 50 ml. This solution was used within 30 min. Ehrlich's reagent was prepared by dissolving 2 g of p-dimethylaminobenzaldehyde in absolute ethanol



containing 3.5% concentrated HCl to a final volume of 250 ml. This solution was stored at 4°C.

To each of the distillation flasks containing hydrolyzed sample were added 5.5 ml of the acetylacetone reagent. The final pH of this mixture was maintained between 9.5 and 10.0. The flasks were tightly stoppered and heated in a vigorously boiling water bath for 20 min. The flasks were cooled in an ice-water bath and then connected to a micro-distillation apparatus. Each flask was heated with a micro-bunsen burner and the steam-volatile chromogen was distilled into a 10 ml volumetric flask containing 7.5 ml of ice cold Ehrlich's reagent. The volumetric flasks were stoppered and the contents mixed. After 30 min, transmission (%) of the solutions was read at 548 nm with a Beckman DK-2A spectrophotometer.

A standard glucosamine solution (50 µg) was treated in the same manner to determine the recovery. Average recovery of the duplicate runs was 90.0%. A standard curve was constructed for glucosamine which spanned the range of zero to 50 µg carbohydrate in 10 µg increments. The blank consisted of one milliliter of 4 N HCl, one milliliter of 4 N NaOH and 2 ml of deionized water.

Sialic Acid

Warren's (1959) thiobarbituric acid method was adopted for the sialic acid determinations. Approximately 5 mg of dried sample was dissolved in 5 ml of 0.1 N H_2SO_4 and hydrolyzed at 80°C in a water bath for one hour to release the bound sialic acid (Svennerholm, 1957). A 0.2 ml aliquot was pipetted in duplicate into a test tube, to which was added 0.1 ml of sodium metaperiodate solution (0.2 M sodium

metaperiodate in 9 M phosphoric acid). The tubes were shaken and allowed to stand for 20 min at room temperature. One milliliter of sodium arsenite solution (10% sodium arsenite in a solution of 0.5 M sodium sulfate and 0.1 N H_2SO_4) was added and the tubes shaken until the yellow-brown color disappeared. Three milliliters of thiobarbituric acid solution (0.6% in 0.5 M sodium sulfate) were added. The test tubes were shaken, capped with marbles, and heated in a vigorously boiling water bath for 15 min. The tubes were removed and placed in cold tap water for five minutes, followed by the addition of 4.3 ml of cyclohexanone which was used for the extraction of the chromophore. The tubes were shaken and the contents were transferred to 15 ml, conically-shaped tubes and centrifuged for five minutes in a clinical centrifuge. The clear, upper cyclohexanone phase was pink or red and more intense than in the aqueous phase. Transmission of the organic phase was measured at 549 nm with a Beckman DK-2A spectrophotometer. A standard curve was prepared from commercially available synthetic N-acetylneuraminic acid in the range of zero to 20 µg per 0.2 ml. A blank was prepared by omitting the sample and following the same procedure.

Fucose

The fucose content of the samples was determined in duplicate utilizing the method described by Dische and Shettles (1948).

Samples (5 to 10 mg) were dissolved in five ml of 0.1 M NaCl. A fucose standard solution, containing approximately 20 µg per ml was prepared. A sulfuric acid-water mixture was made up of six volumes of concentrated sulfuric acid and one volume of deionized water. Duplicate one milliliter aliquots of the sample solutions were pipetted into test

tubes. To these ice water bath chilled tubes (and to 1 ml of 0.1 M NaCl for blank and 1 ml of the fucose standard) were added 4.5 ml of ice cold H_2SO_4-H_2O solution. The solutions were mixed while maintained in an ice bath to prevent rise in temperature. The tubes were transferred to a water bath at room temperature for a few minutes, then to a vigorously boiling water bath which should not cease boiling when the tubes are inserted. After heating for exactly three minutes, the tubes were placed in a water bath at room temperature. Cysteine-hydrochloride solution (0.1 ml of a 3% w/v solution) was added and mixed immediately to one of the duplicate samples, being omitted from the other. This corrects the determination for non-specific color development. The solutions were allowed to stand at room temperature for two hours and the per cent transmission was read at 396 and 430 nm with a Beckman DK-2A spectrophotometer.

The fucose content of the samples were calculated from the differences in the readings obtained at 396 and 430 nm after correcting for the non-specific color increment, i.e., samples without cysteine added.

$$\frac{(OD_{396} - OD_{430})s - (OD_{396} - OD_{430})b}{(OD_{396} - OD_{430})\ std.} \times 0.02 \text{ mg fucose/ml}$$

$$\times 1000 = \mu g \text{ Fucose/ml}$$

Amino Acid

Amino acid analyses were performed on twenty hour acid (hydrochloric) hydrolysates of the samples using a Beckman Amino Acid Analyzer Model 120C (Moore *et al.*, 1958). Five to 30 mg of dried sample (depending on Kjeldahl N value) was weighed and transferred into 10 ml ampoules. Five

milliliters of 6 N constant boiling HCl was then added to the ampoules. The contents of the ampoules were frozen in a dry ice-ethanol bath removed from the bath and evacuated with a high vacuum pump, allowed to melt slowly to remove gasses, refrozen and selaed with an air-propane flame. The sealed ampoules were placed in an oil bath preheated to 110°C in an oven regulated at this temperature. After 20 hr the ampoules were removed and allowed to cool at room temperature.

In order to check the transfer losses, one milliliter of 2.5 μM norleucine solution was added to each ampoule before transferring the hydrolysate to a 25 ml pear-shaped flask fitted with a ground glass joint. After evaporation to dryness on a rotary evaporator, each residue was taken up in a small amount of deionized water and redried until all the remaining hydrochloric acid odor was removed. Finally, the dried hydrolysate was dissolved in 0.067 M citrate-hydrochloric acid buffer, pH 2.2, and made up to five milliliters final volume. An aliquot of 0.2 ml was removed from each sample for the analysis.

At least two or more standard amino acid mixture runs were made with the same buffer and ninhydrin solution within four days.

The amino acid composition of the samples were expressed as moles of amino acid residues per 1000 moles of total amino acid residues.

Folin-Lowry Nitrogen

A method utilizing Folin Phenol Reagent described by Lowry *et al.* (1951) was used to determine the protein concentration for samples that were not lyophilized for experimental purposes. To a one milliliter sample dissolved in deionized water, column buffer or simulated milk salt buffer (Jenness and Koops, 1962) 5 ml of a mixture of 50 ml, 2% Na_2CO_3 in 0.1 N NaOH plus one milliliter of 0.5% $CuSO_4 \cdot 5H_2O$ in 1%

potassium tartrate were added. This mixture was allowed to stand at room temperature for ten minutes. Then 0.5 ml of diluted (1:1 v/v) Folin-Ciocalteu phenol reagent (Fisher Scientific) was added and the tubes were allowed to stand for 30 min. The per cent transmission was determined at 525 nm with a Beckman Model DK-2A spectrophotometer. A standard curve was constructed from bovine serum albumin (Pentex, Inc.) ranging from zero to 0.2 mg/ml dissolved in each of the above three solutions. Blank determinations utilized these solvents minus the sample.

Total Lipids

The total lipid content of the samples was determined by a modification of the method described by Folch *et al.* (1957). Samples (10 to 50 mg) were weighed and transferred to a 15 ml conical centrifuge tube. One milliliter of KCl solution (9.31 g KCl/l) was added to suspend and rehydrate the samples. After thorough mixing, 5 ml of chloroform:methanol (2:1 v/v) was added and the contents again well mixed. The tubes were capped with Saran wrap and cork stoppers and agitated thoroughly for another few minutes. The tubes were then centrifuged for 15 min in a clinical centrifuge with caps in place. The 4 ml lower chloroform-methanol layer was carefully removed with a syringe and transferred to a test tube. A small amount of anhydrous sodium sulfate was added to the chloroform-methanol extract to remove residual moisture. Two milliliters of the extract was pipetted into tared aluminum weighing dishes which were stored in a desiccator under vacuum over P_2O_5. The dishes were heated under mild conditions (50°C) on a hot plate to evaporate the solvent. They were allowed to equilibrate to room temperature under vacuum desiccation. The dishes were retared and the difference times two yielded the total lipid content. Solvent controls were analyzed

using the same procedure to determine their residue content.

Babcock Fat Determination

The Babcock fat test was adopted for determining the fat content of the separated cream.

Physical Methods

Alkaline Polyacrylamide Disc Gel Electrophoresis in a Discontinuous Buffer System

The procedure for preparing and performing the alkaline acrylamide disc gel electrophoresis was adopted with modifications from the method described by Melachouris (1969). The gel system consisted of a running gel and a spacer gel. The running gel was prepared by dissolving 7.5 g cyanogum 41 (a mixture of acrylamide and N,N'-methylene-bisacrylamide) in 0.380 M Tris-HCl buffer at pH 8.9. The volume was made to 100 ml with this buffer but before the mark was reached, 0.1 ml of N,N,N',N',-tetramethylenediamine (TEMED) was added. The spacer gel consisted of 3.75 g of cyanogum 41 dissolved in the same buffer system described above. To promote polymerization, one milliliter of fresh 10% (w/v) ammonium persulfate solution in deionized water was added to each 100 ml of the gel solution. After mixing, the gel solution was added to the stoppered glass disc tubes to a mark etched on the side of the tube. The tubes were secured in a vertical position by means of a Plexiglass supporting frame. A layer of distilled water was placed on top of the gel surface. Polymerization was allowed to occur for 30 min after which the distilled water layer was removed and the side of the tubes were blotted dry. A similar procedure was employed for applying the spacer gel. After polymerization, the gel tubes were removed from the rack

and inserted into a Plexiglas buffer cell which had holes drilled around the perimeter of a circle equally spaced and fitted with rubber grometts. This upper buffer cell was filled with buffer and the gel tubes bridged to a lower buffer cell to complete the circuit. Platinum electrodes were inserted equidistant from all gel tubes.

The electrode vessel buffer consisted of 0.046 M Tris-glycine pH 8.3. The samples which had been dissolved in pH 8.9 Tris-HCl gel buffer were layered on top of the gels. If the sample density was not greater than that of the vessel buffer, a small amount of sucrose was added to the samples to ensure proper sample application at the gel-buffer interface.

The electrodes were connected to a power supply; the lower one being connected to the anode. The current was initiated at 2.5 mAmps per tube for 15 min and increased to 5 mAmps per tube for the remainder of the run. To one sample was added 2 µl of 1% bromophenol blue in water. This agent acted as a marker indicating conclusion of the run. After electrophoresis was completed, the gels were removed from the tubes by means of loosening them from the glass walls with a syringe filled with water. The gels were stained for two hours in a dye solution consisting of 250 ml water, 250 ml methanol, 50 ml glacial acetic acid and five gram of amido black 10B (napthol blue black). The excess dye in the gel was removed by periodic changes of 7% glacial acetic acid in distilled water.

Acid Polyacrylamide Disc Gel Electrophoresis in a Discontinuous Buffer System

The procedure for preparing and performing the acid acrylamide disc gel electrophoresis was adopted from the method described by Butler (personal communication 3/30/70). The gels were run at pH 4.5 using a

7.5% running gel and a 3.75% spacer gel. The acrylamide reagent consisted of 30 g acrylamide plus one gram N,N'-methylenebisacrylamide per 100 ml. The TEMED reagent was made from 48 ml of 1 N KOH, 17.2 ml glacial acetic acid, 4.0 ml TEMED per 100 ml volume. The ammonium persulfate reagent was made fresh each time from 0.28 g ammonium persulfate per 100 ml. The running gel was prepared by mixing two parts TEMED reagent, four parts acrylamide reagent, two parts water and eight parts of the persulfate reagent. The spacer gel was constructed with two parts TEMED reagent, two parts acrylamide reagent, four parts water and eight parts persulfate reagent. The gels were poured and polymerized in the same manner as discussed in the previous section. The electrode vessel buffer was made by dissolving 31.2 g B-alanine in 2.5 l deionized water, adjusting the pH to 4.5 with glacial acetic acid and then adjusting the volume to three liters. The samples were dissolved in TEMED reagent which was lacking the TEMED. The tubes were under-layered in exactly the same manner as described in the previous section. The electrophoretic running conditions were the same except that the gels were electrophoresed for three hours in the acid system toward the cathode instead of the anode. Gel removal and staining were performed as outlined previously.

Immunoelectrophoresis

Immunoelectrophoresis of the samples was performed according to the method developed by Graber and Williams (1953) on glass plates 5 x 10 cm in area. The clean glass plates were coated with a thin layer of 0.1% agar in distilled water. After gellation, the plates were then coated with 8 ml of 1% agar in 0.05 ionic strength veronal buffer. The veronal buffer consisted of 2.797 g barbital and 20.6 g sodium barbital

made up to two liters with a pH of 8.6. The agar solution contained one gram agar, 98 ml veronal buffer and one milliliter of 1% merthiolate to act as a preservative. The agar mixture was heated in a boiling water bath to affect its solution, then applied to the glass plate. After cooling, thin troughs were cut using a razor blade and an index card template. The antigen hole was cut with a twelve gauge needle. The antigen hole was emptied with a disposable pipette under vacuum and filled with 2% samples dissolved in the above veronal buffer. The troughs were not removed. The cells were placed in a laboratory constructed, covered electrophoresis chamber which had the electrode vessels filled with veronal buffer. The plates were wicked to the buffer vessels by means of Whatman No. 42 filter paper strips soaked in the buffer. To one of the samples was added two µl of 1% bromphenol blue to act as a migration marker indicating the completion of the electrophoretic run. The current was applied at the rate of 4 mAmps per cm of gel and electrophoresis was allowed to proceed until the dye marker migrated approximately 4 cm, requiring about one hour. Following electrophoresis, the anti-serum troughs were removed by vacuum and filled with appropriate anti-serum. The plates were then placed in air-tight containers under a moist atmosphere for 48 hr while the precipitin bands developed. The plates were soaked for 24 hr in 0.10 M NaCl to remove any unprecipitated protein, then stained. The staining mixture consisted of 0.1% thiazine red in 1% acetic acid and proceeded for one hour. Destaining was accomplished in 1% acetic acid for approximately one week with periodic changes of acetic acid.

Thin-Layer Chromatography

Thin-layer chromatography was performed according to the method described by Franzen (1967). Clean pyrex glass plates (20 x 20 cm) were coated with a slurry of silica gel G or H at a thickness of 0.5 mm. The silica gel G slurry consisted of 48 g of silica gel G and 98 ml distilled water. This mixture was degassed on a rotary evaporator in preparation for pouring the plates. The silica gel H plates were poured in an analogous manner but consisted of 48 g of support material in 110 ml of 0.001 M sodium carbonate. The plates (silica gel H) were allowed to dry overnight and activated by heating to 110°C for 30 min. The activated plates were stored in a desiccator until used. All plates were spotted with the Folch *et al.* (1957) chloroform-methanol extracts of the samples along with tri-glyceride and cholesterol standards. Silica gel H was used for detection of phospholipids while silica gel G was used to resolve neutral lipids.

The solvent system for phospholipids consisted of 100:50:8:4 (v/v/v/v) chloroform:methanol:acetic acid:water. Neutral lipids were resolved with 80:20:1 (v/v) heptane:diethyl ether:acetic acid. Lipid spots were visualized by spraying the plates with 40% concentrated sulfuric acid followed by charring at 170°C for 30 min in a hot air oven.

Sedimentation Coefficient

The sedimentation-velocity experiments were carried out at 4° and 20°C using rotor speeds of 19,160; 39,460 and 59,780 rpm. These rotor speeds were selected because of the wide range of molecular species observed in other experiments. A capillary-type, single-sector, synthetic boundary cell was used for all determinations. The buffers used in the experiments were (a) 0.15 M NaCl in deionized water containing 0.02%

sodium azide pH 6.8 and (b) Jenness and Koops (1962) simulated milk salt buffer pH 6.6 which contained 0.02% sodium azide. The unlyophilized samples were dialyzed for at least four days with three changes of water made daily at either cold or room temperature before ultracentrifugal analysis. The concentration of protein in the samples was determined by the Lowry *et al.* (1951) procedure, using bovine serum albumin as a standard.

The sedimentation coefficient is defined as the velocity of the sedimenting molecules per unit field as shown by the equation:

$$S = \frac{1}{\omega^2 x} \cdot \frac{dx}{dt}$$

where $\underline{x}$ is the distance of the boundary in centimeters from the axis of rotation, $\underline{t}$ is the time in seconds, and $\underline{\omega}$ is in radians per second (Schachman, 1957).

Thus, by integrating the above equation, the following relation is obtained:

$$S = \frac{2.303}{\omega^2} \cdot \frac{\log x}{t}$$

Upon plotting the logarithm of the distance against time, the sedimentation coefficient is obtained from the slope of the line, using the following formula:

$$S(\text{Svedberg unit}) = s \times 10^{-13}\,\text{sec} = \frac{2.303/60}{(2\pi \cdot \text{rpm}/60)^2 \cdot \text{slope}}$$

This expression yields the observed sedimentation coefficient under the conditions of the centrifuge run. To convert these data to standardized conditions which approximate sedimentation in a solvent with the density

and viscosity of water at 20°C, i.e., $S^{app}_{20,W}$, correction factors were used. These corrected the effects of temperature on the viscosity and density for the NaCl system. To correct for the Jenness and Koops buffer, the assumption was made which presumed that this salt system was affected similarly to the NaCl solution. Thus, observed sedimentation coefficients were corrected to standard conditions according to the following equation:

$$S^{app}_{20,W} = S_{obs} \frac{(\eta_t)}{(\eta_{20})} \frac{(\eta_s)}{(\eta_o)} \frac{(1-\bar{V}\rho_{20,W})}{(1-\bar{V}\rho_t)}$$

where the first term (η_t/η_{20}) is the ratio of the viscosity of water at the experimental temperature to that at 20°C, the second term (η_s/η_o), the relative viscosity of solvent to that of water at any temperature, and the terms $\rho_{20,W}$ and ρ_t the densities of water at 20°C and the solvent at the experimental temperature, respectively. The partial specific volume, $\bar{V}$, of the protein was assumed as a constant value in all solvent systems employed.

The values used in the above equation for water and 0.15 M NaCl were taken from the International Critical Tables (1928 and 1929) and the correction equation for NaCl at 4°C becomes:

$$S^{app}_{20,W} = S_{obs} \frac{(1.5676)}{(1.009)} \frac{(1.574)}{(1.5676)} \frac{(1-0.75 \cdot 0.998)}{(1-0.742 \cdot 1.00651)}, \text{ or}$$

$$S^{app}_{20,W} = S_{obs} \cdot 1.49866.$$

and, for NaCl at 20°C the equation becomes:

$$S^{app}_{20,W} = S_{obs} \frac{(1.009)}{(1.009)} \frac{(1.000081)}{(1.009)} \frac{(1-0.75 \cdot 0.998)}{(1-0.75 \cdot 1.00534)}, \text{ or}$$

$$= S_{obs} \cdot 0.9840.$$

It was assumed that the partial specific volume, $\bar{V}$, decreased slowly with temperature, 0.0005 cm^3/gm/degree as stated by Greenberg (1951), and this correction is indicated for the low temperature conditions. Thus, to correct the observed sedimentation coefficient at 4°C to that of the standard 20°C, the S_{obs} was multiplied by a factor of 1.49866. To correct for the effect of NaCl at 20°C, the S_{obs} was multiplied by a factor of 0.9840.

Creaming Studies

Cream Volume. The creaming property determinations performed in this study were similar to those described by Dunkley and Sommer (1944), an exception being a reduction of sample volume from 100 ml to 10 ml since quantities of isolated immunoglobulins available for study were limited. The fat content of all samples was maintained at a constant level of 4% fat. However, the fat content of normal raw milk, used as a control, was 3.6%.

The procedure employed for assessing cream volumes were as follows: predetermined quantities of separated cream, skim milk and unlyophilized immunoglobulins in Jenness and Koops (1962) simulated, milk salt buffer were weighed into 50 ml Erlenmeyer flasks. The total weight of the ingredients added was 25 g. The quantity of immunoglobulin combined into the system was consistent with the amount usually reported to be present in skim milk (Rose *et al.*, 1970). The mixture was capped with Parafilm and tempered with mild agitation for 30 min in a 40°C water bath. Two 10-ml aliquots were transferred to 10 ml graduated cylinders which were placed in a water bath maintained at 4°C. The cylinders were observed at intervals of 1, 4, and 24 hr to determine the cream volume which was read directly from the calibrations on the cylinders.

Creaming property determinations were performed on (a) normal raw milk, (b) heat-inactivated skim milk with undenatured immunoglobulins and raw cream added to the system, and (c) a model system consisting of Jenness and Koops (1962) buffer, cream washed with, and immunoglobulin fractions dissolved in, the same buffer.

RESULTS AND DISCUSSION

Preparative Procedures

Preparation of Immunoglobulins

The procedure for preparing a crude immunoglobulin fraction from normal cow's milk was adopted with slight modifications from Smith's (1946) ammonium sulfate fractionation method as shown in Figure 1. When a fraction corresponding to Smith's fraction F was passed over a column of Sepharose 6B, two prominent elution peaks were detected on the column monitor at 254 nm, see Figure 2a. The recorder response between these peaks did not return to base line, indicating that protein was being eluted over a broad range. The eluate corresponding to the "void" peak - fraction 1 - was opaque, whereas fraction 2 was only slightly opaque and fraction 3 was clear. A portion of the component contributing to the opacity of the crude immunoglobulin preparation was removed by centrifugation at conditions designed to yield a 100S pellet. Following centrifugation, a fluffy low-density component was concentrated in the top centimeter of the centrifuge tubes. This was removed and discarded. The remaining supernatant was fractionated on a 2.5 x 35 cm column of Sepharose 6B. The 100S pellet material was dissolved in Tris-NaCl column buffer and stored at 4°C for further physical and chemical analyses. The chromatographed supernatant fraction was characterized by the elution pattern shown in Figure 2b.

Figure 2. Elution chromatogram of crude immunoglobulin preparation through Sepharose 6B column with 0.1M Tris-HCl, 1 M NaCl + 0.02% sodium azide, pH 7.2. A-Crude Ig fraction; B-Crude Ig fraction minus 100S pellet.

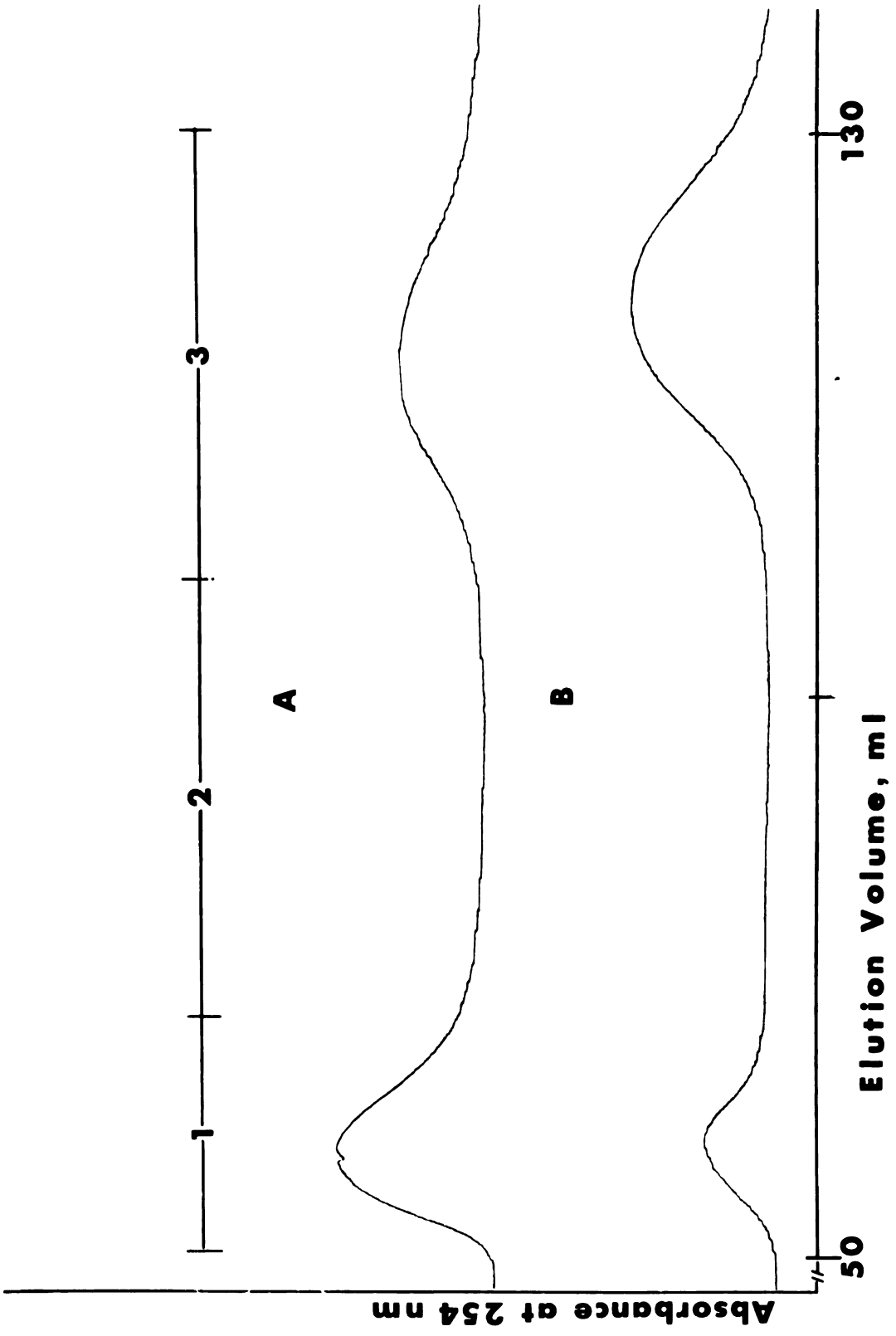


Figure 2

The opacity of fraction 1 was greatly reduced, whereas fractions 2 and 3 appeared to be clear.

The fractions obtained by centrifugation exhibited the following protein contents when analyzed by the method of Lowry *et al.* (1951).

100S pellet	= 10.94%
100S supernatant	= 61.93%
100S supernatant containing low-density components	= 27.10%
Total	99.97%

When the 100S supernatant fraction was resolved into three components over the Sepharose 6B column, their respective protein contents were as follows:

Fraction 1 =	5.60%
Fraction 2 =	8.50%
Fraction 3 =	80.33%
Total	94.43%

Component Assessment by Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide disc gel electrophoretic patterns of acid whey, the crude immunoglobulin preparation and its respective fractions, under both alkaline and acid conditions, are presented in Figures 3a and 3b. In the acid system, the immunoglobulins separate into distinct zones, whereas in the alkaline system, the immunoglobulins tend to form diffuse zones. Similar smearing was also observed in alkaline starch-urea gels (unreported data).

Figure 3. Polyacrylamide disc gel electrophoretic patterns under A, alkaline and B, acid conditions: 1-acid whey; 2-crude Ig prep; 3-100S pellet; 4-fraction 1; 5-fraction 2; 6-fraction 3. Twenty µl of 2% solutions were applied except in alkaline gel 1 which contained 30 µl.

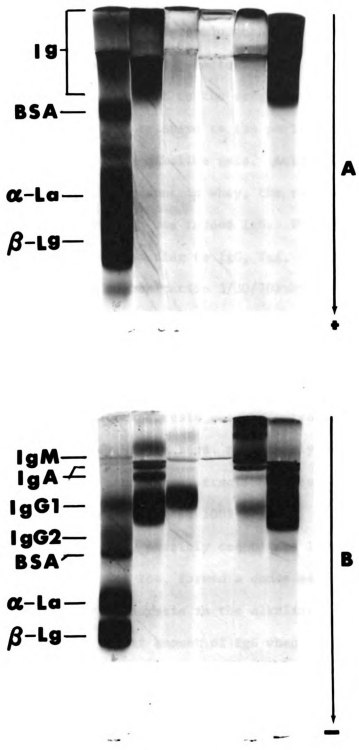


Figure 3

In Figure 3a, gel No. 1 was slightly overloaded with acid whey, so that the immunoglobulins normally present in whey would be concentrated enough to be detectable. The photographic method used was not sensitive enough to pick up the separation of β-lactoglobulin and α-lactalbumin; however, the separation was evident in the original gel. The acid gels (Figure 3b) showed more detail relative to the purity of the immunoglobulin fractions than did the alkaline gels. Acid gel No. 1 showed that the main immunoglobulin present in whey, the raw material for the crude immunoglobulin preparation was indeed IgG. The crude immunoglobulin prep contained proteins corresponding to IgG, IgA, and IgM as described by Butler (1970) (personal communication 3/30/70). The 100S pellet, acid gel No. 3, contained a component which migrated similarly to IgG. However, during the electrophoretic run, a major portion of this material did not enter the gel and diffused through the buffer. This same fraction under alkaline electrophoresis was concentrated at the top of the stacking gel as a narrow, dense band, not clearly evident in the photograph. The void peak components, fraction 1, behaved similarly during acid electrophoresis as did the 100S pellet. Only small amounts of the protein entered the gel, possibly due to its large size or low concentration of protein. It, too, formed a dense narrow band on top of the stacking gel upon electrophoresis in the alkaline system. Fraction 2, gel No. 5, contained a slight amount of IgG when observed in the acid system. However, it contained large amounts of IgM and some IgA. The proteins which formed dense bands in the stacking gel were considered to be large polymers of macroglobulin which, because of their size, could not migrate as the 19S species. Fraction 3, gel No. 6, contained IgG as a major constituent. A small amount of IgA was present. The

original electrophoretogram (alkaline gel No. 6) showed a much lighter smear in the stacking gel than in the running gel, an observation not readily apparent in the photographic reproduction.

Component Assessment by Immunoelectrophoresis

The results of the immunoelectrophoretic analyses of the crude immunoglobulin preparation and the separated fractions are represented by plates A-E in Figure 4. Plates A-D were developed with rabbit anti-sera to whole bovine serum and bovine IgM (plus IgG) which were obtained from Dr. J. E. Butler (U.S.D.A.). Plate E was developed against the above anti-whole bovine serum and, in the opposite trough, to a commercial anti-bovine IgG obtained from Cappel Laboratories.

The crude immunoglobulin preparation, Plate A, showed characteristic precipitation arcs to IgG and IgM when developed against antibodies to whole bovine serum and, when developed against the antibodies to IgM, a characteristic IgM arc and a slight arc to IgG were detected. The 100S pellet, Plate B, revealed precipitin arcs when developed against both anti-sera; however, these were not characteristic of IgM, IgA, or IgG. Due to the short length of the arc and the migration of a portion of this pellet to the IgG region in acid disc electrophoresis (Figure 3b, gel No. 3), it is postulated that this arc represented a protein similar to the 19S IgG reported by Hammer *et al.* (1968). The first fraction recovered from the Sepharose 6B column developed broad bands which were difficult to reproduce photographically (Figure 4, Plate C). They appeared as straight rather broad bands, extending approximately 0.75 cm from the antigen well. The identity of these arcs is not certain and, because of characteristics of this fraction that will be discussed later, it is postulated that they may represent a lipoprotein component. Fraction 2

Figure 4. Immunoelectrophoretic patterns of isolated fractions: Plate A - crude Ig prep; B - 100S pellet; C - fraction 1; D - fraction 2; E - fraction 3.

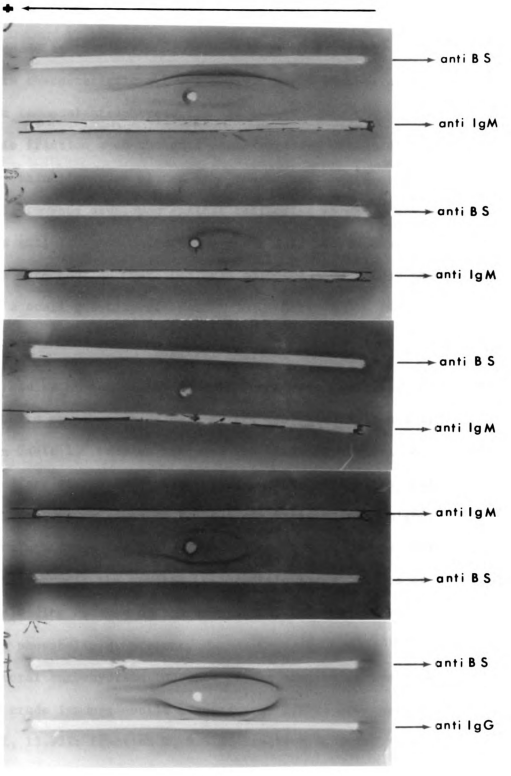

+
A
anti B S
anti IgM
B
anti B S
anti IgM
C
anti B S
anti IgM
D
anti IgM
anti B S
E
anti B S
anti IgG

Figure 4

from the Sepharose 6B column exhibited a characteristic IgM arc when developed against the two antibodies. This fraction also contains IgA as apparent in the acid disc gels. However, because the antibodies used were made against serum proteins, of which secretory IgA is not a member, its characteristic arc was absent in the immunoelectrophoretogram. The immunoelectrophoretogram did not indicate the presence of IgG in this fraction even though a small zone was detected in acid disc gels. The third fraction eluted from the column showed arcs characteristic of IgG. When developed against antibodies to whole bovine serum as well as anti-IgG, the patterns were similar, and no apparent trace of IgA was detected; however, the acid gel data showed that this fraction contained a small quantity of IgA.

Chemical Analyses

The results of the various chemical analyses performed on the crude immunoglobulin preparation as well as the isolated fractions are summarized in Table 1. Samples analyzed for chemical composition were lyophilized and stored under vacuum and over P_2O_5 prior to analysis. Therefore, the values given in Table 1 are based on dry weight.

The values given for the per cent protein obtained by determining Kjeldahl nitrogen in the samples that contain large amounts of lipid may be misleading because of the substantial concentration of nitrogen-containing phospholipids present.

The total carbohydrate contents of the samples analyzed were as follows: crude immunoglobulin preparation, 5.27%; 100S pellet, 12.31%; fraction 1, 13.92%; fraction 2, 9.87%; fraction 3, 2.86%. The carbohydrate data reported for fraction 3 compared very well to that reported by Nolan and Smith (1962) for bovine IgG (i.e., 2-3%) and also with the

Table 1. Chemical composition of immunoglobulin fractions

Constituent	Crude Ig Preparation	100S Pellet	Fraction 1	Fraction 2	Fraction 3
Protein[a]	82.53%	57.10%	40.73%	67.38%	90.37%
Hexose	2.30	5.30	5.70	4.80	1.30
Fucose	0.20	0.33	0.36	0.37	0.10
Sialic Acid	1.05	3.18	3.86	1.80	0.26
Hexosamine	1.72	3.50	4.00	2.90	1.20
Lipid	7.05	29.00	73.00	8.50	0.00
Total	94.85	98.41	127.65	85.75	93.23

[a]Kjeldahl N x 6.25.

2.8% value reported for human IgG by Day (1966). Approximately 10% carbohydrate was found to be present in the second fraction, which contained principally IgM for which Rose *et al.* (1970) reported a value of 12.3% carbohydrate. The value found in this study could be slightly low because of a small quantity of associated lipid, as well as the presence of small amounts of IgA and IgG which have lower carbohydrate contents than IgM.

Considering the amount of lipid present in the 100S pellet and in fraction 1, the carbohydrate values of this preparation appear to be rather high. Thus, these fractions appear to be complex high-density, lipo-glycoproteins which are unlike any other proteins known to be present in bovine milk. It would appear from the amino acid data, to be reported later, as well as from the carbohydrate analyses, that these fractions are polymers or aggregates of macroglobulins which are very stable and

possibly behave as micelles due to their high lipid content.

The amino acid analyses of the various immunoglobulin fractions are presented in Table 2. All fractions were found to contain large amounts

Table 2. Amino acid composition of immunoglobulin fractions

Residue	100S Pellet	Fraction 1	Fraction 2	Fraction 3
	(moles/1000 moles)			
Lysine	59.19	59.65	57.39	57.61
Histidine	19.20	21.73	17.80	16.50
Arginine	37.98	36.30	35.77	30.93
unknown	8.24	12.32	tr.	tr.
Aspartic	86.90	93.05	82.73	80.15
Threonine	83.58	80.00	84.34	100.79
Serine	105.69	100.38	124.27	130.65
Glutamic	86.01	82.80	87.84	79.62
Proline	67.69	59.43	71.61	80.75
Glycine	81.37	71.59	71.49	78.71
Alanine	63.32	66.02	58.55	56.02
Half cystine	20.31	18.23	22.72	25.63
Valine	73.31	70.42	92.23	96.33
Methionine	8.86	8.27	7.27	2.42
Isoleucine	46.01	50.34	38.45	27.20
Leucine	82.33	85.95	80.18	69.33
Tyrosine	26.23	27.57	30.75	39.39
Phenylalanine	46.63	55.88	36.59	27.96
ΣN	999.85	999.93	999.98	999.99

of serine, which is a characteristic of immunoglobulins, that distinguishes them from many other proteins (Day, 1966). The 100S pellet and fraction 1 are quite similar in their amino acid composition, whereas fraction 2

resembles fraction 3 in certain cases and the 100S pellet and fraction 1 in others. Fraction 3 is slightly different from the other fractions with respect to higher contents of threonine and tyrosine, and lower contents of methionine, isoleucine, leucine, and phenylalanine. Due to the close similarities in amino acid composition of all four fractions, it can be assumed that they all most likely belong to the class of proteins called immunoglobulins.

The analytical data indicate that all four fractions are mildly acidic proteins as determined by the ratio of the sum of acidic amino acid residues, i.e., aspartic and glutamic acids, to that of the basic amino acid residues, i.e., arginine, histidine and lysine. This ratio was 1.5 for all fractions. For comparisons, the caseins have a ratio of approximately 2.0 to 2.5.

When the ratio of the hydrophobic residues phenylalanine, proline, methionine, valine, leucine and isoleucine are compared with the hydrophilic residues aspartic, glutamic, tyrosine, lysine, arginine, and histidine, the four fractions are found to be relatively hydrophobic. The ratios for the 100S pellet, and fractions 1, 2 and 3, are 1.04, 1.03, 1.04 and 1.00, respectively. This could possibly explain the affinity to lipid observed in the 100S pellet and fraction 1 and fraction 2.

The 100S pellet and fraction 1 were also observed to have impaired solubility characteristics following lyophilization. It was for this reason, as well as to avoid possible structural and conformational damage, that the physical characterization studies were performed on unlyophilized samples. The large numbers of hydrophobic residues coupled with the lipids present could possibly account for the large

aggregates encountered in the centrifuge studies as well as their low solubility following lyophilization. Putman (1959) reports that disulfide interaction may account for the diminished solubility of human serum macroglobulins in cold and distilled water, as well as for the observation that repeated precipitation or lyophilization of euglobulin tends to induce its insolubility.

Lipids

A qualitative study was undertaken to determine the nature of the neutral and polar lipids present in the various immunoglobulin fractions. The results of thin-layer chromatography of the lipid-containing extracts (Folch *et al.*, 1957) of these samples are presented in Figure 5. Figure 5a indicates the presence of mono- and diglycerides, cholesterol, triglycerides, and cholesterol esters as the neutral lipid components. Spot 6 was a cholesterol standard whereas spot 7 was a triglyceride standard composed of tributyrin. There did not appear to be a large quantity of triglycerides present in the samples; however, the tributyrin spot was very light, which could indicate that the triglyceride did not char properly.

The 100S pellet, fraction 1, fraction 2, and the crude immunoglobulin fraction - spots 1, 2, 3 and 5, respectively - all exhibit a similar neutral lipid composition, with apparent high concentration of cholesterol. Fraction 3, spot 4, appeared to contain a trace of cholesterol esters, present at levels too low to detect by gross analysis.

The phospholipid components present in the samples are illustrated in Figure 5b. Again spots 6 and 7 are standards for cholesterol and triglyceride (tributyrin), respectively. The phospholipid components detected in the samples were sphingomyelins, phosphatidyl choline,

Figure 5. Thin layer chromatographic patterns of A, neutral lipids (silica gel G) and B, phospholipids (silica gel H): Spot 1-100S pellet; 2-fraction 1; 3-fraction 2; 4-fraction 3; 5-crude Ig prep; 6-cholesterol std.; 7-tributyrin std.

Abbreviations: O-origin; SPH-sphingomyelin; PC-phosphatidyl choline; PS-phosphatidyl serine; PE-phosphatidyl ethanolamine; NL-neutral lipids; PL-phospholipids; MDG-mono-diglycerides; C-cholesterol; TG-triglycerides; CE-cholesterol esters; F-solvent front.

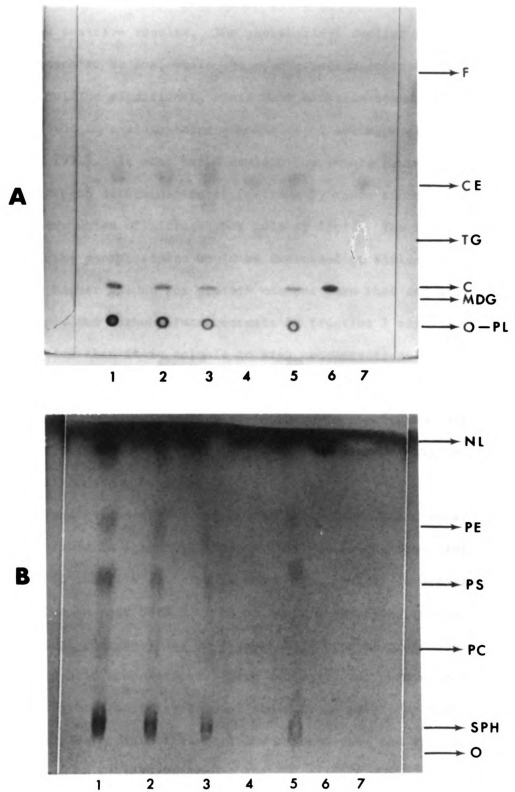


Figure 5

phosphatidyl serine and phosphatidyl ethanolamine. Fraction 3, spot 4, did not appear to contain any phospholipids; however, the other four samples gave positive results. The phosphatidyl choline content of these samples appears to be low, while the sphingomyelin content was very high. This fact could be significant, since many membrane bound lipoproteins have been found to contain large quantities of sphingomyelin (Patton and Keenan, 1971). It also could explain the errors found in the Kjeldahl nitrogen determination of fraction 1, since sphingomyelins contribute two moles of nitrogen per mole of lipid. The lipid nitrogen from all of the phospholipids would be expressed as Kjeldahl nitrogen, thus giving higher values for protein content than that actually present. The high lipid and carbohydrate contents of fraction 1 may also explain the observation that it is soluble in high concentrations of trichloroacetic acid (15%-TCA) and also appears to be stable to boiling for prolonged periods. No precipitation or increase in opacity was apparent upon these treatments. Interestingly, this fraction was highly water soluble, unlike most globulins.

Patton and Keenan (1971) report that 42% of the lipid phosphorous in milk was found in skim milk lipoproteins, while the remaining 58% was present in the milk fat globule membrane. Further investigation led to the conclusion that both sources of lipid phosphorous contained the same individual phospholipids in essentially the same proportions with similar fatty acid compositions. Both contained sphingomyelin and cerebrosides in levels characteristic of those found in plasma membranes; however, their data did not support the concept that the skim milk lipoprotein arises by disintegration of the fat globule membrane.

Physical Analysis

Sedimentation Velocity

Table 3 shows corrected $S^{app}_{20,W}$ values for the different immunoglobulin fractions isolated from the crude immunoglobulin preparation. A NaCl solvent system was chosen because of the prevalence of its use by workers in the field (Richardson and Kelleher, 1970). The Jenness and Koops buffer was chosen, because of its approximation to the indigenous ionic system of milk and, thus, would be useful for comparative work when studying the creaming phenomenon.

As can be seen from these data, many of the fractions were quite heterogeneous. However, in most instances one major boundary gradient, as indicated by an asterisk, was seen to predominate.

Some representative sedimentation-velocity patterns are presented in Figure 6. The protein concentration of the unlyophilized fractions was determined by the Lowry *et al.* (1951) method. All fractions were analyzed at protein concentrations of 0.75% except fraction 3, which was studied at a concentration of 1.1%. The effect of sample opacity on the sedimentation patterns can be seen in Row 1, Figure 6. These samples were highly opaque as indicated by the dark area present at the interference boundary which sedimented to the bottom of the cell as the analysis progressed. Similarly, but not to as great an extent, fraction 1 and fraction 2 exhibited some degree of opacity.

The 100S pellet in NaCl at 20°C resolved into two widely separated boundaries in the centrifuge. However, when this fraction was suspended a second time in Tris-NaCl column buffer and recentrifuged for a 100S pellet, the slower component, with an $S^{app}_{20,W}$ of 15.1, was observed in the supernatant portion of the cell. This indicates that the 15S species is

Table 3. The apparent sedimentation coefficients for separated immunoglobulin fractions centrifuged at 4°C and 20°C in different solvent systems

Fraction	Centrifugation speed (rpm)	Corrected $S^{app}_{20,W}$ values	
		0.15M NaCl 20°C[a]	J&K Buffer 4°C[b]
100S Pellet	19,160	15.1	14.7
		190.1*	152.3*
1	59,780	Lipoprotein*	-----
		-----	8.6
		20.5*	24.2*
		32.8	39.0
		Faster Components	50.5
		-----	70.7
2	c	7.1	-----
		-----	12.7
		20.1*	24.9*
		34.1	42.4
3	59,780	7.1*	8.7*
		0.15M NaCl 4°C[b]	J&K Buffer 20°C[a]
2	59,780	3.8	3.4
		7.8	7.4
		12.3	12.0
		20.8*	19.8*
		35.0	33.3

[a]Correction factor used was 0.9840.

[b]Correction factor used was 1.49866.

[c]20°C at 39,460.
4°C at 59,780.

*Indicates major boundary gradient.

Figure 6. Sedimentation velocity patterns of isolated immunoglobulin fractions: A-in 0.15 M NaCl + 0.02% sodium azide pH 6.8 at 20°C; B-in Jenness and Koops buffer + 0.02% sodium azide pH 6.6 at 4°C. Row 1-100S pellet; 2-fraction 1; 3-fraction 2; 4-fraction 3. Rows 1-3 0.75% protein, Row 4-1.1% protein.

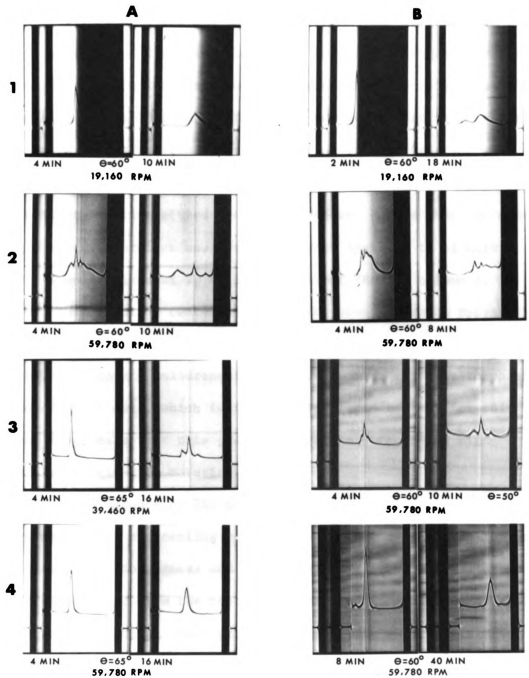

A
B
1
2
3
4
4 MIN Θ=60° 10 MIN
19,160 RPM
2 MIN Θ=60° 18 MIN
19,160 RPM
4 MIN Θ=60° 10 MIN
59,780 RPM
4 MIN Θ=60° 8 MIN
59,780 RPM
4 MIN Θ=65° 16 MIN
39,460 RPM
4 MIN Θ=60° 10 MIN Θ=50°
59,780 RPM
4 MIN Θ=65° 16 MIN
59,780 RPM
8 MIN Θ=60° 40 MIN
59,780 RPM

Figure 6

loosely associated with the larger aggregated species and that the phenomenon is not one of a typical monomer-polymer dissociating system. The twice-centrifuged 100S pellet in NaCl at 20°C is shown in Row 1, column A of Figure 6, while the once-centrifuged 100S pellet in Jenness and Koops buffer at 4°C is shown in Row 1, column B of Figure 6. The larger aggregated species decreased in S value when centrifuged at 4°C in Jenness and Koops buffer. At this time no interpretation of these data is warranted, since the effects of lipids in this fraction are unknown.

The first fraction eluted from the Sepharose column when the crude immunoglobulin preparation was chromatographed appeared to be heterogeneous. When examined at 20°C in NaCl solvent (Row 2, column A, Figure 6), this fraction contained a gradient boundary which appeared to remain near the top of the cell, approaching an equilibrium. Consequently, an accurate measurement could not be made for this component. From the chemical data, which indicate a high lipid content for this fraction, it appears that this gradient could be composed of high-density lipoprotein-type material.

Substantial amounts of 20S protein were also present as were some undetermined faster sedimenting components. When the same fraction was centrifuged at 4°C in Jenness and Koops buffer, the large lipoprotein gradient disappeared from the top of the cell, possibly due to an aggregation of this material. However, one would expect a lipoprotein to rise even faster in the cold due to the increased buoyant density of the solvent. The 20S species apparent in the 20°C run in NaCl increased to a 24S molecule at 4°C in Jenness and Koops buffer. This behavior may have resulted from aggregation of the molecular species causing it to sediment at a faster rate.

Fraction 2 (Row 3, Figure 6), was also increased in S value at 4°C as compared to 20°C in approximately the same proportion as that of fraction 1. Aggregation of the molecular species could also explain this phenomenon.

The third fraction (Row 4, Figure 6) contained one major component with an $S^{app}_{20,W}$ of 7.1 in NaCl at 20°C which increased to 8.7S in Jenness and Koops buffer at 4°C. Both of these samples were water clear and differences in background contrast were due to photographic reproduction.

To determine the effect of solvent on the protein samples, fraction 2 was centrifuged at conditions in opposition to those described above. That is, the sample was centrifuged in NaCl solvent at 4°C and in Jenness and Koops buffer at 20°C. The results are presented in Figure 7 and Table 3. Both runs gave similar results, indicating that both low temperature and the ionic composition of the Jenness and Koops buffer were necessary for the aggregation which was ramified by an increase in the $S^{app}_{20,W}$.

The 3S species observed in this fraction was probably the result of a slight contamination with lipoprotein. The gel electrophoresis data did not indicate any small molecular species that migrated faster than the 7S IgG.

The 7S molecules are IgG, while the small amount of 12S material no doubt reflects the presence of IgA which was present in the acid disc gels, Figure 3. The predominant 20S species is undoubtedly IgM, whereas the small 30S boundary could possibly be a polymeric form of IgM as reported by Day (1966).

Payens and Both (1970) isolated an immunoglobulin fraction from whey by ammonium sulfate precipitation which contained both 7S and

Figure 7. Sedimentation velocity patterns of fraction 2: A-in Jenness and Koops buffer at 20°C; B-in 0.15 M NaCl at 4°C. $\theta = 60°$; 0.75% protein; 59,780 rpm.

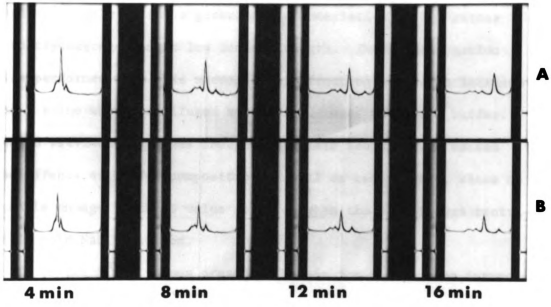


Figure 7

macroglobulin components. The latter component possessed cryoactivity. They stated that cryoaggregation is slow and only partially reversible and that it is strongly enhanced by decreasing the ionic strength of the solution. Their cryoaggregation studies were monitored by sedimentation and turbidity measurements at different temperatures.

A visual increase in turbidity was observed in the present study, when fraction 2 (macroglobulin fraction) was exhaustively dialyzed against deionized water at 4°C. However, the author attributes this phenomenon largely to the euglobulin characteristics of IgM rather than to cryoaggregation at low ionic strength. The centrifugation studies performed with this macroglobulin fraction showed an increase in the S value when centrifuged at 4°C in Jenness and Koops buffer. As stated previously, it was thought that this behavior was caused by the effects of buffer composition as well as temperature, since no noticeable change in the S value occurred when the protein was centrifuged in cold NaCl solution.

All of the $S^{app}_{20,W}$ values presented herein for the various immunoglobulins correlate well with values given in the literature (Rose *et al.*, 1970). It should be mentioned, however, that the S values for these proteins are concentration dependent, thus one would expect a slight change from the apparent S value when expressed as $S^{\circ}_{20,W}$ at infinite dilution.

A review of the dairy science literature revealed that there is little indication that lipoproteins are a part of Smith's (1946) classical immunoglobulin preparations, or that there are higher polymeric forms of immunoglobulins with S values greater than 20. One exception to this was found in the work of Payens (1968), who encountered a large aggregate

in the void-volume elution peak while separating an immunoglobulin preparation (Smith, 1946) on a Sepharose 4B (exclusion 20 x 10^6) column. It was later confirmed in a personal communication with Dr. Payens, 1971, that this fraction was highly opaque and, thus, would seem to correspond to the 100S pellet fraction of the present study.

It is interesting to consider the work of Phelps and Cann (1957) relating to the modification of bovine γ-pseudoglobulin by acid. They found that in nearly neutral solutions the sedimentation behavior was independent of salt concentration over the range of 0.02 to 1.0 M NaCl but that the sedimentation behavior was strongly dependent on salt concentration at pH 3.1. In acidic solutions, the protein sedimented as a single boundary with a decreasing sedimentation rate, i.e., 5.7 to 4.4S, as the ionic strength was varied from 0.1 to 0.02 respectively. At high salt concentrations (0.3 r/2), sedimentation patterns exhibited three boundaries: 6.3S, 9.5S and 12S components. The more rapidly sedimenting components were attributed to aggregation of the basic protein species. They also showed that the pH effects were not completely reversible. After a one hour exposure to pH 3.1, ionic strength 0.1, followed by dialysis against pH 7.0 phosphate buffer and then 0.1 M NaCl, the protein solution had a bluish hue and yielded a precipitate when dialyzed against distilled water. This behavior was unique for a pseudoglobulin. The re-neutralized material was found to contain sedimenting components of 6.69S, 9S, 13S, 24S, and 66S. The water-soluble fraction was found to contain only the 6.51S and 11S molecular species.

It is not possible to assess the effects of acid on the proteins isolated in the crude immunoglobulin preparation in the present study;

however, the above authors stated that γ-globulin sediments at a rate independent of pH over the range of 7.4 to 4.2. Since the lowest pH used in the preparative procedure was 4.5, the effect, if any, would probably be slight.

Creaming Studies

The Effects of Temperature on Creaming

Prior to determining the effects of the isolated immunoglobulin fractions on creaming, an experiment was conducted with raw milk to determine what effect temperature has on the creaming phenomenon. Raw milk samples were observed for cream volume at three different temperatures. All samples were tempered at 40°C for 30 min prior to quiescent storage. The temperatures selected for storage were 4, 22, and 40°C, and the results are shown in Table 4.

Table 4. The effects of temperature on the creaming of raw milk samples

Temperature	Cream Volume[a]		
	1 hr	4 hr	24 hr
4°C	3.20	1.70	1.30
22°C	0.00	0.20	0.40
40°C	0.00	0.10	0.30

[a]Expressed in ml per 10 ml total volume.

The data in Table 4 indicate that a low temperature is prerequisite for normal clustering and creamline formation. Similar findings were also reported by Dunkley and Sommer (1944). Payens *et al.* (1965) showed that

the adsorption of euglobulin to the fat globule membrane was temperature dependent, with a considerable decrease at 45°C and a much greater adsorption at 5° and 10°C. Their findings agree with the above results.

The Effects of the Isolated Immunoglobulin Fractions on Creaming in a Model System

A model system was used which substituted Jenness and Koops buffer for the aqueous phase of milk and cream washed with the same buffer for the non-aqueous phase. The immunoglobulin fractions that were incorporated into the system were dialyzed against Jenness and Koops (1962) buffer. However, the 100S pellet material was not removed from the crude immunoglobulin preparation and, consequently, was present in fraction 1 as collected from the Sepharose 6B column. The data obtained from this experiment are presented in Table 5.

Table 5. The effects of immunoglobulin fractions on a model creaming system of Jenness and Koops buffer plus cream

Fraction	Protein Concentration mg/25 mls	Cream Volume at 4°C[a]		
		1 hr	4 hr	24 hr
Control	0	0.10	0.10	0.15
Fraction 1[b]	2	0.10	0.20	0.60
Fraction 2	2	0.10	0.40	0.85
Fraction 3	14	0.10	0.20	0.20
Crude Ig Prep	20	0.10	0.30	0.60

[a]Expressed in ml per 10 ml total volume.

[b]Includes 100S pellet.

The results of this experiment indicate that fraction 2 had the greatest effect on creaming which, however, did not result in a typical creaming. The creaming observed in this model system seemed to be more typical of a gravity rise of small fat globules than the formation of large clusters, since the cream volumes tended to increase with time. Normal creaming exhibits a reduction in cream volume with time due to the compaction of the large clusters.

Because of the atypical results obtained with the above model system, an alternate system was devised which gave creaming results that agreed more closely with the classical work of Dunkley and Sommer (1944). In this system, the natural clustering agent inherent to separated skim milk was heat inactivated, thereby rendering an almost perfect creaming media since the natural ionic and compositional creaming environment is left intact.

The Effects of Heating on a Recombined Creaming System

The results of creaming experiments showing the effects of heating the separated cream and skim milk to 70°C for 20 min are shown in Table 6. The raw whole milk sample contained 3.6% fat, whereas the recombined samples were adjusted to 4% fat according to Dunkley and Sommer (1944).

The data in Table 6 indicate that the factor responsible for creaming is destroyed by heating at 70°C for twenty min and that most of this factor is located in the skim milk when the milk is separated at 40°C. This conclusion is evidenced by the low cream volumes observed when heated skim milk was recombined with both raw cream and heated cream. These results are atypical of normal creaming where a large cream layer is initially observed which with time compacts to a smaller

Table 6. The effects of heat on the creaming factor in skim milk

Recombined Samples	Cream Volume at 4°C[a]		
	1 hr	4 hr	24 hrs
Raw whole milk (3.6% fat)	2.00	1.30	1.10
Raw cream and raw skim	1.50	1.60	1.45
Raw cream and heated skim	0.00	0.10	0.30
Heated cream and raw skim	1.15	1.35	1.30
Heated cream and heated skim	0.00	0.10	0.20

[a]Expressed in ml per 10 ml total volume.

volume as the fat clusters rise. Closer to normal results were found when raw skim was combined with either heated or raw cream. The larger cream volumes after 24 hours found with the raw skim samples as compared to raw milk are most likely due to differences in fat content between the two samples.

The Effects of the Isolated Immunoglobulin Fractions on a Heated Recombined Creaming System

From the data in Table 6, it was concluded that the best system for determining the effects of the isolated immunoglobulin fractions on creaming would be one which incorporated raw cream, heated skim milk, and immunoglobulin which was unlyophilized and dialyzed against Jenness and Koops (1962) buffer. The immunoglobulin fractions were incorporated into the system at concentrations approximating that found in normal skim milk (Rose *et al.*, 1970). Thus, for the four different immunoglobulin fractions tested, the 100S pellet, fraction 1, and fraction 2 were added

at a rate of 2.0 mg per 25 ml creaming sample volume. Because IgG is the most abundant immunoglobulin present in skim milk, fraction 3 was added at a rate of 14.0 mg to the same volume. The 2.0 mg concentration used for the first three fractions corresponded to the finding by Payens *et al.* (1965), who stated that creaming became constant when 2 mg of euglobulin per gram of fat was present. In some cases, greater than 2 mg quantities of immunoglobulins were added. However, creaming volumes similar to those obtained with the 2 mg level were observed. The effect on creaming of the isolated immunoglobulin fractions are presented in Table 7.

Table 7. The effects of isolated immunoglobulin fractions on the raw cream and heated skim model system

Fraction	Cream Volume at 4°C[a]		
	1 hr	4 hr	24 hrs
100S pellet	0.00	0.10	0.30
Fraction 1	0.00	0.00	0.60
Fraction 2	0.00	1.10	1.20
Fraction 3	0.00	0.00	0.20

[a]Expressed in ml per 10 ml volume.

The data in Table 7 indicate that fraction 2 contained the active creaming factor and yielded cream volumes approaching those normally found when raw cream and raw skim milk are combined. This fraction contained predominantly IgM as monitored by acid disc-gel electrophoresis, immunoelectrophoresis and analytical ultracentrifugation. Fraction 3,

which contained predominantly IgG and a small amount of IgA, as well as the 100S pellet material, had no effect on creaming. Fraction 1 exhibited a slight effect on creaming, possibly due to the presence of a 20S component which was apparent in the sedimentation patterns.

To assess the effects of a smaller quantity of fraction 2 on creaming, an experiment was performed using one mg instead of 2 mg of this fraction. Heated skim milk and raw cream samples were mixed with this fraction and, after 1, 4 and 24 hr at 4°C, cream volumes were 0.0, 0.55, and 1.05 ml, respectively. The reduced cream volumes observed indicate that the quantity of active creaming agent present in a given system is a limiting factor in the creaming process.

An attempt was made to duplicate the work of Gammack and Gupta (1970). They did not state the conditions of centrifugation employed for the sedimentation of the casein pellet; thus an assumption was made which might not have corresponded with their conditions. The heated skim milk used in the above creaming experiments was centrifuged in the Beckman/Spinco, Model L-65 centrifuge at 20°C at conditions designed to produce a 50S pellet. Following centrifugation, the tubes contained a white casein pellet, a clear yellow whey supernatant, and a small cream layer at the top. The cream layer was removed, followed by the removal of the whey fraction. The top of the casein pellet was then carefully scraped out with a spatula. It was assumed that this fraction contained the active lipoprotein fraction described by Gammack and Gupta (1970). It was opalescent as described. The casein pellet was then dissolved in Jenness and Koops buffer and stored for subsequent creaming studies.

The above four fractions were all incorporated into model systems for creaming studies; the centrifuged middle whey fraction was used as the aqueous phase. For example, the casein pellet plus raw cream plus fraction 2 were mixed to determine the effects of the casein fraction on creaming. Similar mixtures using the other fractions were also examined. The results of this experiment are presented in Table 8.

Table 8. The effects of incorporating centrifuged heated skim milk fractions into a creaming system of centrifuged whey, raw cream, and 2 mg fraction 2/25 ml

Sample	Quantity added in grams	Cream Volume at 4°C[a]		
		1 hr	4 hr	24 hrs
Control	0.0	0.00	0.10	0.50
Centrifuged whey containing upper lipid layer	5.0	0.00	0.10	0.40
Lipoprotein layer above casein pellet	5.0	0.00	0.10	0.60
50S casein pellet	5.0	0.00	0.20	0.80

[a]Expressed in ml per 10 ml volume.

The results observed in this experiment do not correspond to those found by Gammack and Gupta (1970). They are also difficult to explain since none of the centrifuged fractions appeared to give normal creaming results as expected for non-centrifuged, heated skim milk. One explanation for atypical behavior is that proportions of centrifuged fractions added to the system were not correct. However, this seems unlikely, because the authors described the lipoprotein fraction above the casein

layer as being very active in the presence of macroglobulin. Another variable which existed between the two different experiments should be mentioned. In this study, heated skim milk was used as the source of lipoprotein, whereas Gammack and Gupta used raw skim milk. Possibly, heating the skim milk could have altered or denatured the lipoproteins or caused the lipoprotein to complex with the casein micelles, thus rendering them unavailable to the creaming phenomenon.

It is also interesting to consider the earlier findings of Hansson (1949), who found that the addition of lecithin and cephalin prepared from cow-brains greatly increased creaming in raw or low-pasteurized milk but not in high-pasteurized milk. This observation correlates with the recent findings of the above authors concerning the creaming activity of the high density lipoprotein.

The results of the present experiments seem to be comparable in certain respects (atypical creaming) with the data obtained when Jenness and Koops buffer was used as the aqueous phase, Table 5. It would therefore seem to indicate that the intact skim milk system is necessary before normal creaming occurs, and would substantiate the finding of the above authors that something other than IgM be present before normal creaming occurs.

The Effects of Neuraminidase on Creaming

Neuraminidase is an enzyme which cleaves terminally bound N-acetyl neuraminic acid (NANA) from glycoproteins which contain this carbohydrate. The objective of this experiment was to determine if NANA played a role in creaming. Ten mg of the fraction 2 protein in Jenness and Koops (1962) buffer was incubated with 1 mg synthetic neuraminidase at 40°C for 2 hr. An aliquot of the resulting mixture was then added to the

normal creaming system and the results are given in Table 9. As can be

Table 9. Creaming as affected by neuraminidase treatment of fraction 2, in a system of raw cream plus heated skim

Sample	Cream Volume at 4°C[a]		
	1 hr	4 hr	24 hrs
Fraction 2 plus Neuraminidase	2.00	1.65	1.35
Fraction 2	2.00	1.75	1.45

[a]Expressed in ml per 10 ml volume.

seen from the data in Table 9, neuraminidase treatment did not affect the creaming ability of the fraction 2 protein. This does not mean that the carbohydrates present on the proteins do not contribute to their activity in cluster formation. In order to ascertain that the enzyme was active, a control hydrolysis was performed which indicated that sialic acid was liberated.

The Effect of Disulfide Reducing Agent on Creaming

The objective of this experiment was to determine the effect of 2-mercaptoethanol (ME) on three different creaming systems. The first system was comprised of whole raw milk which was incubated with 0.5% and 2.5% ME for 1 hr at 40°C. The second system contained heated skim milk, raw cream and fraction 2 and was treated in a similar manner. The third system differed from the others in that fraction 2 in Jenness and Koops (1962) buffer was incubated as above with 0.5% and 2.5% ME which served to reduce disulfide bonds. The sulfhydryl groups were then alkylated by dialyzing for 24 hr at 20°C against an excess of

iodoacetamide (IAc) in Jenness and Koops (1962) buffer. The excess IAc was then removed by further dialysis against Jenness and Koops (1962) buffer for 24 hr at 20°C. The reduced and alkylated fraction 2 protein was then incorporated into a heated skim milk-raw cream model system. The effects of similar concentrations of IAc on fraction 2 were also determined by omitting the presence of ME in the third system, serving to alkylate any free sulfhydryl inherent to this fraction. The results of ME treatment on the above creaming systems are presented in Table 10.

Table 10. Creaming as affected by incorporating mercaptoethanol into raw milk; heated skim model system containing fraction 2; and fraction 2 followed by alkylation and incoporation into the heated skim model system

Sample	% ME	Cream Volume at 4°C[a]		
		1 hr	4 hr	24 hrs
Raw milk	0.0	0.00	3.20	1.15
Raw milk	0.5	0.00	3.50	1.20
Raw milk	2.5	0.00	0.00	0.10
Fraction 2[b]	0.5	0.00	1.30	1.15
Fraction 2[b]	2.5	0.00	0.00	0.15
Fraction 2[c]	0.5	0.00	0.60	0.80
Fraction 2[c]	2.5	0.00	0.00	0.10
Fraction 2[d]	0.0	0.00	1.50	1.30

[a]Expressed in ml per 10 ml volume.

[b]Fraction 2 in heated skim-raw cream system.

[c]Treated with ME and IAc followed by incorporation into heated skim milk model system.

[d]Treated with IAc followed by incorporation into heated skim model system.

The results presented in Table 10 indicate that a mild exposure to mercaptoethanol (0.5%) has no effect on the creaming ability of the raw milk or heated skim milk creaming systems. There was a loss in effectiveness, however, when the fraction 2 protein was subjected to disulfide reduction followed by alkylation. This is probably due to the fewer number of disulfide bonds present in fraction 2 as compared to the first two systems where the large number of disulfides probably diluted the reducing capacity of this reagent. Alkylation alone of fraction 2 had no detrimental effect on its creaming ability. Therefore, the reduction of cream volume observed when fraction 2 was reduced and alkylated was due to the activity of mercaptoethanol.

The effect of a five-fold increase in mercaptoethanol concentration (2.5%) was totally destructive to the creaming capacity of all systems investigated. It was then of interest to determine what effect the reduction and alkylation treatments had on the molecular structure of the IgM present in fraction 2.

Possible alterations in molecular structure were elucidated by acid disc-gel electrophoresis. When the proteins in fraction 2 were reduced in the presence of 0.5% ME and followed by alkylation, the gels indicated the appearance of a band in the running gel approximating the migration of IgG and a corresponding disappearance in the concentration of the IgM zone. When the 2.5% reducing system was observed, the gels showed no band for IgM and all protein migrated as the monomer form which approximated the mobility of IgG.

The findings of the above experiments indicate that the molecular size of IgM is of paramount importance with respect to its function in promoting creaming. The present study therefore considers the apparent

cryo-aggregation of IgM from a 20S molecule to a 25S molecule in the presence of a simulated milk salt environment (Jenness and Koops [1962] buffer) to be a prerequisite to cluster formation and creaming.

SUMMARY

Whey prepared from raw skim milk, which had been acidified to remove the casein, was fractionated by the addition of ammonium sulfate to yield a crude immunoglobulin preparation. This preparation was further fractionated by preparative ultracentrifugation and gel filtration chromatography on a Sepharose 6B column into a 100S pellet and fraction 1, 2, and 3, respectively. The components present in the fractions were analyzed by polyacrylamide disc gel electrophoresis, immunoelectrophoresis, analytical ultracentrifugation as well as various chemical procedures.

The 100S pellet was observed to be very opaque, and contained approximately 29% lipid and 12% carbohydrate. It also appeared to be loosely associated with a 15S species of IgG as evidenced by sedimentation velocity determinations and by acid-disc gel electrophoresis. The major component of this fraction did not enter a 3.75% spacer gel on electrophoresis and was found to sediment as a 190S molecular species in 0.15M NaCl and as a 150S species in Jenness and Koops (1962) simulated milk salt buffer at 4°C.

The eluate corresponding to the void peak of Sepharose 6B column chromatography, fraction 1, also exhibited some opacity. It contained approximately 73% lipid and 14% carbohydrate and was observed to be very heterogeneous in sedimentation velocity determinations. A majority of the components in this fraction, other than the lipoprotein gradient, were found to be in the range of 20-70S. Only a minor portion of the

material was found to enter a 3.75% spacer gel in polyacrylamide disc gel electrophoresis, and unusual precipitin arcs were observed when submitted to analysis by immunoelectrophoresis. From these physicochemical data, these two fractions appeared to be complex high-density lipoglycoproteins.

Fraction 2 contained approximately 8.5% lipid and 10% carbohydrate and contained principally IgM when analyzed by polyacrylamide acid disc gel electrophoresis and immunoelectrophoresis. The sedimentation velocity patterns for this fraction indicated that the major component present was a 20S species which aggregated to a 25S species in 4°C Jenness and Koops (1962) simulated milk salt buffer.

Fraction 3 contained 2.9% carbohydrate and was not found to be associated with lipid. Immunoelectrophoresis indicated that this fraction consisted of IgG. However, polyacrylamide acid disc gel electrophoresis showed a minor quantity of IgA. The sedimentation velocity studies revealed that a 7.1S species predominated in ambient NaCl solvent which aggregated to an 8.7S species in 4°C Jenness and Koops (1962) simulated milk salt buffer.

Amino acid analyses indicated that all fractions studied contained serine as the major amino acid constituent. The proteins were mildly acidic and contained more hydrophobic than hydrophilic residues.

The 100S pellet and fraction 1 contained 29% and 73% lipid, respectively. A qualitative lipid analysis of these high lipid-containing fractions indicated that the neutral lipids consisted mainly of cholesterol, while the principal phospholipid present was sphingomyelin. Fraction 2, which contained 8.5% lipid, had a similar distribution of lipid components.

The results of creaming experiments, utilizing raw milk, indicate that a low temperature, i.e., 4°C, is prerequisite for normal clustering and creamline formation. When Jenness and Koops (1962) simulated milk salt buffer was substituted for the skim milk aqueous phase, normal creaming did not result. Instead, a gradual increase in cream volume with time resulted, rather than a cream volume reduction which normally occurs with time.

When a heat-inactivated creaming system was used, into which the unheated immunoglobulin fractions were incorporated, normal creaming resulted only in the case of fraction 2. This fraction was shown to contain mainly IgM. The result of the enzymatic removal of terminally bound sialic acid from fraction 2 by the action of neuraminidase had no effect on its creaming ability.

Following partial reduction of disulfide bonds with 0.5% 2-mercapto-ethanol and alkylation with iodoacetamide, a decrease in creaming ability was observed. In the presence of 2.5% 2-mercaptoethanol, the creaming ability of fraction 2 was totally destroyed. This was attributed to the IgM being reduced to its monomeric species.

It was concluded from the above observations that IgM cryoaggregates from a 20S to a 25S molecule in the presence of a cold milk salt ionic environment. This aggregation is postulated as being prerequisite to fat globule clustering and creaming. The size characteristics of IgM are therefore considered of fundamental importance in the creaming phenomenon.

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APPENDIX

APPENDIX

Composition of Jenness and Koops' (1962) Simulated Milk Salt Buffer

Solution I

The following gram quantities of salts were made to 9.0 l with deionized water:

KH_2PO_4	15.80
K_3citrate$\cdot H_2O$	5.08
Na_3citrate$\cdot 2H_2O$	17.90
K_2SO_4	1.80
Mg_3citrate$\cdot H_2O$	5.02
K_2CO_3	3.00
KCl	10.78

Solution II

A 13.20 g of $CaCl_2 \cdot 2H_2O$ was dissolved in 500 ml deionized water.

Solution II was slowly added to Solution I while stirring the mixture vigorously.

Two grams of sodium azide was then added and the pH was adjusted to 6.6 with 1 N KOH. The final volume was adjusted to 10 l with deionized water.

Electrophoretic Mobility

The electrophoretic mobilities of the various isolated fractions were determined in veronal buffer at pH 8.6, ionic strength 0.1. The buffer was composed of 5.6 g veronal plus 41.2 g sodium veronal made to 2 l with deionized water. The mobilities were calculated from the descending channels and are expressed as Electrophoretic mobility = $X10^{-5}$ cm^2 $volts^{-1}$ sec^{-1}.

Two boundaries were observed when the crude immunoglobulin preparation was analyzed, a minor leading boundary migrating at a rate of -7.15, and a major boundary with a rate of -3.68.

When the void peak from Sepharose 6B, which had not been centrifuged to remove the 100S pellet, was analyzed, one boundary was observed with a mobility of -7.6. Fraction 2 exhibited two migrating boundaries with mobilities of -6.5 and -2.75. Fraction 3 appeared as a single migrating boundary with a mobility of -3.60. The crude immunoglobulin prep and fraction 3 were analyzed at 1% protein concentration, and the fraction 1 and fraction 2 at 0.5% protein concentration.