ISOLATION AND CHARACTERIZATION OF LACTEAL IMMUNOGLOBULINS

Thesis for the Degree of Ph. D.

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KHEE CHOON RHEE

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This is to certify that the

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF
LACTEAL IMMUNOGLOBULINS

by Khee Choon Rhee

The immunoglobulin fraction of cow's milk contains two principal components; a water-insoluble euglobulin and a water-soluble pseudoglobulin. Euglobulin consists mainly of IgG2-like globulins and small amounts of the IgG1-like globulins, IgA, and IgM, whereas pseudoglobulin consists mostly of IgG1 and a small amount of "secretory IgA". Each of these protein groups comprises about three per cent of the total whey proteins.

This investigation was directed toward a better understanding of the role of euglobulin in the creaming phenomenon of cow's milk, using both chemical and physical methods of assessment. In addition, some of the physico-chemical properties of euglobulin and pseudoglobulin were determined to reevaluate or confirm the existing data on these proteins. To isolate electrophoretically pure euglobulin and pseudoglobulin fractions from other milk components, gel filtration in Sepharose 6B and DEAE-cellulose anion exchange column chromatographic techniques were employed. Enrichment of the starting material was achieved by salting-out with ammonium sulfate.

Chemical analyses revealed that euglobulin and pseudoglobulin are identical in composition. They contained

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fucose, galactose, galactosamine, glucosamine, mannose, and sialic acid in their carbohydrate moiety. Phosphorus was absent in both proteins. Both proteins were similar in amino acid content and quite like other milk proteins except for lower glutamic acid and proline and higher serine and threonine contents.

The electrophoretic mobilities of euglobulin and pseudoglobulin were -1.82 and -2.02 x 10^{-5} cm^2 sec^{-1} $volt^{-1}$, respectively. The isoelectric point of euglobulin was pH 6.03 and that of pseudoglobulin was pH 5.54. Euglobulin had a partial specific volume of 0.712 ml/g while pseudoglobulin had a value of 0.710 ml/g. The diffusion coefficients of euglobulin and pseudoglobulin in veronal buffer (pH 8.6, μ = 0.1) were 3.20 and 4.20 x 10^{-7} cm^2 sec^{-1}, respectively. Euglobulin contained two sedimenting species whose sedimentation coefficients were 6.24S and 19.04S, while pseudoglobulin showed a single sedimenting boundary of 6.00S, as determined in veronal buffer at pH 8.6 with an ionic strength of 0.1. The weight average molecular weights of these two proteins were approximately 175,000 in veronal buffer and 90,000 in 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol.

Both the sedimentation coefficient and the weight average molecular weight of euglobulin increased at low temperatures. The weight average value in milk salt solution at 5° C was approximately equal to a dimer weight of 350,000. The hydrogen ion concentration and ionic strength

of buffers also had significant effects on the polymerization of euglobulin. As the hydrogen ion concentration or ionic strength decreased, both the sedimentation coefficient and the weight average molecular weight decreased owing to the slow dissociation of the euglobulin molecules.

The necessity of the euglobulin for the "creaming" of cow's milk was demonstrated in experiments with model systems. As the concentration of euglobulin was increased, up to about 0.04%, the creaming ability was improved gradually close to that of normal milk. At constant euglobulin concentration, the creaming ability was significantly improved by lowering the creaming temperature to 5° C. The effects of hydrogen ion concentration and ionic strength of the system on the creaming ability of the model system were similar to those on the sedimentation coefficient and weight average molecular weight of euglobulin.

ISOLATION AND CHARACTERIZATION OF LACTEAL IMMUNOGLOBULINS

By

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INTRODUCTION

The lactoglobulin fraction of cow's milk is classically defined as that portion of the whey proteins precipitated by saturation with magnesium sulfate or half-saturation with ammonium sulfate. Upon dialysis this fraction separates into two components, a precipitate called euglobulin and a protein remaining in the solution called pseudoglobulin. These proteins possess the immunological properties and are often called the "immunoglobulins". They each comprise about three per cent of the total whey proteins and are present in much higher concentration in colostrum than in normal milk.

Previous studies indicated that euglobulin might have an important role in the fat globule clustering or creaming of milk, but little has been elucidated in this regard. Very little information is available presently on the temperature directed association and dissociation phenomenon which euglobulin is believed to undergo. This phenomenon has been thought to be one of the fundamental causes of the "creaming" of cow's milk.

The main purpose of this research is to elucidate the role of milk euglobulin in the creaming of milk. Particular emphasis has been devoted to exploring the nature of association and dissociation of this protein in model

systems at different protein concentrations, temperatures, ionic strengths, and hydrogen ion concentrations in relation to fat globule clustering and the creaming abilities of this protein fraction.

Although reports have been made of the procedures for isolating the immunoglobulins and of their physical and chemical properties, they differ widely depending on the purpose of the experiments, the methods of analyses, and the source of the milk, both in the chemical and physical properties. Therefore, an extensive reinvestigation on the properties of these particular protein fractions is needed for elucidating the physico-chemical characteristics.

REVIEW OF LITERATURE

Nomenclature of Immunoglobulins in Cow's Milk and Colostrum

The Committee on Milk Protein Nomenclature, Classification and Methodology of the Manufacturing Section of American Dairy Science Association (Rose et al., 1969) recommended a complete revision of classical bovine immunoglobulin nomenclature to correspond with that used for more extensively studied species in accord with the World Health Organization Report (1964). The term "immunoglobulin" replaces terms like "immune lactoglobulin", "gamma globulin", "euglobulin", "pseudoglobulin", and "T-globulin" which can be found in the early dairy science literature.

Accordingly, three distinct classes of immunoglobulins occur in the lacteal secretions of the cow: IgG, IgA, and IgM. IgG immunoglobulins which have sedimentation constant of 7S can be further divided into at least two subclasses, IgG1 and IgG2. IgG1, with a sedimentation coefficient of 6.6S, is the principal immunoglobulin of milk and colostrum while IgG2, with a value of 6.3S, is primarily a serum component. IgG immunoglobulins are relatively low in carbohydrate (Smith et al., 1946; Nolan and Smith, 1962; Groves and Gordon, 1967; Kickhoefen et al., 1968). IgA of the lacteal secretions is a 10-12S molecule probably composed

of a dimer of subunits plus glycoprotein-a. It is best referred to as "secretory IgA". IgM occurs in milk and colostral whey. This immunoglobulin has a sedimentation constant of 19S, is sensitive to 2-mercaptoethanol and has been reported to contain 12.3% carbohydrate (Gough et al., 1966).

The new nomenclature of immunoglobulins can be correlated with the early preparations of Smith (1946a) in the following manner. Although antigenically heterogeneous, Smith's pseudoglobulin and plasma T-globulin contain mostly IgG1. The pseudoglobulin fraction also contains "secretory IgA". Smith's euglobulin consists of IgG2-like globulins, slower IgG1 globulins, IgA, and IgM. His serum gamma globulin corresponds to the IgG2 subclass.

However, the old nomenclature is used throughout this thesis for the following reasons: (a) the new system recommended by the committee has not yet been announced formally and (b) more importantly, the nature of this study was so designed that the classical euglobulin and pseudoglobulin classification of immunoglobulins was more suitable for comparing with the presently available information and in explaining the gross effects of euglobulin on the fat globule clustering.

Isolation Procedures of Immunoglobulins from Cow's Milk and Colostrum

Until the 1880's, milk was known to contain casein as the predominant protein and smaller quantities of other

protein with properties similar to those of blood serum albumin and blood serum globulin. It was found that after casein was removed by isoelectric precipitation at pH 4.6, the whey contained about 0.5% protein or about 20% of the total protein of skim milk. The milk serum proteins were then classified as lactalbumin and lactoglobulin according to their solubilities in one-half saturated ammonium sulfate or saturated magnesium sulfate.

The presence of globulin in milk was first recognized by Eugling (1880). He isolated globulin from milk by prolonged treatment of diluted whey with carbonic acid. A few years later, Hammersten (1883) observed that after separating casein from milk the filtrate contained albumin and a substance separated by saturation with magnesium sulfate. He suggested that this precipitate was a globulin.

Sebelien (1885) introduced the name "lactoglobulin" for a flocculent precipitate obtained by saturating whey with magnesium sulfate. This material possessed characteristics identical with serum globulin. He proved that albumin was not precipitated by saturating whey with magnesium sulfate, but that it remained in solution from where it could be precipitated by the addition of acetic acid. Halliburton (1890), to the contrary, claimed the substance isolated from milk by saturating with magnesium sulfate was lactalbumin. Schlossmann (1896-1897) observed that globulin, after prolonged standing at room temperature, collected on the surface of the liquid. The procedures of

Sebelien (1885) and Schlossmann (1896-1897) were reexamined by Simon (1901) and the globulin fractions obtained proved to be identical in their compositions and solubilities.

Storch (1897) isolated globulin from freshly drawn milk by saturating it with sodium sulfate. The salt concentration was about 14-19% at this temperature (ca. 38° C).

Crowther and Raistrick (1916) employed anhydrous magnesium sulfate for precipitating globulin. They observed that, like serum globulin, milk globulin could be separated into water-soluble pseudoglobulin and water-insoluble euglobulin by exhaustive dialysis.

Applying the sodium sulfate fractionation procedures of blood proteins to milk proteins, Howe (1921; 1922) found that the material precipitated up to 14.2% salt at 34° C was euglobulin. He designated the material precipitated between 14.2-18.4% salt as pseudoglobulin I and that precipitated at 18.4-21.5% salt as pseudoglobulin II. The yield of the last fraction was very small and there was no positive evidence that this was a separate protein.

Smith (1946b) observed that globulin separated by repeated precipitations with saturated magnesium sulfate gave preparations which showed complex electrophoretic patterns in moving-boundary electrophoresis. Thus, he devised a method of preparing an electrophoretically homogeneous globulin by stepwise fractionation with ammonium sulfate. With his method, the crude globulin was precipitated by half-saturation of the whey with ammonium sulfate.

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 immunoglobulins were precipitated from the supernatant at 0.4 saturation with ammonium sulfate at pH 6.0. The precipitate was reworked by dissolving in water at 1° C, adjusting to pH 4.5, and removing the insoluble residue by filtration. The supernatant was brought to 0.3 saturation with solid ammonium sulfate. The resulting precipitate was removed. A final adjustment to pH 6.0 and 0.4 saturation with ammonium sulfate resulted in yet another salted-out fraction. Upon dialysis, this fraction separated into electrophoretically homogeneous specimen of euglobulin and pseudoglobulin.

To isolate immunoglobulins from colostrum, a slightly different procedure was described (Smith, 1946a; Smith and Greene, 1948). After casein was removed by isoelectric precipitation at pH 4.5, a globulin fraction was salted-out from the acid whey with ammonium sulfate added to 0.3 saturation, and another fraction was obtained when the salt concentration was raised to half-saturation. These two fractions contained most of the colostral protein. By salting-out with ammonium sulfate at 0.4 saturation and pH 6.0, followed by resolution and precipitation with ammonium sulfate, he was able to obtain electrophoretically pure globulin from colostral serum. This protein fraction completely accounted for all the immune properties of the

colostrum. Apparently this procedure is not applicable to the isolation of the globulin from normal milk.

Another method for isolating lactoglobulins from milk and colostrum was introduced by Kenyon et al. (1959), as an adaptation of a method developed by Horejsi and Smetana (1956) for the isolation of gamma-globulin from the blood serum. In this method, rivanol (2-ethoxy-6,9-diamino-acridine lactate) was used to form a metal-combining globulin complex which remained in solution. The precipitated albumins were filtered off. Rivanol was removed from the supernatant by adsorption on activated charcoal, leaving a crude globulin fraction in solution.

A column chromatographic technique employing anion exchange cellulose for the fractionation of the blood proteins (Sober et al., 1956) was modified by Yaguchi et al. (1959) to fractionate milk proteins. Filtered acid whey was adjusted to pH 6.8 with dilute sodium hydroxide and the traces of casein were removed by reacidifying to pH 4.6. The whey was filtered, adjusted to pH 7.0 and dialyzed against a large volume of phosphate buffer for 48 hr at 2-3° C. At least eight whey protein components were eluted from the column developed with a linear gradient of sodium chloride (0 to 0.2 M) in phosphate buffer. The immunoglobulin fractions were eluted in the first and second peaks (Yaguchi et al., 1961).

Characteristics of Immunoglobulins in Cow's Milk and Colostrum

In comparing milk with colostrum, the marked difference in the physical and biological properties of the colostrum can be ascribed primarily to its high protein content, particularly globulins, and atypical protein distribution (Sebelien, 1885; Bauer and Engel, 1921; Smith, 1946a).

Crowther and Raistrick (1916) did a comparative study of the proteins of the colostrum and milk of the cow and their relationship to the blood serum proteins. They arrived at the following conclusions: (a) casein, lactoglobulin and lactalbumin are distinct proteins and have the same composition whether prepared from colostrum or normal milk; (b) globulin fractions obtained from colostrum and milk, although occurring in small amounts in milk, are alike and closely allied to or identical with serum globulin; (c) euglobulin and pseudoglobulin are identical insofar as their composition is concerned. Dudley and Woodman (1918) and Woodman (1921) suggested that euglobulin and pseudoglobulin were structurally identical. They investigated the optical rotational properties of these two proteins in alkaline solution and made a comparative study of the optical properties of the amino acids derived from racemized euglobulin and pseudoglobulin by hydrolysis.

A series of outstanding studies and contributions to our knowledge of the lactoglobulin fraction was made by Smith (1946a; 1946b; 1948). The globulin character of the

isolated protein fractions was indicated by their precipitation at low concentrations of ammonium sulfate, low solubility near the isoelectric point, and by a marked increase in solubility in the presence of neutral salts. Smith also reported that exhaustive dialysis of the lactoglobulin fraction resulted in the separation of a water insoluble euglobulin and a water soluble pseudoglobulin and that the immune activity was associated with both of these proteins. Furthermore, he found by electrophoresis that the lactoglobulin of milk was identical to that found in colostrum. He also reported the absence of phosphorus and the presence of sulfur and carbohydrates in lactoglobulin fractions. Hansen et al. (1947) reported high contents of proline and glutamic acid, while Smith and Greene (1947) reported high threonine content and that cystine and methionine accounted for all the sulfur of these proteins. The carbohydrate content of lactoglobulins in milk and colostrum was contributed by the protein-bound hexose and hexosamine (Smith et al., 1946; Smith, 1946b).

Smith (1946a; 1946b) was the first to use electrophoresis and ultracentrifugation as well as chemical analysis in comparing the properties of lactoglobulin fractions. He reported values of pH 6.05 and 5.60 for the isoelectric points of euglobulin and pseudoglobulin, respectively. He recorded the electrophoretic mobilities (Tiselius units) of euglobulin and pseudoglobulin of milk and colostrum of -1.7 and -2.5, and -1.9 and-2.2, respectively. Murthy and

whitney (1958) reported values of -1.76 and -2.04 for similar protein fractions isolated from milk.

The sedimentation coefficient of the principal component (about 84-89%) of milk and colostral immunoglobulins was reported to be approximately 7 Svedberg units (Smith, 1946a & 1946b; Smith and Brown, 1950). The identity of these molecules to those of the serum immunoglobulin IgG was confirmed by many investigators (Larson and Gillespie, 1957; Carroll, 1961; Dixon et al., 1961; Micusan and Buzila, 1965; Pierce and Feinstein, 1965; Milstein and Feinstein, 1968). However, some evidence was given that a small change might occur in the molecule during its transport from serum to milk (Kickhoefen et al., 1968). The carbohydrate content of these proteins seems to be relatively low--approximately 2-3% (Smith et al., 1946; Nolan and Smith, 1962; Groves and Gordon, 1967; Kickhoefen et al., 1968). The second most concentrated component had a sedimentation coefficient of 10-12S and was very sensitive to 2-mercaptoethanol (Smith et al., 1946; Jenness et al., 1965). This component had a carbohydrate content of 8-9% (Gough et al., 1966) and was reported to be identical to serum immunoglobulin IgA (Hanson and Johansson, 1959; Murphy et al., 1964; Jenness et al., 1965; Baglioni and Fioretti, 1967). A third component which was found only in the euglobulin fraction had a sedimentation coefficient of 19-20S (Smith et al., 1946; Gough et al., 1966), was sensitive to 2-mercaptoethanol, and was reported to contain 12.3 per cent

carbohydrate (Gough et al., 1966). This macroglobulin was reported to have the same physico-chemical and biological properties as the IgM immunoglobulin from the blood of other species (Murphy et al., 1964; Jenness et al., 1965; Gough et al., 1966; Baglioni and Fioretti, 1967; Coffey and Reithel, 1968).

The molecular weight of immune lactoglobulin was reported at about 160,000 to 190,000 by Smith et al. (1946) while the much higher values of 252,000 for euglobulin and 289,000 for pseudoglobulin were reported by Murthy and Whitney (1958). Smith (1946a) reported a diffusion coefficient of 3.6×10^{-7} cm^2 sec^{-1} for the immunoglobulin fraction.

The ultraviolet absorption spectra of electrophoretically homogeneous immunoglobulins were obtained by Smith and Coy (1946). No evidence was found for the presence of light-absorbing groups other than the three aromatic amino acids, phenylalanine, tyrosine and tryptophan.

The effect of heat treatment of milk on the denaturation of globulin fractions had been studied by many investigators. Hetrick et al. (1950) observed that the extent of denaturation of globulin with time at constant temperature of 170° F followed closely the first order law but the first order law was no longer obeyed at 230° F. Steam-injection produced more severe effects at short-time and low-temperature combinations than have been reported for other heat exchange methods, but less severe effects than

other methods at high temperatures (Dill et al., 1964). Globulins showed a rapid increase of denaturation even at low temperatures of heating, showing that this is the most heat-labile protein fraction of milk (Melachouris and Tuckey, 1966).

Changes in the whey protein fraction during cold storage of raw milk were studied by Lindquist and Storgards (1966). They observed some changes on pseudoglobulin and blood albumins during the first 24 hr of storage of raw milk at 2° C. The decomposition products of both proteins appeared in the alpha-lactalbumin peak in free-boundary electrophoresis diagrams. However, they could not determine at what stage this change took place.

The Role of Immunoglobulins on the Clustering of Fat Globules in Creaming of Cow's Milk

The process by which fat globules rise to the top of milk upon standing, forming a fat-rich layer, is known as "creaming". This phenomenon represents one of the fundamental physical properties of the fat emulsion. The formation of a cream layer on milk involves two steps: (a) clustering of the fat globules and (b) rising of the clusters to form the cream layer. The manner in which fat clusters rise and pack in the cream layer has been extensively investigated and is well understood. While it has been established experimentally that the clustering of fat globules is essential for the rapid creaming and the formation of deep cream layer on milk, the fundamental mechanism involved is still

a matter of controversy.

Babcock (1889) was the first to study the phenomenon of fat globule clustering in freshly secreted milk. He advanced the theory that clustering resulted from coagulation of fibrin, which he thought to be a normal constituent of milk, and that the coagulated fibrin entangled the fat globules and weighed them down enough to prevent the "clots" from rising. Although his conclusions were proved incorrect, many investigators give him credit for being the first to indicate the significance of fat globule clustering in the creaming of milk.

Since the work of Babcock in 1889, several theories were proposed to explain fat globule clustering: (a) gravitational rise of fat globules; (b) electrokinetic potential of fat globules; (c) fat-serum interfacial tension; (d) stickiness and state of hydration of the adsorbed fat globule membrane; and (e) fat clustering considered as an agglutination process.

Dunkley and Sommer (1944) reviewed extensively all the pertinent references concerning the above proposed theories, performed a series of carefully designed experiments, and drew the following conclusions: (a) that the gravitational rise of the fat in milk favors the formation and increase in size of fat globules, but the variability in creaming properties of milk can not be explained on the basis of differences in the rates of rise of fat globules and clusters; (b) that the salts normally present in milk are

sufficient to reduce the surface potential on the fat globules below the critical level which permits cluster formation, thus the creaming properties of milk are not determined by the variability of the charge on the fat globules; (c) that the interfacial tension at the fat-serum interface does not determine the creaming properties of milk and that the free energy at the surface of the fat globules is not the cause of clustering; and (d) that milk euglobulin promotes the clustering of fat globules.

There are many publications favoring the theory that the mechanism for the clustering of the fat globules in milk is the same as that of a bacterial agglutination reaction in which euglobulin is involved.

Babcock (1889) was the first to report that the addition of blood serum to milk caused an aggregation of the fat globules. Van Dam et al. (1922) and Hansson (1949) reported similar results. However, when blood serum, heated to above 62°C, was added to milk no improvement in creaming was observed (Van Dam et al., 1922; Hekma and Sirks, 1923; Rowland, 1937). Therefore, it was suggested that the substance responsible for the enhancement of creaming is thermolabile at the same temperature as are bacterial agglutinins.

The substance responsible for the improvement in creaming in milk was precipitated with the globulin fraction (Hekma and Sirks, 1923). Brouwer (1924) fractionated the globulin into euglobulin and pseudoglobulin and

demonstrated that the euglobulin was effective in improving the creaming properties of milk but that the pseudoglobulin had only a slight beneficial effect. Palmer et al. (1926) and Troy and Sharp (1928) also observed that the agglutinin material followed the globulin fraction when the proteins of milk were separated.

Sharp and Krukovsky (1939) considered the clustering of fat globules as an agglutination process and observed that the agglutinating substance normally present in milk was adsorbed on the surface of solid fat globules but not on liquid fat globules. They also found that the agglutinin was concentrated in cream separated at low temperatures and was relatively absent in the corresponding skim milk. The opposite was true for milk separated at high temperatures.

Dunkley and Sommer (1944) proposed a possible explanation for the clustering of fat globules based on Marrack's theory of the mechanism of bacterial agglutination (Marrack, 1939). Accordingly, clustering would be promoted by: (a) a partial dehydration of the adsorbed membrane on the fat globules as affected by a specific polar adsorption of the euglobulin; (b) an aggregation of fat globules resulting from the adsorption of a single globulin molecule by two or more fat globules; and (c) a 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, from which it was precipitated by adding sodium chloride or gum arabic. This material consisted of two components, one inactivated by homogenization and the other by heating in water, whey or milk to 70-75° C for 15 seconds. In systems containing cream mixed with water, why 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 one or the other of these treatments, no creaming resulted. Similar observations were made by Kenyon and Jenness (1958) and Jenness and Patton (1959). They observed that either homogenization or heat treatment of skim milk prevented agglutination of fat globules, but a mixture of equal parts of homogenized and heated skim milk produced normal creaming. They also found that the addition of very small amounts of colostral euglobulin to treated skim milk restored the creaming ability of the heated but not that of the homogenized.

Payens (1964) and Payens et al. (1965) reported that I^{131}-euglobulin was adsorbed on the surface of the fat globules after homogenization and this adsorption was accompanied by small amounts of casein, particularly κ-casein. With similar results, Koops et al. (1966) speculated that euglobulin-casein complex was formed during the

homogenization, which was still capable of adsorbing onto the fat globule surface but unable to effect clustering, and that the adsorption of κ-casein would screen off the clustering sites of the adsorbed euglobulin.

A model of the euglobulin-fat globule complex was postulated by Dunkley and Sommer (1944) and Payens et al. (1965). They reported that agglutination was brought about by the formation of euglobulin bridges between two fat globule molecules. The surface layer with the peptide chains projecting into the plasma phase would fit best into this picture. Kenyon et al. (1966) suggested the mechanism for the Brucella ring test to explain the model; specific agglutinins are adsorbed on the globules in such a way that the reactive sites of the Brucella agglutinins are available to combine with Brucella cells.

EXPERIMENTAL

Apparatus and Equipment

The milk used in this study was collected in five- or ten-gallon stainless steel cans and separated with a DeLavel disc-type separator. Stainless steel, plastic or Pyrex containers were used for performing all experiments. A Beckman Model 115 or a Sargent Model DR pH meter, equipped with glass electrodes was used to measure pH values. For weighings, top-loading, direct reading Mettler Type K-7 and H-16, a Sartorius series 2400 balance, and a Cahn electrobalance were used.

Low-speed centrifugations were performed with an International Model V, size 2 centrifuge. Intermediate-speed centrifugations were performed with a Sorvall RC2-B refrigerated centrifuge, using Type SS-34 and Type GSA rotors. For high-speed separations, a Beckman Model L-65 refrigerated preparative ultracentrifuge, equipped with Type 21, 30 and 65 fixed-angle rotors, was used.

Sephadex laboratory columns, Type K25/45, equipped with cooling jackets were used for packing the supporting materials. The eluates from the columns were monitored either at 280 nm by Gilson Medical Electronics' Adsorption Meter and recorded by a recording milliammeter manufactured by Esterline Angus Instrument Company or at 254 nm

with a recording ultraviolet analyzer manufactured by Instrumentation Specialties Company, Incorporated.

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 temperature controlled Cenco vacuum oven in which vacuum was obtained with a Cenco Pressovac 4 vacuum pump.

Laboratory-constructed Plexiglas electrophoretic cells were used for polyacrylamide gel electrophoresis. Power was supplied by a Heathkit Model IP-32 and a Beckman/Spinco constant voltage supplier.

A Perkin-Elmer Model 38-A Tiselius electrophoresis apparatus was used for free-boundary electrophoresis. The resistances of all buffers and protein solutions were determined with an Industrial Instruments' Model RC conductivity bridge.

Amino acid analyses were performed with a Beckman/Spinco Model 120 C amino acid analyzer. A Beckman DK-2A ratio recording spectrophotometer, equipped with quartz cells, was used for all colorimetric determinations. All viscosity measurements were made using a Hewlett Packard Model 5901B Auto-viscometer equipped with Thompson's thermostatic water bath and circulator, Model TV-40. The densities of buffers were measured pycnometrically at controlled temperatures.

A Beckman/Spinco Model E analytical ultracentrifuge equipped with a RTIC temperature control unit and phase

plates as schlieren and interference diaphragms was used for the sedimentation coefficient, diffusion coefficient, molecular weight, initial protein concentration, and partial specific volume determinations. A capillary-type, synthetic-boundary cell was used for sedimentation studies, and a double-sector cell was used for determining the partial specific volume and molecular weight. A capillary-type, double-sector synthetic-boundary cell was used for the determination of initial protein concentration and diffusion coefficient. In all determinations, 12 mm filled Epon centerpieces with quartz windows were used. An-D and An-J Duralumin rotors were used for centrifugation and a General Electric AH-6 mercury lamp served as the light source.

Kodak metallographic and Type II-G spectroscopic glass plates were used for recording the schlieren and interference patterns, respectively. A Nikon Model 6 Shadowgraph microcomparator was used for measuring the recorded patterns.

A Brice-Phoenix Differential Refractometer Model BP-2000-V was used to adjust the refractive index of buffers for interference centrifugation and to check protein concentrations.

Chemicals and Materials

The principal chemicals used in this research and the suppliers are given below.

Tris-hydroxymethyl aminomethane (Sigma 121 and Trisima

Base) was used as a primary standard and Sigma 7-9 was used to prepare buffers. Both were obtained from Sigma Chemical Company. Diethylbarbituric acid and sodium diethylbarbituric acid for veronal buffer, monosodium phosphate and disodium phosphate for phosphate 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.

p-Dimethylaminobenzaldehyde, 2-thiobarbituric acid and N,N,N',N'-tetramethylethylenediamine were obtained from Eastman Organic Chemicals. Reagent grade ammonium sulfate and urea were purchased from Fisher Scientific Company.

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

Bio Gel P-series and 1% solution of dimethyldichlorosilane in benzene were obtained from Bio-Rad Laboratories. Sephadex G-series and Sepharose 6B were obtained from Pharmacia Fine Chemicals. Cellulose N,N-diethylaminoethyl ether was acquired from Fisher Scientific Company.

Fucose, galactosamine and glucosamine hydrochloride were obtained from Nutritional Biochemicals Corporation. Galactose was purchased from Pfanstiehl Laboratories, Incorporated and mannose from Fisher Scientific Company.

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Triphenyltetrazolium chloride was obtained from Eastman Organic Chemicals and penicillin "G" from Allied Chemicals Corporation. N-acetyl neuraminic acid, tryptophan, and streptomycin sulfate were acquired from Calbiochem.

All other chemicals used in this study were of reagent grade.

Preparative Procedures

The milk used in this study was obtained from Michigan State University dairy herd which consisted of Brown Swiss, Jersey and Holstein cows. All milk was collected immediately at the afternoon milking and separated immediately while still warm.

Preparation of Euglobulin and Pseudoglobulin

The euglobulin and pseudoglobulin fractions were prepared by combining the ammonium sulfate precipitation method described by Smith (1946a; 1946b) and column chromatographic technique using Sepharose 6B. The isolation procedure for euglobulin and pseudoglobulin 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 fat phase was separated, the resulting skim milk was adjusted to pH 4.6 with 0.1 N HCl and the precipitated casein was removed by filtering through double layered

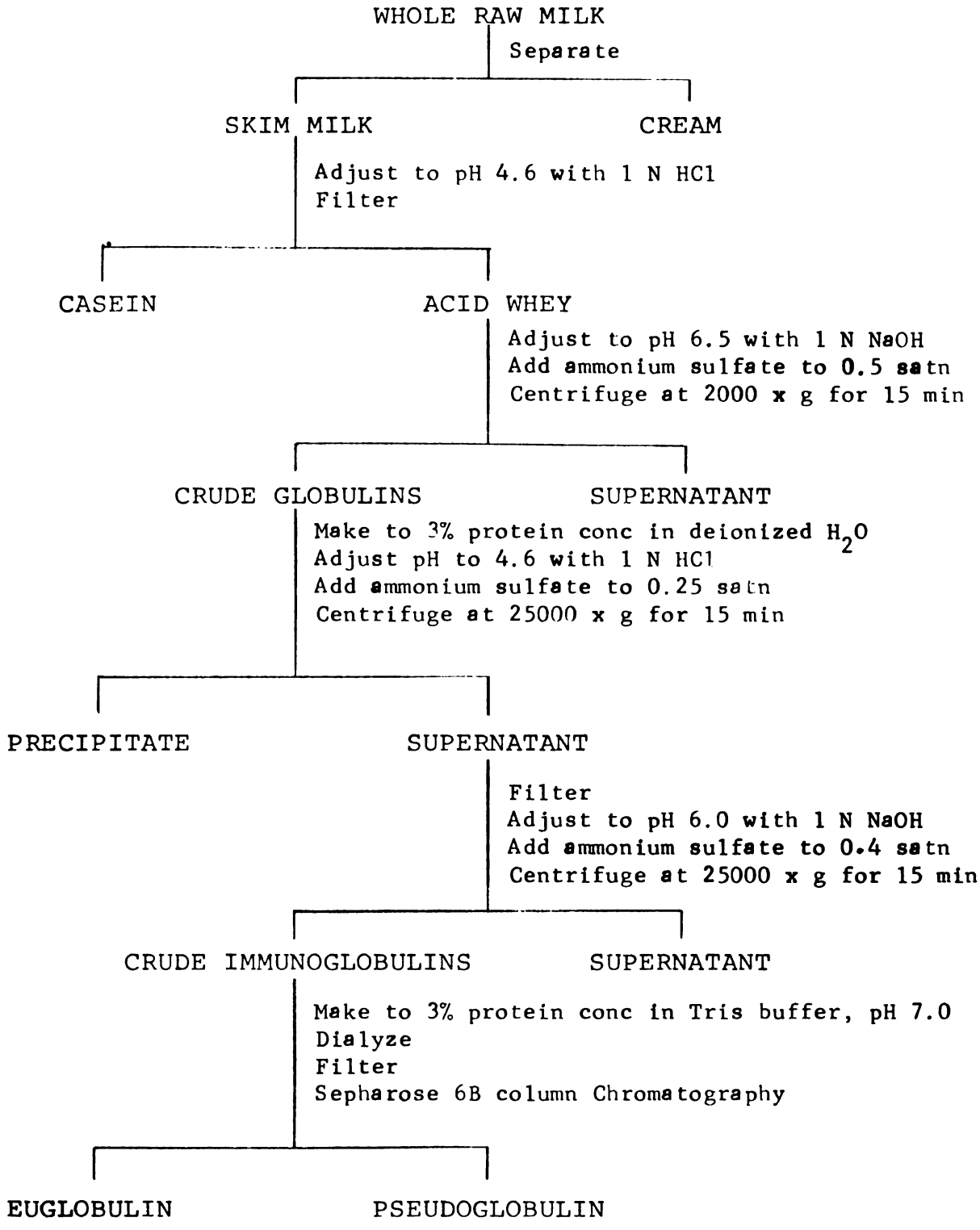

Figure 1. Schematic for the fractionation of euglobulin and pseudoglobulin from cow's milk.

cheese cloth. The acid whey was adjusted to pH 6.5 with 0.1 N NaOH, and solid ammonium sulfate added to 0.5 saturation to salt-out the crude globulin fraction. This precipitate was collected by centrifugation at 2000 x g for 15 minutes and redissolved to about 3% protein concentration in deionized water. The pH was adjusted to 4.6 and ammonium sulfate added to 0.25 saturation. After centrifuging at 25,000 x g for 15 minutes to remove a suspended precipitate, the supernatant was filtered through a thick layer of glass wool. The immunoglobulins were precipitated from this supernatant with ammonium sulfate added to 0.4 saturation at pH 6.0. The precipitated globulins were collected by centrifugation at 25,000 x g for 15 minutes.

Separation of Euglobulin and Pseudoglobulin by Gel Filtration. The principle of gel filtration employing Sepharose 6B was used for separating euglobulin and pseudoglobulin fractions from the enriched immunoglobulin fraction.

Sepharose 6B has a molecular weight exclusion limit of approximately 400,000. The column support was washed with deionized water three times followed by equilibration with 0.02 M tris-HCl buffer, pH 7.0, before packing the column. Chromatographic columns (2.5 x 45 cm) equipped with up-flow adaptors were filled with eluting buffer. A funnel was attached on top of the column and the equilibrated beads were poured into the column with constant stirring. Following the formation of a thin layer of beads at the bottom

of the column, the outlet was opened to allow the bed to pack more tightly and to complete the packing. The final column height was 30 cm. A void volume of 60 ml was determined by eluting a solution of Blue Dextran 2000 through the column. The flow rate was approximately 200 ml/hr.

The immunoglobulin fraction obtained by salting-out with ammonium sulfate was dissolved in 0.02 M tris-HCl buffer, pH 7.0, at about 3% protein concentration and dialyzed against a large volume (4 l) of the same buffer for 48 hr with constant stirring at 3° C. The dialyzing buffer was changed every 12 hr.

The dialyzed protein solution was filtered through a thick layer of glass wool and a 10 ml aliquot of this solution was applied to the column by siphon action through the bottom of the column. The column effluent was monitored at 254 nm. Each fraction was collected separately and concentrated by pervaporation to one quarter of its volume.

Purification of Euglobulin and Pseudoglobulin Fractions. The euglobulin and pseudoglobulin fractions separated by gel filtration were purified using an anion exchange cellulose column chromatographic technique. N,N-Diethylaminoethyl ether-cellulose (DEAE-cellulose) was first soaked in 1 N NaOH for an hour to remove yellow impurities and to ensure complete regeneration. The regenerated adsorbent was washed with a large volume of deionized water to remove NaOH, following several volumes of 0.02 M phosphate buffer (pH 7.0). The resulting suspension

was allowed to stand for 30 min to permit settling of large cellulose particles. The fine particles remaining in the supernatant were discarded. The suspension of sedimented particles was poured as slurry into a Sephadex chromatographic column (2.5 x 45 cm). The adsorbent was allowed to settle under the flow conditions induced by gravity, a filter disc was placed on the top of the column to prevent erosion of the supporting material by the incoming buffer solution. The adsorbent was further compacted by nitrogen pressure at 5-6 p.s.i. until the column height was constant--approximately 30 cm. The column was washed with the eluting buffer at pH 7.0 to obtain equilibrium.

Approximately 10 ml of the pervaporated protein solution was loaded on the column and eluted with 0.02 M phosphate buffer. The elution pattern was monitored at 254 nm and the flow rate adjusted to about 200 ml/hr. The eluted, purified proteins were desalted by passing the column cut through a Bio Gel P-10 column (5.5 x 30 cm) equilibrated with deionized water, lyophilized, and stored at -15° C in a tightly sealed brown bottle.

The lyophilized proteins were dried in a vacuum oven at room temperature over P_2O_5 for 24-48 hr before any chemical and physical analyses were conducted. Dry weights were used as the concentration basis for all samples.

Preparation of Washed Cream

Washed cream was prepared according to a method

described by Swope (1968). After separating the cream, it was diluted with warm (40° C) deionized water (1:3 dilution) and reseparated. This process was repeated five times to remove all of the whey proteins. Aliquotes of cream and corresponding washings were collected after each separation to check the amounts of whey proteins left in the cream. This washed cream was preserved for subsequent experiments by adding penicillin "G" and streptomycin sulfate at the ratio of 50 mg and 5,000 units per liter, respectively.

Samples taken from skim milk, whey and each cream washing were dialyzed against deionized water for 24 hr with constant stirring to remove milk salts. Following lyophilization, 5% suspensions of the residues were analyzed for whey proteins by polyacrylamide gel electrophoresis according to a method described by Melachouris (1966).

Chemical Methods

Nitrogen

A micro-Kjeldahl apparatus was used for the nitrogen determination. The digestion mixture consisted of 5.0 g $CuSO_4 \cdot 5H_2O$ and 5.0 g SeO_2 in 500 ml of concentrated sulfuric acid. Approximately 15 mg of dried protein in duplicate for each sample were digested with 4 ml of the digestion mixture over a gas flame for 1 hr. After cooling, 1 ml of 30% H_2O_2 was added to each flask and digestion continued for one additional hour. Each digestion flask was cooled and rinsed with 10 ml of deionized water. Then,

each digested mixture was neutralized with approximately 25 ml of a 40% sodium hydroxide solution. The released ammonia was steam distilled into 15 ml of a 4% boric acid solution containing five drops of indicator. The indicator consisted of 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was continued until a final volume of 60 ml of the solution was collected in the receiving flask. The ammonia-borate complex was titrated with 0.020 N HCl which had been standardized with trishydroxymethyl aminomethane serving as a primary standard.

The average recovery for a tryptophan standard was 98.83%.

Phosphorus

Phosphorus was determined in duplicate for each sample of protein according to the method described by Sumner (1944). About 10 mg of dried protein were digested with 2.2 ml of a 50% sulfuric acid in Pyrex test tubes. Digestion was carried out for 20 min on a sand bath heated by an electric heater at a temperature of 160 to 170° C until the protein was completely charred. After cooling, 1.0 ml of 30% H_2O_2 was added to each test tube and the heating continued for 15 min. This step was repeated to obtain colorless solutions. The complete removal of all remaining H_2O_2 residue is very important to determine phosphorus content correctly. The digested mixture was cooled and transferred

to a 25 ml volumetric flask. Five milliliters of a 6.6% $(NH_4)_6MoO_{24}\cdot 4H_2O$ solution and enough deionized water were added to give a volume of approximately 15 ml. Then 4 ml of a freshly prepared solution of 5 g $FeSO_4\cdot 7H_2O$ in 50 ml of water plus 1 ml of 7.5 N sulfuric acid were added and quickly mixed. The volumetric flask was made up to volume with deionized water and mixed. Color was allowed to develope for 30 min. Percentage transmittance was read at 660 nm with a Beckman DK-2A spectrophotometer.

Ten milliliters of a stock solution containing 1.3613 g of KH_2PO_4 dissolved in 1,000 ml of deionized water were diluted to 100 ml to give 0.031 mg phosphorus/ml of standard solution. A standard curve covering the range of zero to 0.28 mg phosphorus was prepared.

Carbohydrate

The components comprising the carbohydrate moieties of euglobulin and pseudoglobulin fractions were identified by a paper chromatographic technique. Approximately 25 mg of dried sample were placed in a 20 ml ampoule and 10 ml of 2 N hydrochloric acid were added. After the solution was frozen in a dry ice-ethanol bath, the ampoule was evacuated to remove dissolved air from the sample and immediately sealed. The sealed ampoule was placed in a 110° C oil bath for five hours.

The partial hydrolysate was transferred to a 50 ml round-bottom flask fitted to a rotary evaporator and dried.

The residue was taken up in a small amount of deionized water and redried. This process was repeated two more times. The final residue was dissolved in 10 ml of deionized water and passed through a 2 x 20 cm column of Dowex resin. The column was prepared as follows: Dowex 1 x 8 in Cl^- form was washed with 1 N NaOH to convert to the OH^- form. Then, the resin was converted to acetate form by washing with 1 N acetic acid. Next, the resin was washed with deionized water until the pH of the washing was between 5.0 and 9.0. A second resin consisting of Dowex 50 x 8 was converted to the H^+ form by washing with 1 N HCl and rinsing with deionized water as above. The column was packed with Dowex 50 x 8 (H^+ form) in the bottom half and with Dowex 1 x 8 (acetate form) in the top half.

The eluate collected from this column was lyophilized and extracted with 10 ml of redistilled pyridine at 100° C for 20 min according to the procedure described by Block et al. (1958). The resulting pyridine mixture was cooled, filtered, and the solvent removed with a rotary evaporator at 40° C. The resulting residue was dissolved in 2 ml of 10% isopropanol. This solution of unknown carbohydrates was spotted on Whatman No. 1 (10 x 42 cm) chromatography paper and developed as an ascending chromatogram for 20 hr with a butanol-acetic acid-water (4:1:5) solvent system (Bezkorovainy, 1963). Authentic standards of fucose, glucose, galactose, mannose, glucosamine, and galactosamine were analyzed concurrently with the unknown samples.

The carbohydrate constituents were detected by spraying the dried chromatogram (60° C for 15 min) with a mixture of equal volumes of a 2% aqueous solution of triphenyltetrazolium chloride and 1 N NaOH (Block et al., 1958). The sprayed chromatograms were placed in a moist atmosphere at about 60-70° C until the red spots appeared.

Fucose

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

Approximately 2 mg of dried protein samples were placed in test tubes (15 x 150 mm) and 5 ml of a 95% ethanol was added. After mixing, the tubes and their contents were centrifuged for 15 min, the supernatant decanted and the precipitates suspended in 5 ml of 95% ethanol. The solutions of protein were again centrifuged and the supernatants decanted. The precipitated proteins were dissolved in 1 ml of 0.1 N NaOH and 4.5 ml of ice cold H_2SO_4-H_2O mixture (6 volumes of concentrated c.p. H_2SO_4 and 1 volume of H_2O) were added. The contents were well mixed while maintaining a low temperature in an ice bath. Then, the tubes and their contents were heated for exactly 3 min in a boiling water bath, then cooled in tap water. To this mixture 0.1 ml of the cysteine reagent (3 g of cysteine hydrochloride in 100 ml of water) was added and the contents mixed immediately. The addition of the cysteine

reagent was omitted from one of the duplicate samples to correct for nonspecific color development. After 60 to 90 min at room temperature, optical density readings were made at 396 and 430 nm with distilled water set at zero.

The fucose content of the proteins was calculated from the differences in the readings obtained at 396 and at 430 nm after correcting for the nonspecific color increment, i.e., samples without cysteine added:

$$\frac{(OD_{396}-OD_{430})_s - (OD_{396}-OD_{430})_b}{(OD_{396}-OD_{430})_{std}} \times 0.02 \times 1000$$

= mg of fucose per 100 g of protein

The standard solution was prepared by dissolving 20 mg of fucose per liter of distilled water.

Hexose

Hexose analyses were performed colorimetrically by the method of Dubois et al. (1956).

Two to three milligrams of protein were placed in a test tube and 3 ml of deionized water and an equal volume of 5% phenol solution were added and mixed. Then, 15 ml of concentrated sulfuric acid were added rapidly while stirring simultaneously with a Mini-shaker to obtain maximum mixing and heat generation which is critical to the reproducibility of the assay. After 10 min of standing, the tube was stirred again and placed in a water bath at 25 to 30° C for 30 min. The percentage transmittance was

monitored at 490 nm.

A standard curve was constructed covering the range of zero to 200 µg of galactose-mannose (1:1, w/w) per 3 ml of water.

Hexosamine

The method described by Johansen et al. (1960) was used to determine hexosamine content of the proteins.

About 3 mg samples of protein were placed directly into 5 ml ampoules. After adding 1 ml 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 6 hr at 100° C in a hot air oven. After cooling, the hydrolyzed samples were transferred to distillation flasks. The empty ampoules were rinsed with 1 ml of 4N NaOH, followed by two 1 ml rinsings with deionized water.

The acetylacetone reagent was prepared by dissolving 1 ml of freshly distilled 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 recrystallized p-dimethylaminobenzaldehyde (Rondle and Morgan, 1955) in absolute ethanol containing 3.5% of concentrated HCl. This solution was diluted to a final volume of 250 ml.

To each of the distillation flask containing the hydrolyzed sample were added 5.5 ml of the acetylacetone

reagent. The final pH of this mixture must be maintained between 9.5 and 10.0. The flasks were tightly stoppered and heated in a boiling water bath for 20 min. Then, each cooled flask was heated with a Bunsen burner until 2.5 ml of the distillate containing the steam-volatile chromogen was condensed in 7.5 ml of Ehrlich's reagent. The volumetric flasks were stoppered and the contents mixed. After 30 min, the transmittance (%) of the solutions was read at 548 nm with the spectrophotometer.

Standard glucosamine and galactosamine solutions (25 µg) were treated in the same manner to check the recovery. The average recovery of the duplicate runs was 98.79%.

Equal amounts of glucosamine and galactosamine were combined and used to construct a standard curve which spans the range of zero to 50 µg carbohydrates per 3 ml. The blank consisted of 1 ml of 4 N HCl, 1 ml of 4 N NaOH and 2 ml of deionized water.

Sialic Acid

Warren's (1959) thiobarbituric acid method as modified for milk proteins by Marier et al. (1963) was adapted for the sialic acid determination.

Approximately 1.5 mg of dried proteins were placed in test tubes. Following the addition of 0.5 ml of 0.1 N sulfuric acid to each tube, digestion was performed at 80° C for 40 min to release the sialic acid. To each hydrolyzed sample was added 0.1 ml of the periodate solution

(0.2 M meta-sodium periodate in 9 M phosphoric acid) and the mixture was incubated at 25° C for 20 min. Following the addition of 1 ml of arsenite solution (10% sodium arsenite in a solution containing 0,6 M sodium sulfate and 0.1 N sulfuric acid), the tubes were shaken until the solution became colorless. Three milliliters of a 0.6% of recrystallized 2-thiobarbituric acid solution in 0.5 M sodium sulfate were added and the tubes shaken, covered tightly and placed in a boiling water bath for 15 min. After cooling, 7 ml of cyclohexanone were added and the tubes shaken vigorously. The contents were centrifuged in a clinical centrifuge for 10 min to obtain a clear cyclohexanone phase. The transmittance (%) was measured at 549 nm.

A standard curve was contructed using N-acetyl neuraminic acid in the range of zero to 50 µg in 0.5 ml of water.

Tryptophan

Tryptophan content of a protein must be determined separately from the rest of the amino acids since it is labile to acid hydrolysis.

In this study tryptophan was analyzed spectrophotometrically as described in Procedure W by Spies (1967). A 3 mg sample of protein was weighed directly into a 1.5 ml glass vessel. To each sample were added 100 µl of freshly prepared pronase solution, containing 10 mg of pronase per milliliter of 0.1 M phosphate buffer (pH 7.5), and a drop of toluene. Each vessel was placed in a vial, and the

vials were stoppered and then incubated for 24 hr at 40° C. After cooling, the vessels were placed in 25 ml Erlenmeyer flasks containing 9.0 ml of 21.1 N sulfuric acid and 30 mg p-dimethylaminobenzaldehyde which had been prepared immediately before use. To each vessel was added 0.9 ml of a 0.1 M phosphate buffer solution. The vessels were tipped over and the contents were quickly mixed by gentle swirling. The flasks were placed in the dark at room temperature for 6 hr. After adding 0.1 ml of 0.045% sodium nitrate solution, the reaction mixtures were shaken and the color was allowed to develope for 30 min in the dark at room temperature. The transmittance (%) was read at 590 nm.

Duplicate samples of pronase solution, without sample protein, were treated and analyzed as described above. The tryptophan content of pronase solution was subtracted from the total tryptophan content. The blank solution contained everything but protein and pronase.

A standard curve was prepared using zero to 120 µg of tryptophan in phosphate buffer solution as described in Procedure E and F by Spies and Chambers (1948).

Amino Acid

The amino acid analyses were performed on 20 and 70 hr hydrolysates of the protein employing a Beckman Amino Acid Analyzer Model 120 C (Moore and Stein, 1954; Moore et al., 1958).

About 4 mg portions of the dried proteins were weighed

directly into 10 ml ampoules. Six milliliters of 6 N HCl were added to the ampoules. The contents of the ampoules were frozen in a dry ice-ethanol bath, evacuated with a high vacuum pump, allowed to melt slowly to remove gases, re-frozen and sealed 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 either 20 or 70 hr hydrolysis the ampoules were removed from the oil bath and cooled to room temperature (25° C).

In order to check the transfer losses, 1 ml of 2.5 μM norleucine solution was added to each ampoule before transferring the hydrolysate to a pear-shaped evaporating flask. 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 residue was removed. Finally, the dried hydrolysate was dissolved in 0.067 M citrate-hydrochloric acid buffer, pH 2.2, and the volume was made up to 5 ml. 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 a week.

The amino acid composition of the protein samples was expressed as gram residues of amino acids per 100 g protein, number of amino acid residues per mole of methionine residue, and moles of amino acid residues per 1000 moles of total amino acid residues.

Physical Methods

Free-boundary Electrophoresis

A Perkin-Elmer Model 38-A Tiselius electrophoresis apparatus was used to determine the electrophoretic mobilities of the protein fractions in free solutions.

Electrophoretic mobilities were calculated for the ascending and descending patterns using the following equation:

$$\mu = \frac{d\ a\ k}{t\ i\ m\ r}$$

where μ is the electrophoretic mobility in 1×10^{-5} cm^2 sec^{-1} $volt^{-1}$, d the distance the boundary migrated from the initial boundary in cm, a the cross sectional area of the electrophoretic cell in cm^2, k the conductivity cell constant, t the time of electrophoresis in seconds, i the current applied in amperes, r the resistance of the protein solution or buffer used in dialyzing the protein solution in ohms determined in a conductivity cell at the same temperature as the electrophoresis run, and m the magnification factor of the optical system.

Veronal buffer, pH 8.6, $\mu = 0.1$, was used as the solvent system for the electrophoretic mobility determinations. Electrophoretic mobilities determined at pH 4.0, 5.0, 6.0, 7.0, and 8.6 ($\mu = 0.2$) in the buffers described by Miller and Golder (1950) were used to determine isoelectric points.

The composition of these buffers is presented in Appendix. The isoelectric points were estimated from a plot of the average ascending and descending mobilities against the pH values.

Protein samples were dissolved in the buffers at the concentration of approximately 1.0 to 1.5% and the solutions were dialyzed against 600 ml of the same buffer for 24 hr at 2-3° C and with constant stirring. All free-boundary electrophoretic analyses were performed at ≈1° C. The boundaries in the Tiselius cell were photographed both at the beginning and termination of the run.

Polyacrylamide Gel Electrophoresis in a Discontinuous Buffer System

The procedure for preparing and performing the discontinuous acrylamide gel electrophoresis was adopted from the method described by Melachouris (1969). The discontinuous gel system, consisting of a running gel and a spacer gel, was prepared by using two working solutions. The running gel solution was prepared by dissolving 45 g of Cyanogum 41, a mixture of acrylamide and N,N'-methylenebisacrylamide, in 0.38 M tris-HCl buffer, pH 8.9, and making up the volume to 500 ml. To this solution, 0.5 ml of N,N,N',N'-tetramethylethylenediamine (TEMED) was added. Cyanogum 41 at 5% concentration in 0.062 M tris-HCl buffer, pH 6.7, was prepared as the spacer gel. The catalyst, TEMED, was then added in the ratio of 0.1 ml TEMED per 100 ml gel solution. The working gel solutions were filtered and stored at 2-3° C.

All solutions were brought to room temperature before use.

The running and spacer gels were cast in a Plexiglas gel bed (26 x 12 x 0.4 cm). A Plexiglas divider was placed 15 cm from one end of the gel bed and silicon grease used to prevent seepage of the gel-forming solution. The large area of the gel bed was filled with 190 ml of running gel solution containing 2 ml of a freshly prepared 10% (w/v) solution of ammonium persulfate in deionized water. The divider was removed after polymerization of the running gel solution. Then, the smaller area of the gel bed was filled with 90 ml of the spacer gel solution containing 1 ml of the ammonium persulfate solution. The slot former (1 x 0.1 x 0.3 cm) was inserted into the spacer gel solution 0.5 cm from the edge of the running and spacer gel interface.

The protein samples were dissolved in the spacer gel solution diluted 1:1 with deionized water at approximately 5% protein concentrations. Bromophenol Blue was added to each sample slot as a marker.

Approximately 15 µl samples were applied to the slots with the aid of a micro-syringe and the slots were covered with mineral oil. The gel was covered with Saran Wrap to reduce evaporation and connected to electrode vessels filled with 1,600 ml of 0.046 M tris-glycine buffer, pH 8.3. Platinum electrodes were inserted into the buffer tanks and electrophoresis was carried out at 15° C at a constant voltage of 180 to 200 volts (instrument) until the buffer front migrated 12 cm from the sample slots.

Upon completion of the electrophoretic run, the gel was removed from its frame and stained for 10 min in a dye solution consisting of 250 ml of water, 250 ml of methanol, 50 ml of glacial acetic acid and 5 g of amido black 10B (naphthol blue black). The excess dye in the gel was removed electrically in an electric destaining cell containing 7% acetic acid.

The electrophoretogram was photographed using a Polaroid MP-3 Land Camera for a permanent record.

Ultracentrifugation

Protein Concentration. Protein samples were weighed on a microbalance and dissolved in appropriate solvents. Following dialysis against the solvent, the concentration was rechecked by measuring the refractive index of the solution with a differential refractometer. The protein concentration was determined from a standard curve of refractive index differences and concentration of protein.

In many cases, the concentration was determined by measuring the area of the schlieren pattern for the initial boundary formed in a double-sector cell. The amount of protein was then determined from a plot of area versus known concentration of protein from the data obtained in a similar manner.

Density. The solvent densities were measured with 25 ml pycnometer at 20° C. The densities of solutions were

calculated according to the relation given by Fujita (1962):

$$\rho_{solution} = \rho_{solvent} + (1 - \bar{v}\rho_{solvent}) \cdot C$$

where ρ is the density in g cm^{-3}, $\bar{v}$ the partial specific volume of the protein in ml g^{-1}, and C its concentration in g cm^{-3}. The pycnometers were calibrated with freshly boiled, redistilled water at 20° C. The densities of the solvent used are presented in Appendix.

Viscosity. The viscosities of solvent and solution were measured in a standard Ubbelohde Dilution Suspended Level Type ASTM D 445 viscometer. The viscosity equation applicable to capillary type viscometers is written as follows:

$$\eta = A\rho t - \frac{m\beta\rho}{t}$$

where η is the absolute viscosity in g sec^{-1} or Poise, A the viscometer constant, t the efflux time in seconds, m the kinetic energy coefficient, and β the kinetic energy constant.

To calibrate a capillary viscometer, the efflux times of freshly boiled, redistilled water at two temperatures were measured. With the efflux times measured and the densities and viscosities known for the two liquids, the viscometer calibration constants A and $m\beta$ were calculated from the above equation.

The absolute viscosity for an unknown solvent or solution was then calculated from the factors A and mβ and the measured efflux time using the same equation. All solutions were filtered through a fine sintered glass filter before measuring the efflux time.

The relative viscosity was then calculated according to the relation:

$$\frac{\eta}{\eta_o} = \frac{t}{t_o}\,\frac{\rho}{\rho_o}$$

where η, t, ρ are the viscosity, efflux time, and density of the solution and η_o, t_o, ρ_o represent the corresponding values of the solvent. The viscosities of the solutions used relative to those for solvent are presented in Appendix Table 1.

Partial Specific Volume. The partial specific volume for the protein was determined by performing two parallel sedimentation equilibrium experiments of a given substance in H_2O and D_2O solutions at the same temperature and protein concentration (Edelsteine and Schachman, 1967).

Two double-sector cells were used for this purpose. The solution containing 0.1 mg of protein in 0.1 M veronal buffer in H_2O, pH 8.6, was placed in one compartment of a double-sector cell and the buffer in the other compartment. The second cell contained the analogous solution of protein in the above buffer prepared with 90% D_2O in one compartment and the corresponding buffer in the other.

For this experiment an ultracentrifuge speed of 16,000 rpm was used and the resulting interference pattern was photographed after 24 hr. The fringe measurements were made from the recorded pattern and the data were plotted as the logarithm of the fringe displacement against the square of the distance, r, from the axis of rotation. The partial specific volume was then calculated according to the equation:

$$\bar{v} = \frac{k - [(d\ln c/dr^2)_{D_2O}/(d\ln c/dr^2)_{H_2O}]}{\rho_{D_2O} - \rho_{H_2O}\,[(d\ln c/dr^2)_{D_2O}/(d\ln c/dr^2)_{H_2O}]}$$

where k is the ratio of the molecular weight of the protein in the deuterated buffer to that in the nondeuterated one and the term, $d\ln c/dr^2$, represents the slope of the plot.

Diffusion Coefficient. The diffusion coefficient was determined in a double-sector synthetic boundary cell at a low centrifuge speed of about 4,000 rpm and computed by using the relation:

$$D_{app} = (A/H)^2\ (1/4\pi t)\ (1 - \omega^2 st)$$

where D_{app} is the apparent diffusion coefficient in $cm^2\ sec^{-1}$, A the area enclosed by the sedimentation boundary curve above its base line in cm^2, H the maximum height of the boundary in cm, t the time measured from the start of centrifugation in seconds, and ω the angular velocity of

rotation in radians sec^{-1} (Lamm, 1929).

The sample was dissolved in appropriate solvents at different concentrations. The cell was filled with 0.15 ml of the solution in one compartment and the solvent in the other. Schlieren photographs were taken at preset time intervals. After measuring the plates, the data were plotted as $\underline{(1/4\pi)(A/H)^2}$ versus $\underline{t}$ to obtain the apparent diffusion coefficient (D_{app}) which is equal to the slope of the line. The observed diffusion coefficient (D_{obs}) was obtained by plotting the values of apparent diffusion coefficient against the protein concentrations. The intercept of this linear plot at infinite dilution is the observed diffusion coefficient.

Since the value of observed diffusion coefficient obtained as above relates to the given solvent and temperature, it is common practice to report the diffusion coefficient corresponding to a temperature of 20° C in a solvent with the viscosity of water, $D_{20,w}$. The correction of the observed diffusion coefficient to this standard condition was made by using the following equation:

$$D_{20,w} = D_{obs} \cdot [293/(273 + t)] \cdot (\eta_s/\eta_w)(\eta_t/\eta_{20})_w$$

where $\underline{t}$ is the temperature of the diffusion experiment in degrees centigrade, (η_s/η_w) the relative viscosity of solvent to that of water, and $(\eta_t/\eta_{20})_w$ the relative viscosity of water at the temperature of the experiment to

that at 20° C.

Sedimentation Coefficient. The sedimentation-velocity experiments were carried out at 20° C using a rotor speed of 59,780 rpm (259,700 x g). The low sedimentation coefficients observed for the proteins in the presence of dissociating agents necessitated the use of the synthetic boundary cell in this study. Accurate sedimentation coefficients are obtained with this cell for solutes whose coefficients are less than one Svedberg unit because the problem of restricted diffusion at the meniscus is eliminated (Schachman, 1959). Furthermore, the concentrated guanidine hydrochloride solution forms gradients at the meniscus making the observation of the protein boundary difficult unless a synthetic boundary cell is used. Buffers with an ionic strength of 0.1 or greater were used to dampen the charge effects inherent to proteins in solution (Schachman, 1957).

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

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

where r is the distance of the boundary from the axis of rotation in cm, t the sedimenting time in seconds, and ω the angular velocity in radians sec^{-1}. By integrating the above equation, the following relation is obtained:

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

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

$$S = 1 \times 10^{-13} \text{ sec} = \frac{2.303/60}{(2\pi \cdot \text{rpm}/60)^2} \cdot \text{slope}$$

Sedimentation coefficients are usually reported as $S_{20,w}$, which is the value the protein would have in a solvent with the density and viscosity of water at 20° C. Therefore, the observed sedimentation coefficients were corrected to this standard condition according to the following equation:

$$S_{20,w} = S_{obs} \cdot \left(\frac{\eta_t}{\eta_{20}} \right) \left(\frac{\eta_s}{\eta_o} \right) \left(\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t} \right)$$

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 the same in all solvent systems used in this experiment.

The standard sedimentation coefficient at infinite dilution, $S^{\circ}_{20,w}$, was obtained by plotting the values of $S_{20,w}$ versus the concentrations of protein and extrapolating to zero concentration (infinite dilution).

Low-speed Sedimentation-equilibrium. Molecular weights were determined using the low-speed short-column sedimentation-equilibrium technique described by Van Holde and Baldwin (1958). This method covers the protein concentration range of 0.2 to 1.2%. Analyses were carried out in double-sector cells using a column depth of 1 to 2 mm. The rotor speed was estimated from the relation:

$$\ln 4 = \frac{M\,(1 - \bar{v}\rho)\,\omega^2}{RT}\,\frac{b^2 - a^2}{2}$$

where M is the estimated molecular weight of the protein, R the gas constant, T the absolute temperature, and a and b the distances from the axis of rotation to the air-solution meniscus and to the bottom of the solution, respectively.

The time required to achieve equilibrium condition was approximated by the equation:

$$t_{0.1\%} = 0.7 \cdot \frac{(b - a)^2}{D}$$

where D is the diffusion coefficient and $t_{0.1\%}$ the time required to achieve the concentration difference across the solution column within 0.1% of the equilibrium value.

The experiment was conducted as follows: first, an amount of fluorovarbon FC 43 oil was introduced into both sectors of the cell to provide a false bottom; secondly, enough protein solution was added to give a column depth of 1 to 2 mm. Finally, the solvent was introduced into the other sector of the cell to provide a column of solvent greater in depth than the solution. The solvent column extended above the air-solution meniscus and below the oil-solution meniscus of the protein solution.

The schlieren pattern was photographed and measured, and the apparent weight- and z-average molecular weights were calculated according to the equations:

$$\bar{M}_{w,app} = \frac{c(b) - c(a)}{c^\circ} \frac{2}{b^2 - a^2} \frac{RT}{\omega^2 (1 - \bar{v}\rho)}$$

$$\bar{M}_{z,app} = \frac{RT}{\omega^2 (1 - \bar{v}\rho)} \frac{[\Delta Y(b)/b] - [\Delta Y(a)/a]}{c(b) - c(a)}$$

where $\bar{M}_{w,app}$ and $\bar{M}_{z,app}$ are the apparent weight- and z-average molecular weights, respectively, $\underline{c(b)-c(a)}$ the difference in protein concentration between the bottom of the solution and the air-solution meniscus, $\underline{c}^\circ$ the initial protein concentration, and $\underline{\Delta Y(b)}$ and $\underline{\Delta Y(a)}$ the net vertical displacement of the gradient curve at the bottom of the solution and that at the air-solution meniscus. The values of both apparent weight- and z-average molecular weights are

plotted against the protein concentrations and extrapolated to infinite dilution to obtain the corresponding true molecular weights, $\bar{M}^{\circ}_{w}$ and $\bar{M}^{\circ}_{z}$.

Creaming Studies

Most of the creaming property determinations were made on a series of samples in which the fat content was kept as uniform as possible to 3.6% according to the procedures described by Dunkley and Sommer (1944).

Creaming Properties of Normal Milk

The creaming properties of normal milk under various conditions were determined as reference to those of recombined milk samples as well as the model systems employed in this study. Both the cluster index and cream volume were used in expressing the creaming ability.

Cluster Index. The clustering ability of the fat was expressed in terms of a "cluster index". Creaming cells with the inside dimensions of 76 x 20 x 1 mm were constructed with thin microscope slides. Two cells were mounted on a thick glass base plate.

In order to eliminate the decrease in clustering properties resulting from storage of milk at low temperatures, 2-4° C, the samples were prewarmed to 40° C prior to the cluster index determinations. The procedures used for the prewarming treatment and for the determination of cluster index were as follows: approximately 200 ml of

fresh raw milk were measured into rubber stoppered bottles and held in a warm water bath at 40° C for 30 min with gentle agitation. The specially designed creaming cells were filled with warm milk and placed in a water bath at 4° C. The milk in the creaming cells was examined by transmitted light at one minute intervals until the clustering of the fat first became visible. The cluster index was calculated by dividing into 100 the time in minutes required for clustering to become visible. The milk samples which did not show clustering within 100 min were arbitrarily given a cluster index of zero.

Cream Volume. The volume of cream layer formed on milk under standardized conditions was used as a measure of the creaming ability of milk sample.

The milk was first prewarmed in the same manner as for the cluster index determinations. The milk was then transferred to 100 ml graduated cylinders and placed in a water bath maintained at 4° C. The creaming time was measured from the time the cylinders were placed in the 4° C bath. The cream volumes were divided by the percentage of fat in the milk in order to express the results as cream volume percentages per 1% fat.

Creaming Properties of Recombined Milk

The creaming properties of recombined milk samples were determined in the same manner as described for the normal milk samples. The recombined milk samples consisted of

three time-separated skim milk and five time-washed cream mixed in various combinations.

Creaming Properties of Model System

A model system consisting of purified euglobulin fraction and washed cream in simulated milk dialysates was developed to study the effects of protein concentration, temperature, hydrogen ion concentration and ionic strength on the creaming properties under controlled conditions. The cluster index and cream volume were determined as described before. The composition of Jenness and Koops' (1962) milk salt solution is presented in Appendix.

RESULTS AND DISCUSSION

Preparative Procedures

Preparation of Euglobulin and Pseudoglobulin

The isolation procedure for preparing a crude immunoglobulin fraction from normal cow's milk (Figure 1) was adapted essentially from Smith's (1946a) ammonium sulfate fractionation method. By following his original procedure, an electrophoretically homogeneous pseudoglobulin fraction was obtained by prolonged dialysis of an immunoglobulin fraction against large volume of distilled water. However, the euglobulin fraction always contained a small amount of pseudoglobulin even after repeated dialysis (3X). In addition to this pseudoglobulin contamination in euglobulin preparations, the dialysis procedure is so tedious that there exists a possibility for the denaturation of these two proteins during the purification process. Furthermore, the yields of these proteins were so small that large quantities of milk had to be processed to obtain sufficient working material. Therefore, his method was modified to obtain workable amounts of electrophoretically pure euglobulin and pseudoglobulin fractions in a relatively shorter time.

In this study, the final three steps of Smith's

procedure, i.e., 0.3 saturation of the crude immunoglobulin fraction (Fraction D) with ammonium sulfate at pH 4.6 to remove pseudoglobulin and other components (Fraction E), 0.4 saturation of the supernatant with ammonium sulfate at pH 6.0 to obtain immunoglobulin fraction (Fraction F), and exhaustive dialysis to separate euglobulin and pseudoglobulin, were replaced by Sepharose 6B, DEAE-cellulose and Bio Gel P-10 column chromatographic techniques. Among the buffer systems tested for their effectiveness in separating and purifying these proteins, the following systems were found to be most effective: 0.01 M tris-HCl buffer, pH 7.0, for the fractionation of crude immunoglobulins into euglobulin and pseudoglobulin through Sepharose 6B columns and 0.01 M phosphate buffer, pH 7.0, in purifying the separated immunoglobulin fractions on DEAE-cellulose anion exchange columns. The desalting process was carried out with deionized water through a Bio Gel P-10 column.

The elution pattern of the crude immunoglobulin fraction through Sepharose 6B column is presented in Figure 2. The first peak corresponds to euglobulin and the second to pseudoglobulin. The two small peaks were not characterized. Polyacrylamide gel electrophoretic patterns of skim milk, whey, crude immunoglobulin fraction, and euglobulin and pseudoglobulin fractions purified through DEAE-cellulose columns are presented in Figure 3. The free-boundary electrophoretograms of the purified euglobulin and pseudoglobulin in veronal buffer, pH 8.6, $\mu = 0.1$, are shown in

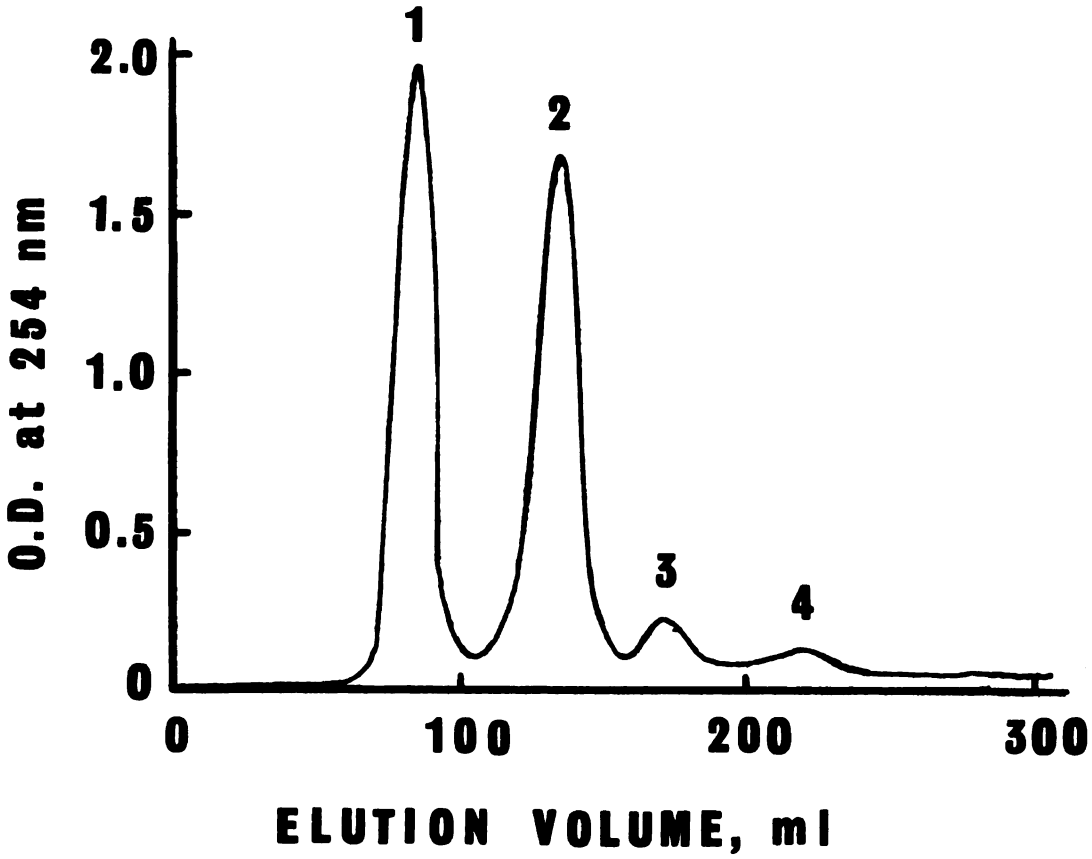

Figure 2. Elution diagram of crude immunoglobulin fraction through Sepharose 6B column with 0.01 M tris-HCl buffer, pH 7.0: Peak 1, euglobulin; Peak 2, pseudo-globulin; Peaks 3 and 4, unidentified.

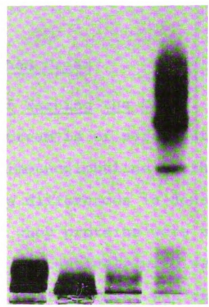

Figure 3. Polyacrylamide gel electrophoretic patterns of skim milk, whey, crude immunoglobulin fraction and purified euglobulin and pseudoglobulin: A-whey; B-crude immunoglobulin fraction; C-euglobulin; D-pseudoglobulin.

Figure 4. Electrophoretically pure euglobulin and pseudoglobulin fractions were obtained after once passing the eluates containing peaks 1 and 2 from the Sepharose 6B column through DEAE-cellulose column (see Figures 3 and 4). The yields of purified euglobulin and pseudoglobulin were approximately 20 and 30 mg per liter of skim milk, respectively.

Preparation of Washed Cream

The Effects of Washing the Cream. Removal of the whey proteins from the cream is of paramount importance for studying the role of immunoglobulins in the fat globule clustering. Due to the differences in density between the whey and the cream, the whey proteins were adequately removed by diluting the cream successively with water (1:3 dilution) and reseparating. Their removal was checked by polyacrylamide gel electrophoresis on each dried washing. The gel patterns are shown in Figure 5. Although equal amounts of a 5% concentration of the dried materials were applied to the gel slots, the band intensities decreased progressively to the fifth washing. Undoubtedly, the erosion products from the fat globule membrane, principally membrane lipoprotein complexes, made up the majority of the material recovered in the fourth and fifth washings. This material neither migrates in the gel nor stains well with amido black dye.

The concentration of the whey proteins decreased

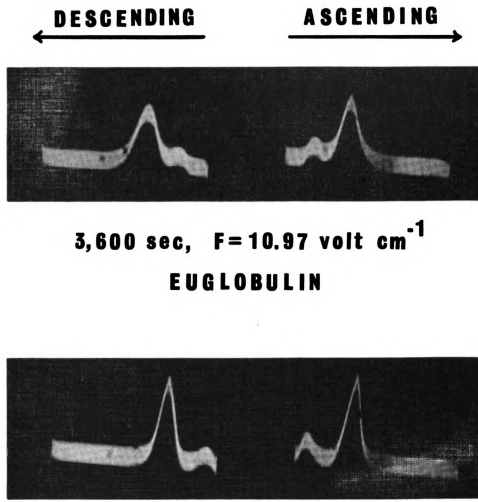

Figure 4. Free-boundary electrophoretic patterns of purified euglobulin and pseudoglobulin in veronal buffer, pH 8.6, ionic strength of 0.1.

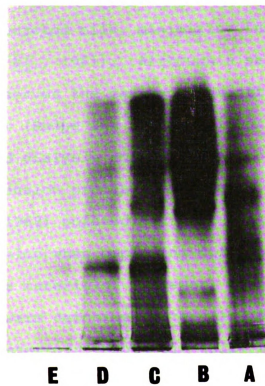

Figure 5. Polyacrylamide gel electrophoretic patterns of skim milk, whey and cream washings: A-skim milk; B-whey; C-first washing; D-second washing; D-third washing. Samples were applied at 5% concentrations.

considerably from the second to third washing and then became constant over the next several washings. Hence, three washings were sufficient to obtain a whey protein-free cream. The wash medium used was warm (40° C) deionized water. With this temperature of the wash medium, the separations were essentially carried out at the temperature of freshly secreted milk, minimizing induced lipolysis (Thompson et al., 1961). Although a saline-sucrose wash solution preserves the membrane integrity better than deionized water (Swope and Brunner, in preparation), the need for prolonged dialysis and the threat of bacterial growth in this suspension precluded its use.

Swope (1968) pointed out that the membrane is susceptible to erosion when employing dilution techniques to remove plasma materials. However, washing cream three times with warm deionized water (1:3 dilution) removed the plasma materials adequately while restricting erosion to acceptable levels.

The Effects of Antibiotics on Fat Globule Clustering. As mentioned previously in experimental section, the washed cream was preserved by adding streptomycin and penicillin "G" at the ratio of 50 mg and 5,000 units per liter, respectively. The gross effect of these antibiotics on the creaming phenomenon was tested by measuring the cluster index and the cream volume of milk samples with or without added antibiotics. As evident from the results in Table 1,

these antibiotics did not alter the creaming properties of these milk samples.

TABLE 1. The effect of antibiotics on the creaming properties of milk

Sample	Cluster Index	% Cream Volume[a] After	
		1/2 Hr	4 Hr
Normal Milk			
Untreated	8.3	11.8	9.0
Treated[c]	8.6	11.4	9.5
Recombined Milk			
Untreated	7.7	8.3	7.4
Treated[c]	7.4	8.1	6.9
Model System[b]			
Untreated	4.3	4.4	3.6
Treated[c]	4.4	4.1	3.7

[a]Per cent cream volume per 1% fat.

[b]Euglobulin concentration was 0.01%.

[c]Antibiotics, penicillin "G" and streptomycin sulfate, were added to samples in the ratio of 5,000 units and 50 mg per liter, respectively.

Chemical Analyses

Various chemical constituents of the purified euglobulin and pseudoglobulin fractions were analyzed and the results are summarized in Table 2. In addition, the corresponding values reported by other investigators are given

TABLE 2. Chemical compositions of euglobulin and pseudoglobulin

Constituent	Euglobulin			Pseudoglobulin		
	This Study[a]	Smith[b]	Veiss[c]	This Study[a]	Smith[b]	Veiss[c]
	(%)			(%)		
Nitrogen	13.86	16.05	15.83	14.14	15.58	15.58
Phosphorus	none	none	none	none	none	none
Sulfur	----	1.01	----	----	1.00	----
Fucose	0.94	----	0.86	0.88	----	0.73
Hexose	3.72	2.94	3.24	3.15	2.49	2.15
Hexosamine	3.00	1.32	2.56	3.15	1.27	2.15
Sialic Acid	3.58	----	2.80	3.46	----	2.12

[a]Average of duplicates.

[b]Smith et al (1946).

[c]Veiss (1961).

for comparison. The immunoglobulins prepared for this study possessed lower nitrogen and higher carbohydrate contents than those reported previously (Smith et al., 1946; Veiss, 1961). A meaningful explanation for these differences is lacking but it should be mentioned that the source of the milk and the methods employed for preparing and analyzing these fractions were quite different. Surprisingly, the euglobulin and pseudoglobulin fractions prepared here show nearly identical chemical compositions.

The nitrogen contents of euglobulin and pseudoglobulin were 13.86 and 14.14%, respectively, slightly lower than those of most milk proteins which contain between 15.0 and 16.0% nitrogen. This difference is related to the presence of higher concentrations of carbohydrates in the immunoglobulins. The values of 13.86% for euglobulin and 14.14% for pseudoglobulin are somewhat lower than the corresponding values of 16.05 and 15.29% reported by Smith et al. (1946) and 15.53 and 15.58% by Veiss (1961). No phosphorus was present in either of the preparations isolated in this study.

The protein portion comprised 86.6% of euglobulin and 88.4% of pseudoglobulin, the remainder being essentially carbohydrates. Thus, the total carbohydrate content accounted for approximately 11% of these two glycoproteins. Euglobulin contained slightly higher amounts of fucose, hexose, and sialic acid and lower amount of hexosamine than pseudoglobulin, but the differences were small.

A summation of the residue weights of the amino acids together with the total carbohydrate values accounted for 97.84 and 99.04% of the total weight for euglobulin and pseudoglobulin, respectively.

Paper chromatogram of the carbohydrates released from euglobulin and pseudoglobulin is shown in Figure 6. Both protein fractions contained fucose, galactose, mannose, galactosamine and glucosamine. Like most glycoproteins, no glucose was detected. The presence of galactosamine and glucosamine in both euglobulin and pseudoglobulin fractions was also confirmed in the chromatograms obtained from the amino acid analyzer on 20 hr hydrolysates.

The amino acid compositions of the euglobulin and pseudoglobulin fractions are presented in Tables 3 and 4, respectively. The data are tabulated as gram residues of amino acid per 100 gram of protein and the number of amino acid residues per mole of methionine. The weight percentage of the amino acid residues was based on the nitrogen content as determined by the micro-Kjeldahl method. The latter method of presentation describes the molar ratios of the amino acid residues present.

Under the conditions employed in preparing the samples for amino acid analyses, about 5% of threonine, cystine and tyrosine and about 10% of serine were lost in the 20 hr hydrolysis. Therefore, the values for these amino acids were corrected for the loss according to the equation given by Hires et al. (1954):

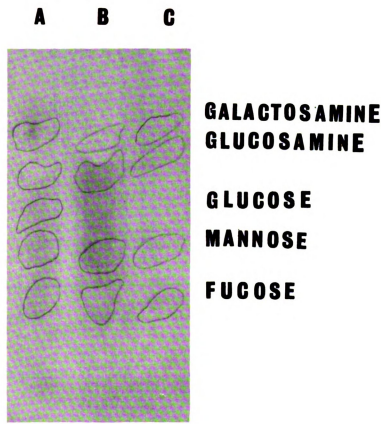

Figure 6. Paper chromatogram of authentic carbohydrate mixtures and of carbohydrates released from euglobulin and pseudoglobulin: A-authentic carbohydrate mixture; B-euglobulin; C-pseudoglobulin.

TABLE 3. Amino acid composition of euglobulin [a]

Residue	Gram Residues per 100 g Protein[b]	Number of Residues per Mole of Methionine[c]
Lysine	5.70 ± 0.09	13
Histidine	1.79 ± 0.06	4
Arginine	4.26 ± 0.11	8
Aspartic Acid	7.26 ± 0.13	19
Threonine	7.92 ± 0.07	23
Serine	9.36 ± 0.09	33
Glutamic Acid	8.23 ± 0.10	19
Proline	5.61 ± 0.21	13
Glycine	3.24 ± 0.01	10
Alanine	3.02 ± 0.01	13
Half Cystine	2.10 ± 0.04	6
Valine	7.34 ± 0.12	22
Methionine	0.44 ± 0.02	1
Isoleucine	2.51 ± 0.03	7
Leucine	6.32 ± 0.08	17
Tyrosine	4.99 ± 0.12	9
Phenylalanine	3.41 ± 0.05	7
Tryptophan	2.90 ± 0.10	5
Amino Acid Residue (Weight %)	86.60	
Total Carbohydrate (Weight %)	11.24	
Total (Weight %)	97.84	

[a]Amino acid content based on four replicate analyses of 20 and 70 hr hydrolysates with values corrected for destruction during acid hydrolysis according to the equation given by Hires et al. (1954).

[b]Weight percentage of i^{th} amino acid residue, based on the nitrogen content of 13.86%, with the standard deviation from the mean for each value.

[c]Number of residues of amino acids based on a single methionine residue per mole of protein.

TABLE 4. Amino acid composition of pseudoglobulin[a]

Residue	Gram Residues per 100 g Protein[b]	Number of Residues per Mole of Methionine[c]
Lysine	6.33 ± 0.07	13
Histidine	2.02 ± 0.10	4
Arginine	3.83 ± 0.08	7
Aspartic Acid	6.99 ± 0.10	17
Threonine	8.82 ± 0.59	24
Serine	9.65 ± 0.09	30
Glutamic Acid	8.91 ± 0.11	19
Proline	6.06 ± 0.25	17
Glycine	3.10 ± 0.12	15
Alanine	2.97 ± 0.07	11
Half Cystine	2.28 ± 0.08	7
Valine	6.77 ± 0.07	19
Methionine	0.48 ± 0.03	1
Isoleucine	2.60 ± 0.07	6
Leucine	6.51 ± 0.19	16
Tyrosine	5.03 ± 0.09	9
Phenylalanine	3.27 ± 0.06	6
Tryptophan	2.78 ± 0.01	4

Amino Acid Residue (Weight %)	88.40
Total Carbohydrate (Weight %)	10.64
Total (Weight %)	99.04

[a]Amino acid content based on five replicate analyses of 20 and 70 hr hydrolysates with values corrected for destruction during acid hydrolysis according to the equation given by Hires et al. (1954).

[b]Weight percentage of i^{th} amino acid residue, based on the nitrogen content of 14.14%, with the standard deviation from the mean for each value.

[c]Number of residues of amino acids based on a single methionine residue per mole of protein.

$$\log A_o = \frac{t_2}{t_2 - t_1} \cdot \log A_1 - \frac{t_1}{t_2 - t_1} \cdot \log A_2$$

where $\underline{A}_1$, $\underline{A}_2$, $\underline{A}_o$ are the quantities of amino acids after $\underline{t}_1$, $\underline{t}_2$, and $\underline{t}_o$ hr hydrolysis, respectively.

These two immunoglobulins were similar in amino acid content to other milk proteins except for lower glutamic acid and proline and higher serine and threonine values. As shown in Figure 7, there were no marked differences in the amino acid composition of these two proteins when the number of moles of each amino acid residue per 1000 moles of total amino acid residues was compared. One of the characteristic properties of the euglobulin was its insolubility in the low ionic strength aqueous buffer systems commonly employed to dissolve proteins. The quantity of hydrophobic amino acid residues in euglobulin was no greater than that in pseudoglobulin which exhibited a high degree of solubility. Therefore, it is presumed that the sequential arrangement of amino acids and/or the conformational differences in peptide chains might be the significant factors in determining the solubility properties of proteins. It should be noted, however, that proteins do not necessarily show a marked difference in amino acid content in order to reflect considerable differences in chemical and physical properties.

These two immunoglobulins contained relatively high

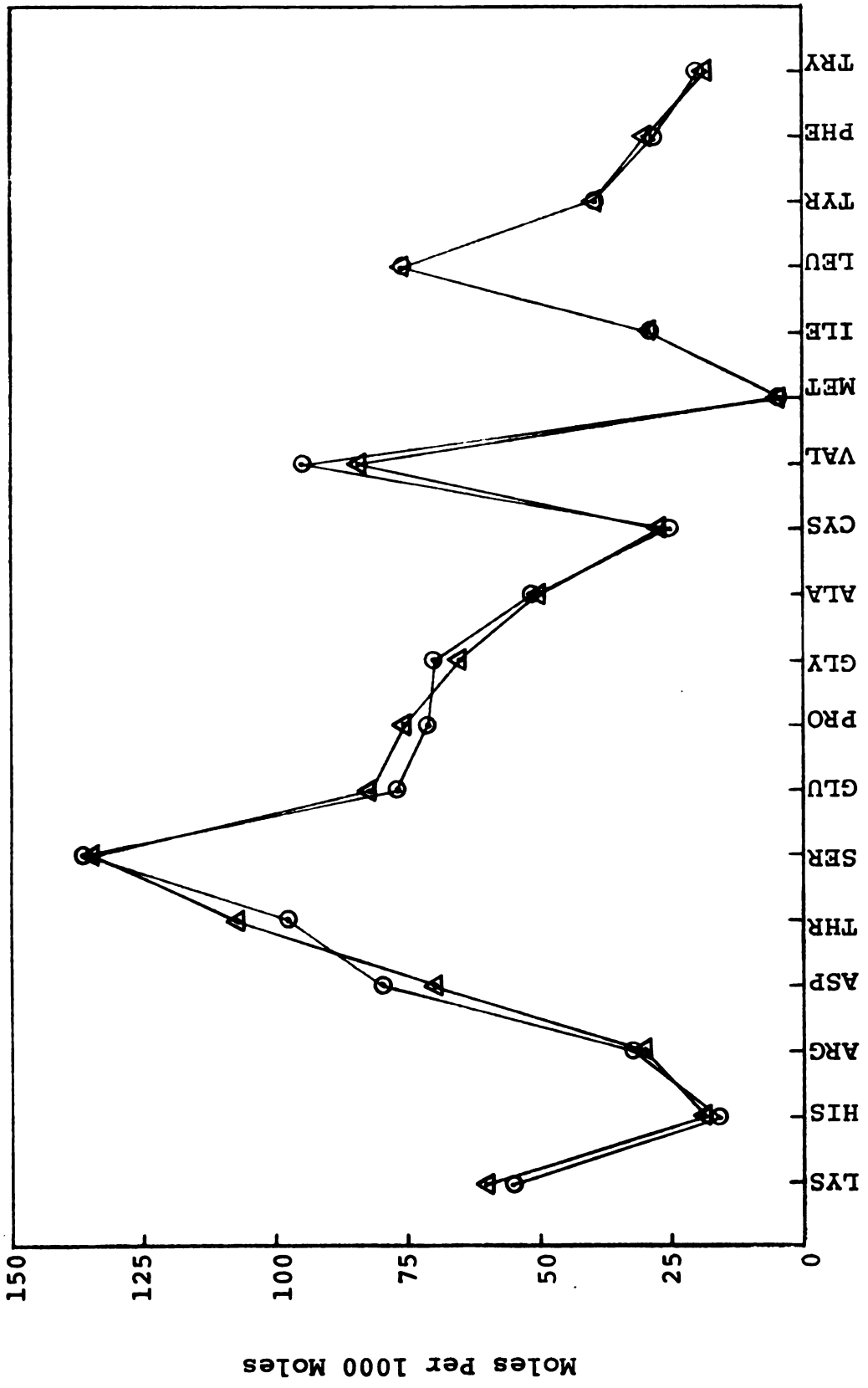

Figure 7. Amino acid profile of euglobulin (—O—O—) and pseudoglobulin (—Δ—Δ—).

contents of hydroxyl amino acids as well as large amounts of valine, proline, leucine, glutamic acid, aspartic acid, and lysine. Methionine, histidine, isoleucine, phenylalanine and tryptophan represented the amino acid residues present in relatively small concentrations. Values of the minimum molecular weights of these proteins were calculated from the molar ratios of the amino acid residues when one mole of methionine was used as the limiting residue (Tables 3 and 4). The molar ratios of methionine, fucose, hexose, hexosamine and sialic acid in euglobulin and pseudoglobulin were approximately 1:2:5:5:3 and 1:2:6:5:3, respectively. Assuming that molecular weights of about 175,000 for these two proteins are approximately correct, the residue number per mole would be increased by a factor of six.

The analytical data indicate that both euglobulin and pseudoglobulin are acidic proteins as determined by the ratio of the sum of acidic amino acid residues, aspartic acid and glutamic acid, to that of basic amino acid residues, arginine, histidine and lysine. This ratio was about 1.30 for both proteins. This value is much lower than those for caseins which have the ratio of approximately 2.0 to 2.5.

Smith et al. (1946) reported limited amino acid data for euglobulin and pseudoglobulin fractions which were determined by microbial and chemical methods. Consistently higher values than those found in this study were reported.

Physical Properties

Electrophoretic Characteristics

Electrophoretic Mobility. The electrophoretic mobilities of the purified euglobulin and pseudoglobulin fractions were determined in veronal buffer at pH 8.6, ionic strength 0.1. A single migrating boundary was observed for each of the proteins. The free-boundary patterns are shown in Figure 8 and the mobilities calculated from the ascending and descending channels are listed in Table 5.

TABLE 5. Electrophoretic mobilities of euglobulin and pseudoglobulin[a]

Protein	Mobility(μ) x 10^5, cm^2 sec^{-1} $volt^{-1}$		
	Ascending	Descending	Average
Euglobulin	-1.86	-1.77	-1.82
Pseudoglobulin	-2.10	-1.93	-2.02

[a]Average of duplicate runs. Each run consists of three measurements at various time intervals.

The average mobilities of the ascending and descending patterns were -1.82 and -2.02 Tiselius units for euglobulin and pseudoglobulin, respectively. The value for euglobulin is in close agreement with that reported by Smith (1946), i.e., -1.80 Tiselius units, but the value for pseudoglobulin is somewhat lower than his value of -2.20 Tiselius

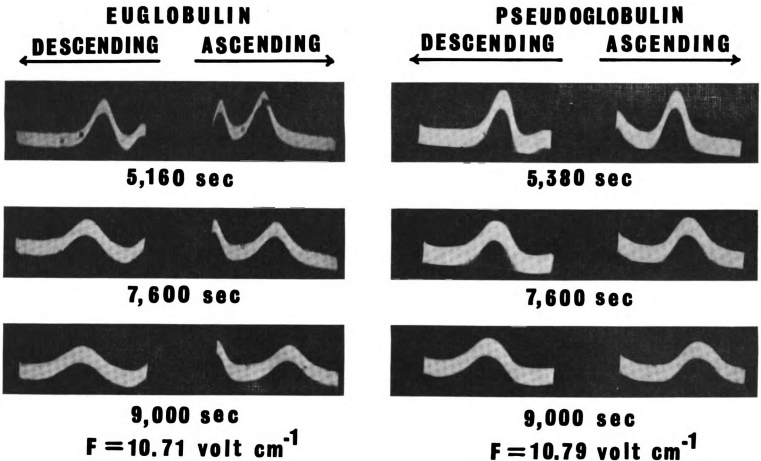

Figure 8. Free-boundary patterns of euglobulin and pseudoglobulin at various time intervals in veronal buffer, pH 8.6 and ionic strength of 0.1.

units. It is, however, possible that the electrophoretic mobility of a protein would vary slightly depending on the purity of the preparation and the state of its occurrence, i.e., dissociation or association with other protein components. Equilibrium dialysis of the protein solution against the buffer is also an important factor in determining the electrophoretic mobility.

Isoelectric Point. Isoelectric points of the immunoglobulins were estimated from the electrophoretic mobilities of the proteins in buffers covering a pH range of 4.0 to 8.6 at an ionic strength of 0.2. The results are tabulated in Table 6. Mobility determinations were made in duplicate with each run consisting of three measurements at various time intervals. At all values of pH tested, these preparations appeared as a single homogeneous boundary. The isoelectric point of pH 6.03 for euglobulin and that of pH 5.54 for pseudoglobulin were estimated from the plot of the average of ascending and descending electrophoretic mobilities against the corresponding pH values as shown in Figure 9.

The isoelectric points for both immunoglobulin fractions are in close agreement with those reported by Smith et al. (1946),i.e., pH 6.05 for euglobulin and pH 5.60 for pseudoglobulin.

Ultracentrifugal Characteristics

Partial Specific Volume. Figure 10 shows typical

TABLE 6. Electrophoretic mobilities of euglobulin and pseudoglobulin in various buffer systems

Buffer[a]	pH	Mobility (μ) x 10^5 cm^2 sec^{-1} $volt^{-1}$					
		Euglobulin[b]			Pseudoglobulin[b]		
		Ascending[c]	Descending[c]	Average[c]	Ascending[c]	Descending[c]	Average[c]
Na-acetate	4.0	+2.22	+2.07	+2.15	+2.07	+1.96	+2.02
Na-acetate	5.0	+1.15	+1.05	+1.10	+0.66	+0.64	+0.65
Na-phosphate	6.0	+0.06	+0.04	+0.05	-0.58	-0.51	-0.55
Na-phosphate	7.0	-0.90	-0.81	-0.86	-1.51	-1.36	-1.44
Na-veronal	8.6	-1.90	-1.80	-1.85	-2.66	-2.47	-2.57

[a]Ionic strength of 0.2.

[b]Protein concentration of 1.25%.

[c]Average of duplicate runs. Each run consisted of three measurements at different time intervals, i.e., 3600, 5400 and 7200 sec.

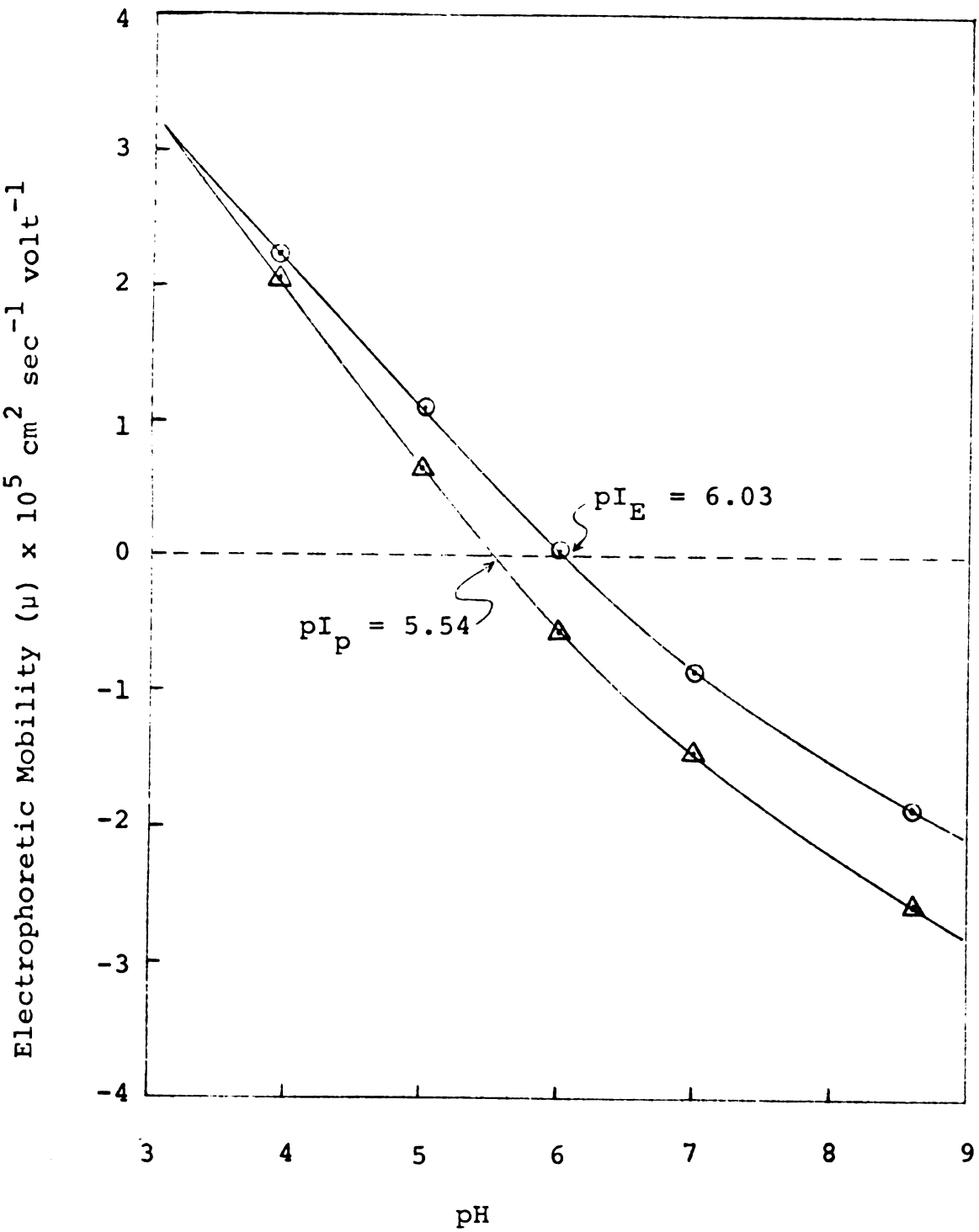

Figure 9. Electrophoretic mobility as a function of pH for the euglobulin (⊙—⊙) and pseudoglobulin (△—△).

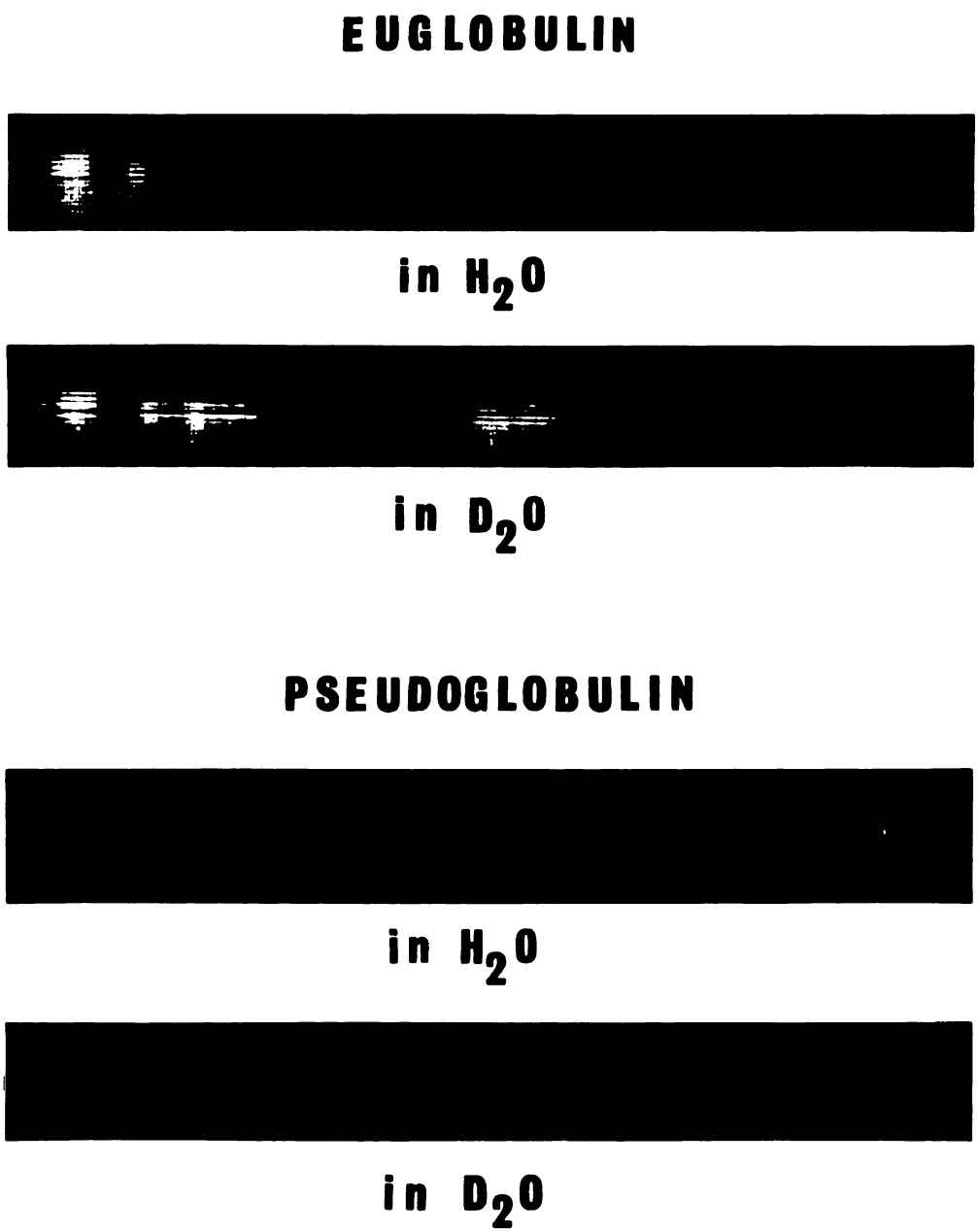

Figure 10. Interferograms obtained in high-speed sedimentation-equilibrium experiment with euglobulin and pseudoglobulin in H_2O and D_2O solutions. Protein concentration was 0.5 mg/ml.

interferograms obtained in high-speed sedimentation equilibrium experiments with euglobulin or pseudoglobulin in H_2O in one double-sector cell and the same protein in D_2O in the other. As shown in the patterns the concentration change across the cell was much less for the cell containing euglobulin or pseudoglobulin in D_2O. The representative data from these experiments are illustrated in Figure 11. The plots of the logarithm of net fringe displacement, which is proportional to concentration, with respect to the distance from the axis of rotation were of upward curvature. This is a typical characteristic for specimens possessing molecular heterogeniety. The slopes of these plots for euglobulin were 0.762 (H_2O) and 0.606 (D_2O) and for pseudoglobulin corresponding values were 0.789 (H_2O) and 0.628 (D_2O).

The ratios of the slope in D_2O to that in H_2O were 0.795 for euglobulin and 0.796 for pseudoglobulin. The value of $\underline{k}$, the ratio of the molecular weight of deuterated protein to that of the nondeuterated species, was assumed as 1.0155 since the value should be relatively constant for all proteins (Martin et al., 1959; Hvidt and Nielsen, 1966). The values of $\bar{v}$, the partial specific volume, evaluated from the experiments as illustrated in Figures 10 and 11 were 0.712 ml/g for euglobulin and 0.710 ml/g for pseudoglobulin.

Diffusion Coefficient. Apparent diffusion coefficients for euglobulin and pseudoglobulin fractions were

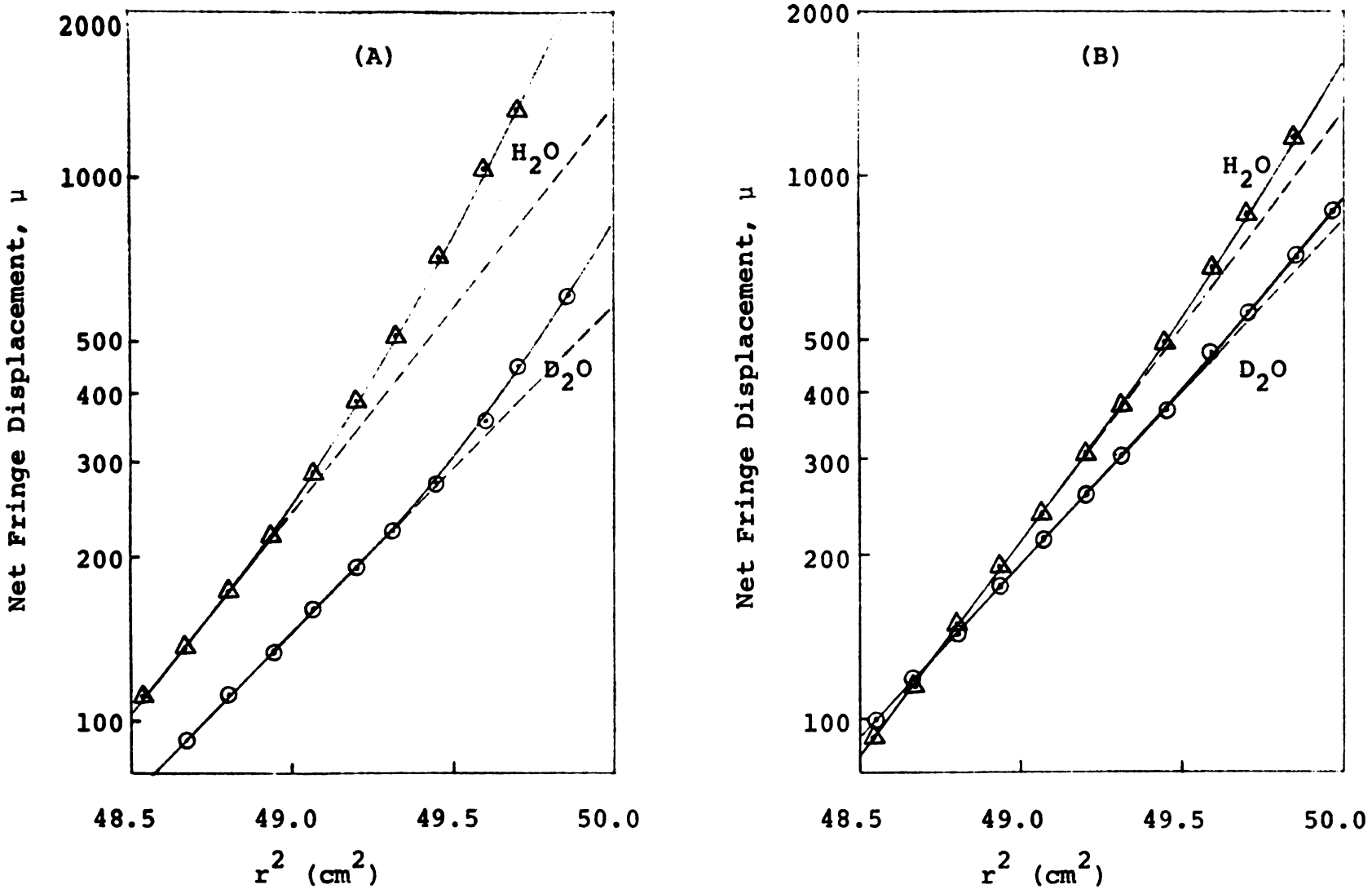

Figure 11. Sedimentation equilibrium of euglobulin and pseudoglobulin in H_2O and D_2O solutions: A-Euglobulin and B-Pseudoglobulin.

determined at various protein concentrations in veronal buffer (pH 8.6, $\mu = 0.1$), milk salt solution (pH 6.6) and 6 M guanidine hydrochloride plus 0.02 M 2-mercaptoethanol solution. The results of these experiments are recorded in Table 7. The concentration dependency of the apparent diffusion coefficients ($D^{app}_{20,w}$) of euglobulin and pseudoglobulin is illustrated by the plots in Figure 12.

TABLE 7. The apparent diffusion coefficients of euglobulin and pseudoglobulin in veronal buffer, milk salt solution and 6 M guanidine-HCl plus 0.02 M 2-mercaptoethanol solution

Protein	Protein Concentration (mg/ml)	Diffusion Coefficient[a] x10^7		
		Veronal	Milk Salt	Guanidine
Euglobulin				
	0.0[b]	3.20	3.20	37.90
	4.0	3.33	----	27.10
	5.0	----	3.31	-----
	7.0	3.40	----	18.43
	7.5	----	3.40	-----
	10.0	3.53	3.45	10.54
Pseudoglobulin				
	0.0[b]	4.20	3.62	30.00
	4.0	----	3.20	24.04
	5.0	3.62	----	-----
	7.0	----	3.15	19.01
	8.0	2.98	----	-----
	10.0	2.79	2.42	15.04

[a]Values corrected to water at 20° C.

[b]Values obtained by extrapolating to infinite dilution using least squares linear regression method.

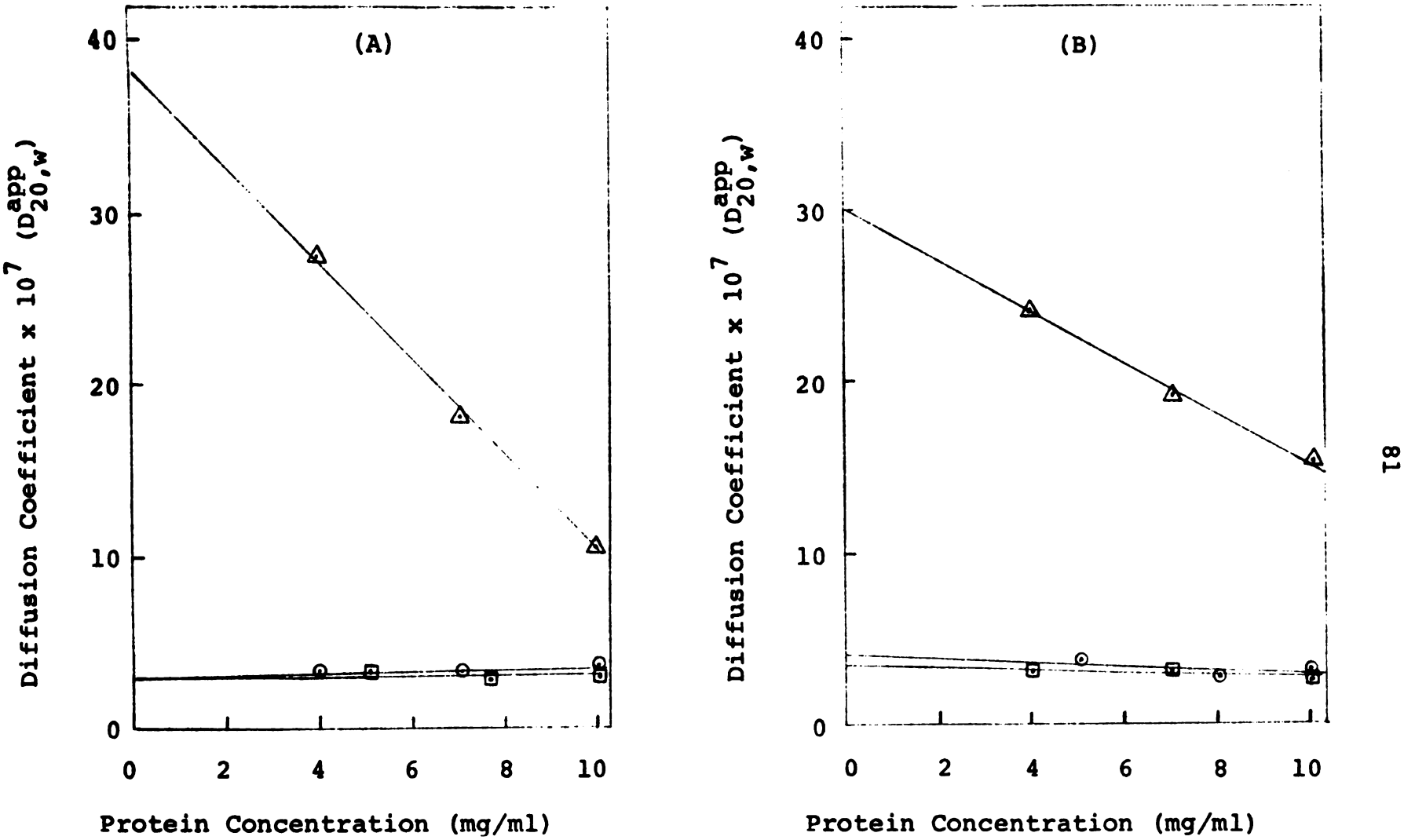

Figure 12. Diffusion properties of euglobulin and pseudoglobulin in veronal buffer (○—○), milk salt solution (□—□) and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol (△···△): A-Euglobulin and B-Pseudoglobulin.

In veronal buffer, the apparent diffusion coefficients of euglobulin remained nearly constant over the entire range of protein concentrations. Only a slight increase in the diffusion coefficient with increasing protein concentrations was perceptible. Similar characteristics were observed in the milk salt solution. Pseudoglobulin showed somewhat different results. In both veronal buffer and milk salt solution, the apparent diffusion coefficients increased slightly with decreasing protein concentrations. However, these slight changes in the diffusion characteristics of both euglobulin and pseudoglobulin seemed insignificant when compared to their behavior in the 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol dissociating system. Here, the apparent diffusion coefficients of both euglobulin and pseudoglobulin showed a high degree of dependency on the protein concentration, increasing 5- to 12-fold over the concentration range observed. It seems that both proteins are extremely sensitive to dissociating agent, particularly to 2-mercaptoethanol (Gough et al., 1966). These data indicate that low molecular species or monomer units of these proteins are present in this dissociating system. Smaller species would tend to give higher diffusion rates than the larger molecular polymers.

Corrected diffusion coefficients at infinite dilution of euglobulin in veronal buffer, milk salt solution and the 6 M guanidine hydrochloride solution containing 0.02 M

2-mercaptoethanol were 3.20, 3.20 and 37.90 x 10^{-7} cm^2 sec^{-1}, respectively. Corresponding values for pseudoglobulin were 4.20, 3.62 and 30.00 x 10^{-7} cm^2 sec^{-1}. Values of 3.20 x 10^{-7} cm^2 sec^{-1} for euglobulin and 4.20 x 10^{-7} for pseudoglobulin in veronal buffer are comparable with reported values of 3.41 and 3.84 x 10^{-7} cm^2 sec^{-1}, respectively (Smith et al., 1946).

The effects of various hydrogen ion concentrations on the diffusion coefficients of euglobulin are shown by the data presented in Table 8.

TABLE 8. The effects of various hydrogen ion concentrations on the diffusion coefficients of euglobulin

Buffer[a]	pH	Diffusion Coefficient[b,c] (Ficks)
Na-acetate	4.0	4.98
Na-phosphate	6.0	3.92
Na-veronal	8.6	3.53

[a]Ionic strength of 0.1.

[b]Protein concentration of 10.0 mg/ml.

[c]Values corrected to water at 20° C.

The apparent diffusion coefficients of euglobulin at 10.0 mg/ml concentration increased as the hydrogen ion concentration decreased. Euglobulin is quite stable within the pH range of 6.0 to 8.6, as the differences in the apparent diffusion coefficient at these pH values are not

significant. The somewhat higher apparent diffusion coefficient obtained at pH 4.0 would indicate that euglobulin might dissociate into smaller molecular species at low values of pH.

Effects of the electrolyte concentration of veronal buffers on the diffusion rates of a euglobulin preparation are illustrated by the data reported in Table 9.

TABLE 9. The effects of electrolyte concentrations on the apparent diffusion coefficients of euglobulin

Ionic Strength	Diffusion Coefficient[a,b] (Ficks)
0.01	4.63
0.05	3.94
0.10	3.53
0.20	3.38
0.50	3.31

[a]Protein concentration of 10.0 mg/ml.

[b]Values corrected to water at 20° C.

As the concentration of electrolytes in the buffers decreased, the apparent diffusion coefficients of euglobulin increased gradually. The rate of change was pronounced in the lower ionic strength buffers than in the higher salt concentrations. Timascheff (1964) stated that some proteins tend to dissociate slowly in low ionic strength buffer systems.

The effects of various temperatures on the apparent diffusion coefficients of euglobulin were studied in veronal buffer and milk salt solution. The results are

summarized in Table 10.

TABLE 10. The effects of temperatures on the apparent diffusion coefficients of euglobulin in veronal buffer and milk salt solution[a]

Buffer	Protein Concentration (mg/ml)	Diffusion Coefficient[b] (Ficks)		
		5° C	20° C	32° C
Veronal				
	0.0[c]	3.01	3.20	3.41
	4.0	3.17	3.33	3.59
	7.0	3.29	3.40	3.75
	10.0	3.41	3.53	3.89
Milk Salt Solution				
	0.0[c]	2.93	3.20	3.36
	5.0	3.09	3.31	3.51
	7.5	3.23	3.40	3.62
	10.0	3.37	3.45	3.67

[a]Rotor was precooled or prewarmed to the temperature slightly lower or higher than the desired.

[b]Values corrected to water at 20° C.

[c]Values obtained by extrapolating to infinite dilution using the least squares linear regression method.

The diffusion coefficients of euglobulin in both buffer systems decreased slightly with decreases in temperature. However, the concentration dependency of the apparent diffusion coefficients remained unchanged over the range of all temperatures studied. The slightly lower diffusion coefficients obtained in the milk salt solution,

as compared to those in veronal buffer, are noteworthy.

Sedimentation Coefficient. The apparent sedimentation coefficients of euglobulin and pseudoglobulin in veronal buffer, milk salt solution and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol, at various protein concentrations are summarized in Table 11.

The sedimentation-velocity studies in veronal buffer revealed that the euglobulin preparation contained only two components -- a 7S and a 19S molecular species. Approximately 85-90% of the protein consisted of the 7S component while the 19S species accounted for from 10 to 15%. Smith (1946a) reported three molecular species, 7S, 10S and 19S in the ratio of approximately 8:1:1, for a similar preparation obtained by ammonium sulfate precipitation.

The apparent sedimentation coefficients of the 7S component were essentially independent of protein concentration. The 19S component showed more of a concentration dependency, increasing slightly with decreasing protein concentrations (see Figure 13-A). This type of concentration dependency in the apparent sedimentation coefficient is typical for a slow association-dissociation reaction of a protein (Gilbert, 1959 and 1963; Gilbert and Jenkins, 1959). Nearly identical results were obtained in milk salt solution. The sedimentation coefficients of these two molecular species decreased considerably in the presence of dissociating agents, indicating that the polymer-dimer equilibrium was shifted toward the low molecular weight

TABLE 11. The sedimentation coefficients of euglobulin and pseudoglobulin in veronal buffer, milk salt solution and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol

Protein	Protein Concentration (mg/ml)	Sedimentation Coefficient[a]		
		Veronal	Milk Salt	Guanidine
Euglobulin				
Slow Boundary	0.0[b]	6.24	6.15	4.63
	4.0	----	6.13	4.13
	5.0	6.22	----	----
	7.0	----	6.06	3.75
	7.5	6.21	----	----
	10.0	6.20	6.03	3.37
Fast Boundary	0.0[b]	19.04	19.16	13.66
	4.0	-----	19.00	11.81
	5.0	18.82	-----	-----
	7.0	-----	18.68	10.43
	7.5	18.67	-----	-----
	10.0	18.55	18.71	9.04
Pseudoglobulin				
	0.0[b]	6.00	5.91	4.61
	4.0	----	6.33	4.10
	5.0	6.53	----	----
	7.0	----	6.75	3.72
	8.0	6.89	----	----
	10.0	7.10	7.04	3.34

[a]Values corrected to water at 20° C.

[b]Values obtained by extrapolating to infinite dilution using the least squares linear regression method.

species. The sedimentation coefficients, corrected for density and viscosity of the solvent and at infinite dilution, were approximately 19.04S, 19.16S and 13.66S for the 19S component in veronal buffer, milk salt solution and dissociating system, respectively. For the 7S component, the corresponding values were 6.24S, 6.15S and 4.63S.

The apparent sedimentation coefficients of pseudoglobulin as function of protein concentrations are shown in Figure 13-B. In both veronal buffer and milk salt solution, the sedimentation values were concentration dependent, increasing with increasing protein concentrations. In the dissociating system, however, the usual trend of concentration dependency of the sedimentation coefficients was reversed, decreasing with increasing protein concentrations. The sedimentation coefficients obtained in veronal buffer, milk salt solution and dissociating system were approximately 6.00S, 5.91S and 4.61S, respectively.

The effects of various temperatures on the sedimentation behavior of euglobulin were studied in veronal buffer and milk salt solution. The results are shown in Table 12.

The sedimentation behavior of the 7S component was not affected significantly by the temperature changes. However, the 19S component showed noticeably higher sedimentation values as the temperature decreased, although not as drastically as expected from the results of sedimentation-equilibrium experiments. Nevertheless, the noticeable

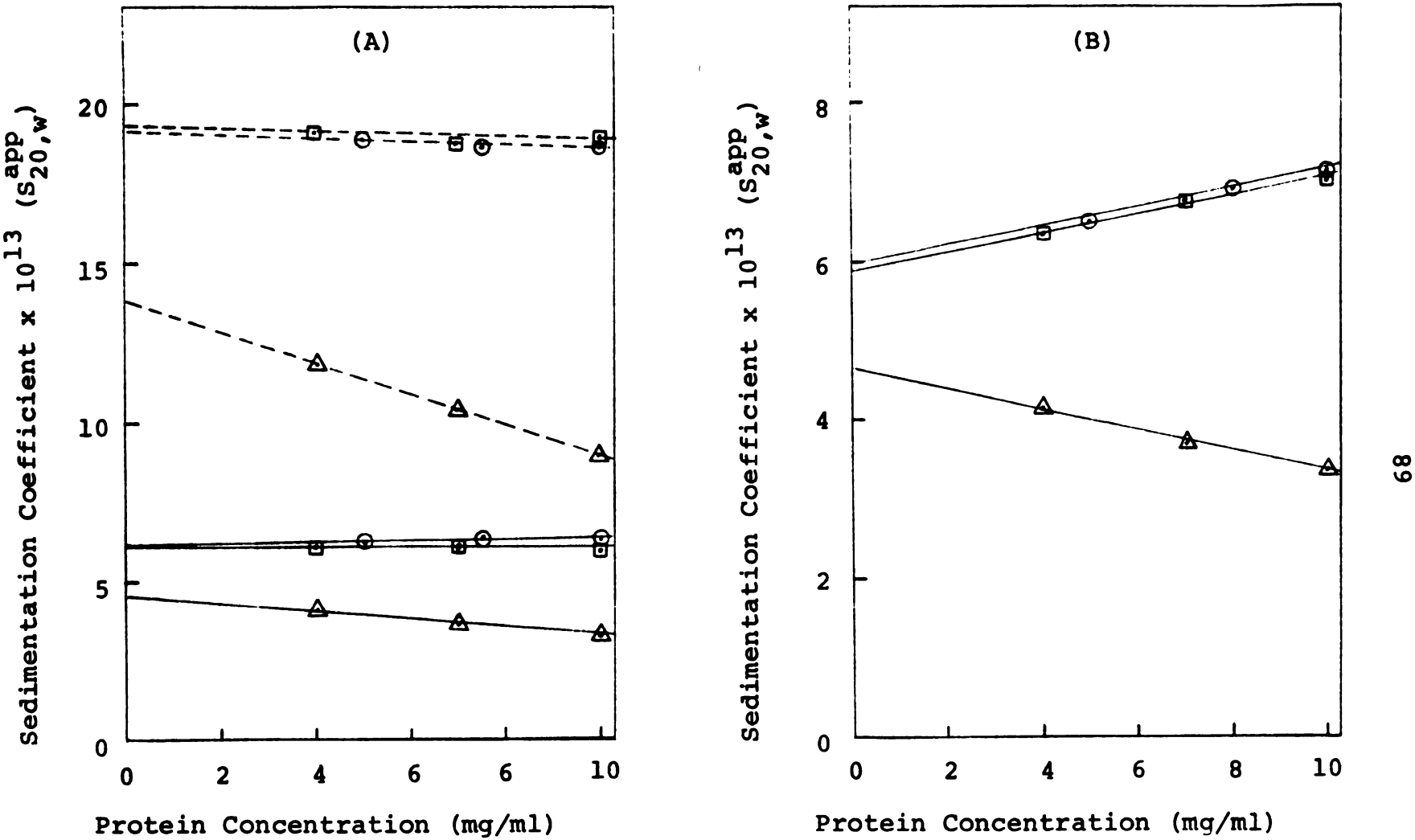

Figure 13. Sedimentation behavior of euglobulin and pseudoglobulin in veronal buffer (-o-o-), milk salt solution (-□-□-) and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol (-△-△-): A-Euglobulin (----, fast moving boundary; ———, slow moving boundary) and B-Pseudoglobulin.

TABLE 12. The effects of temperatures on the sedimentation coefficients of euglobulin in veronal buffer and milk salt solution

Buffer	Component[a]	Protein Concentration (mg/ml)	Sedimentation Coefficient[b] 5° C	20° C	32° C
Veronal					
	7S	0.0[c]	6.40	6.24	6.19
		5.0	6.33	6.22	6.17
		7.5	6.26	6.21	6.21
		10.0	6.22	6.20	6.18
	19S	0.0[c]	22.61	19.04	18.90
		5.0	20.64	18.82	18.41
		7.5	19.69	18.67	18.04
		10.0	18.63	18.55	17.97
Milk Salt Solution					
	7S	0.0[c]	7.05	6.15	6.10
		4.0	6.67	6.13	6.06
		7.0	6.31	6.06	6.04
		10.0	6.02	6.03	5.98
	19S	0.0[c]	23.55	19.16	18.84
		4.0	21.67	19.00	18.57
		7.0	20.73	18.68	18.49
		10.0	18.88	18.71	18.11

[a] 7S denotes the slow moving boundary and 19S the fast moving boundary.

[b] Values corrected to water at 20° C.

[c] Values obtained by extrapolating to infinite dilution using the least squares linear regression method.

increase in the sedimentation coefficients at lower temperatures, particularly in milk salt solution, suggests that a slow association or polymerization reaction of the 19S component occurred. In fact, slow association reactions of proteins require a much longer time than the residence time during a centrifuge run. Indeed, the detection of the association phenomenon could escape during the short time required for the sedimentation-velocity experiments.

The effects of various hydrogen ion concentrations on the sedimentation behavior of euglobulin are represented by the data in Table 13.

TABLE 13. The effects of various hydrogen ion concentrations on the sedimentation coefficients of euglobulin

Buffer[a]	pH	Sedimentation Coefficient[b,c]	
		7S Component	19S Component
Na-acetate	4.0	4.82	14.96
Na-phosphate	6.0	6.00	18.83
Na-veronal	8.6	6.20	18.55

[a]Ionic strength of 0.1.

[b]Protein concentration of 10.0 mg/ml.

[c]Values corrected to water at 20° C.

The sedimentation coefficients of both the 7S and 19S components of euglobulin decreased with decreasing hydrogen ion concentrations. However, the differences in

sedimentation values between the pH 6.0 and 8.6 buffer systems were not great. This observation suggests that both the 7S and 19S components are stable in this pH range but dissociate at pH values much lower than their isoelectric points.

Table 14 summarizes data showing the effects of ionic strength on the sedimentation behavior of euglobulin.

TABLE 14. The effects of electrolyte concentrations on the sedimentation coefficients of euglobulin

Ionic Strength	Sedimentation Coefficient[a,b]	
	7S Component	19S Component
0.01	5.13	15.70
0.05	5.47	16.06
0.10	6.20	18.55
0.20	6.27	18.72
0.50	6.23	18.79

[a]Protein concentration of 10.0 mg/ml

[b]Values corrected to water at 20° C.

The concentration of electrolytes in veronal buffer had only slight effects on the sedimentation coefficients of the 7S and 19S components of euglobulin preparation which decreased with decreasing ionic strength. This observation coincides with Timasheff's (1964) observation that the dissociation of β-lactoglobulin is decreased by

increased ionic strength. Buffers with ionic strength lower than 0.01 could not be tested because of the insolubility of euglobulin in these systems. When the concentration of the electrolytes reached a critical level, the sedimentation coefficients were no longer affected by further increases in the ionic strength.

Molecular Weight. The apparent weight average molecular weights of euglobulin and pseudoglobulin were determined by the low-speed sedimentation-equilibrium method at various protein concentrations in veronal buffer, milk salt solution and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol. The results of these experiments are summarized in Table 15. The ratios of the apparent z- to weight-average molecular weights ($\bar{M}_z^{app}/\bar{M}_w^{app}$) are also shown in the same table. It was extremely difficult to make accurate measurement of the apparent $\bar{M}_z$ of these samples, particularly euglobulin because of the viscous layer of sedimented material which collected at the bottom of the cell. It is very unlikely that the concentration gradient curve would ever reach the bottom meniscus during the sedimentation run. Considerably high $\bar{M}_z/\bar{M}_w$ ratios suggest that these proteins consist of highly heterogeneous molecular species. The extent of heterogeniety or polydispersity increases considerably as the protein concentration increases (Tanford, 1961). Polydispersity is particularly evident in the dissociating buffer system.

TABLE 15. Molecular weights of euglobulin and pseudoglobulin in veronal buffer, milk salt solution and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol

Protein	Protein Concentration (mg/ml)	Veronal		Milk Salt		Guanidine	
		$\bar{M}_w$	$\bar{M}_z/\bar{M}_w$	$\bar{M}_w$	$\bar{M}_z/\bar{M}_w$	$\bar{M}_w$	$\bar{M}_z/\bar{M}_w$
Euglobulin							
	0.0[a]	175,250	1.11	243,752	1.15	89,200	1.45
	4.0	179,639	1.32	-------	----	64,836	3.71
	5.0	-------	----	259,638	1.53	------	----
	7.0	190,047	2.28	-------	----	40,436	5.21
	7.5	-------	----	275,872	2.53	------	----
	10.0	194,968	3.12	287,149	3.67	24,517	7.35
Pseudoglobulin							
	0.0[a]	166,857	1.10	173,981	1.14	86,073	1.38
	4.0	-------	----	170,026	1.33	64,081	3.81
	5.0	152,863	1.28	-------	----	------	----
	7.0	-------	----	161,198	2.13	42,946	5.51
	7.5	150,621	1.96	-------	----	------	----
	10.0	142,013	2.65	159,921	2.98	26,505	7.70

[a]Values obtained by extrapolating to infinite dilution using the least squares linear regression method.

As illustrated in Figure 14, the apparent weight average molecular weights of euglobulin and pseudoglobulin were concentration dependent. In veronal buffer and milk salt solution, euglobulin showed a stronger dependency on the concentration than did pseudoglobulin. It is important to note that in veronal buffer and milk salt solution the apparent weight average molecular weights of euglobulin decreased with decreasing concentrations of protein, whereas the opposite behavior was observed for pseudoglobulin. This behavior of euglobulin is typical for an interacting system in contrast to a noninteracting system provided that sufficient time is allowed for the attainment of equilibrium at constant environmental conditions (McKenzie, 1967). According to this theory, pseudoglobulin could be a noninteracting material since the apparent average molecular weights decreased as the protein concentration increased.

In the dissociating buffer system, both euglobulin and pseudoglobulin behaved similarly. The apparent weight average molecular weights of both proteins increased with decreasing protein concentration.

The weight average molecular weights of both proteins in various buffers were estimated by extrapolating the apparent weight average values to infinite dilution. The values obtained were approximately 175,000; 244,000; and 89,000 for euglobulin and 167,000; 174,000; and 86,000 for pseudoglobulin in veronal buffer, milk salt solution and dissociating buffer system, respectively. The values of

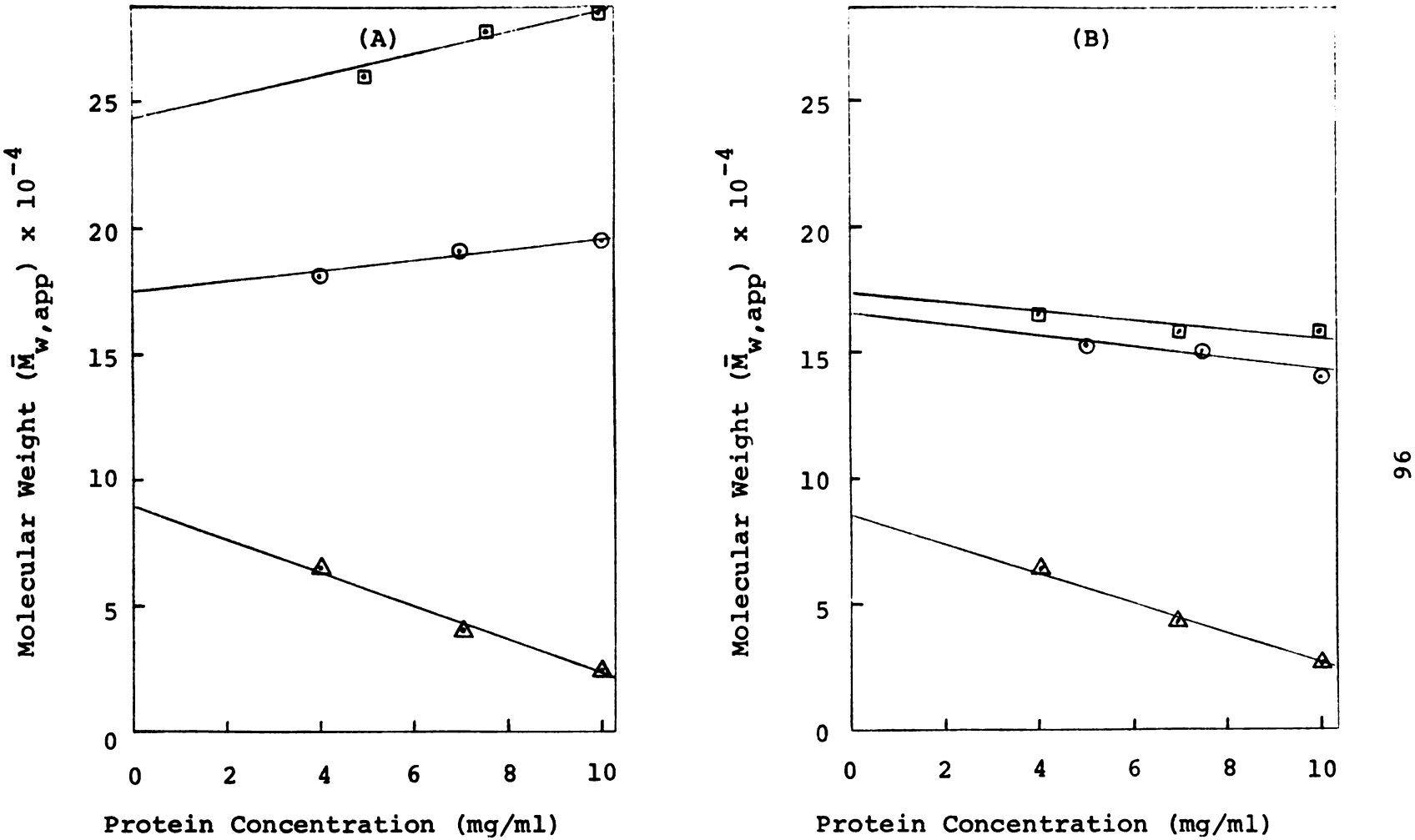

Figure 14. Concentration dependency of the apparent weight average molecular weights of euglobulin and pseudoglobulin in veronal buffer (—⊙—⊙—), milk salt solution (—⊡—⊡—) and 6 M guanidine hydrochloride solution containing 0.02 M 2-mercaptoethanol (-△-△-): A-Euglobulin and B-Pseudoglobulin.

175,000 for euglobulin and 167,000 for pseudoglobulin in veronal buffer are in the range of 160,000 to 190,000 for both proteins reported by Smith et al. (1946) but somewhat lower than the values of 252,000 for euglobulin and 289,000 for pseudoglobulin reported by Murthy and Whitney (1958).

Why euglobulin exhibits a substantially higher weight average molecular weight in the milk salt solution than in veronal buffer is not clearly understood. Environmental conditions such as hydrogen ion concentration, species and amounts of salts in the milk salt solution possibly favor the polymerization of euglobulin molecules during the long equilibrium experiments, i.e., ≈20-30 hr.

The effects of various temperatures on the apparent weight average molecular weights of euglobulin were studied in veronal buffer and milk salt solution at three different protein concentrations. The results are shown in Table 16. The apparent weight average values for euglobulin in both buffer systems were greatly affected by temperature changes. At all temperatures studied, the pattern of the concentration dependency of the apparent weight average values in both buffers was not changed. As clearly illustrated in Figure 15, the weight average molecular weights increased cosiderably as the temperature decreased. Presumably, polymerization of euglobulin was greatly enhanced at low temperatures. The $\bar{M}_w$ of euglobulin at 5° C in veronal buffer was almost one and one-half times that observed at 20° C in the same buffer, suggesting that a

TABLE 16. The effects of temperatures on the apparent weight average molecular weights of euglobulin in veronal buffer and milk salt solution

Buffer	Protein Concentration (mg/ml)	Molecular Weight[a]		
		5° C	20° C	32° C
Veronal				
	0.0[b]	275,918	175,250	104,027
	4.0	294,013	179,639	117,663
	7.0	323,781	190,047	138,819
	10.0	337,619	194,968	144,492
Milk Salt Solution				
	0.0[b]	387,932	243,752	175,339
	5.0	413,781	259,638	204,013
	7.5	440,035	275,872	224,917
	10.0	449,910	287,149	230,281

[a]Apparent weight average molecular weight.

[b]Values obtained by extrapolating to infinite dilution using the least squares linear regression method.

slow polymerization occurred at the lower temperature. The $\bar{M}_w$ at 5° C in milk salt solution was slightly more than twice that observed in veronal buffer at 20° C. A dimerization reaction might have been favored by these conditions. It is, therefore, presumed that the polymerization interaction of euglobulin has a negative enthalpy and a large negative entropy. Thus, the polymerization is favored by low temperatures.

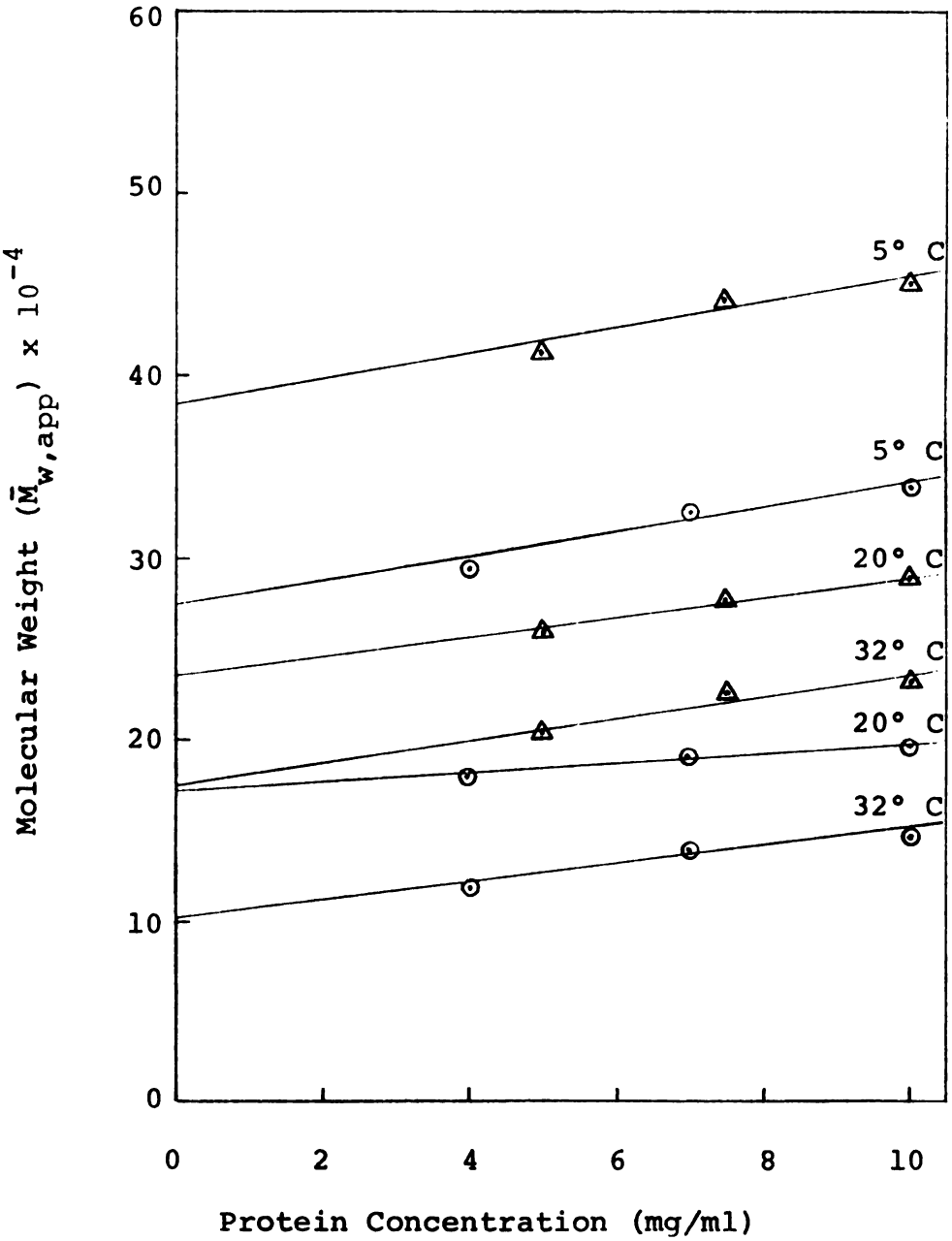

Figure 15. The effects of temperatures on the weight average molecular weight of euglobulin in veronal buffer (-⊙—⊙-) and milk salt solution (-△—△-).

The effects of hydrogen ion concentrations on the apparent weight average molecular weights of euglobulin are shown in Table 17.

TABLE 17. The effects of hydrogen ion concentrations on the apparent weight average molecular weights of euglobulin

Buffer[a]	pH	Molecular Weight[b,c]
Na-acetate	4.0	130,715
Na-phosphate	6.0	174,033
Na-veronal	8.6	194,968

[a]Ionic strength of 0.1.

[b]Protein concentration of 10.0 mg/ml.

[c]Apparent weight average molecular weight.

The apparent weight average molecular weights of euglobulin, at 10.0 mg/ml concentration, decreased considerably with decreasing hydrogen ion concentration in the buffer. As mentioned previously concerning the sedimentation behavior of this protein, the significant reduction of the apparent weight average molecular weight at pH 4.0 compared to those at pH 6.0 and 8.6 may be explained on the basis that dissociation is favored by low values of hydrogen ion concentration.

Results illustrating the effects of the ionic strength of veronal buffer on the apparent weight average molecular

weights of euglobulin (20° C) are found in Table 18.

TABLE 18. The effects of ionic strengths on the apparent weight average molecular weights of euglobulin in veronal buffer, pH 8.6, at 20° C

Ionic Strength	Molecular Weight[a,b]
0.01	190,937
0.05	190,293
0.10	194,968
0.20	193,254
0.50	195,798

[a]Protein concentration of 10.0 mg/ml.

[b]Apparent weight average molecular weight.

As was the case for the apparent sedimentation coefficients of euglobulin, the apparent weight average molecular weights decreased slightly with decreasing ionic strength. This slight tendency for decreasing $\bar{M}_w$ values could be a ramification of slow, apolar dissociation in the protein.

Creaming Studies

The Effects of Euglobulin Concentrations on Creaming

The effects of different concentrations of euglobulin on the creaming abilities of normal and recombined milk samples and model system are summarized in Table 19. The model system was prepared by resuspending thrice-washed fat globules (3.6%, w/v) in milk salt solution.

TABLE 19. The effects of euglobulin concentrations on creaming

Sample	Added Euglobulin (%)	Cluster Index	Cream Volume[a,b]	
			1 Hr	4 Hr
Normal Milk				
	None	8.5	10.2	8.3
	0.01	8.9	11.0	8.4
	0.02	8.4	10.9	8.6
Recombined Milk				
	None	7.7	8.3	7.4
	0.01	8.9	10.9	8.7
	0.02	8.7	11.0	8.4
Model System				
	None	0.6	0.3	0.6
	0.01	4.3	4.4	3.6
	0.02	6.7	6.6	5.0
	0.03	7.9	8.3	7.0
	0.04	7.7	8.1	7.1

[a]Average of duplicates.

[b]Per cent of cream volume per 1% fat (v/v).

In the model system, no measurable cream layer was formed in the absence of euglobulin. The cluster index and cream volume increased markedly when increasing amounts of euglobulin were added, indicating that this protein plays a definite role in the creaming phenomenon. The slight tendency for fat globules to cluster in the absence of added

euglobulin may have been the result of small amounts of adsorbed euglobulin on the surface of fat globules.

Even with the addition of 0.04% of euglobulin to the model system, its net creaming ability seeded inferior to that of normal milk. Obviously, then, the best simulated milk salt solution does not provide the optimum conditions for creaming when compared to natural milk.

The enhancement of the creaming ability of recombined milk by the addition of euglobulin supports the contention that euglobulin is a principal component in the creaming phenomenon. The slightly poorer creaming ability of the recombined milk minus added euglobulin suggests that the wild agitation of the milk system during the process of cream separation may have produced a partial denaturation of the euglobulin. Additionally, the cream separation process could have caused mechanical injury to the fat globules, altering the plasma/lipid interfacial surface in such a manner that the adsorption of euglobulin was impeded.

The addition of euglobulin to normal milk did not enhance its creaming property significantly. There seems to be a definite relationship between euglobulin concentration and fat globule surface area involved in the creaming phenomenon.

The Effects of Temperatures on Creaming

The effects of various temperatures on the creaming property of normal and recombined milk samples and model

systems are shown in Table 20.

In all three systems, creaming was greatly improved at the lower temperatures, i.e., ≈5° C. This observation correlates with the behavior of euglobulin in the sedimentation experiments. Recall that euglobulin, especially its 19S component, exhibited extensive intermolecular association in the low temperature runs. However, no direct evidence is available at present to claim that the cold association of euglobulin molecules contributes significantly to the enhancement of the creaming properties at low temperatures. Further extensive studies are needed to verify this hypothesis.

The Effects of Electrolyte Concentrations on Creaming

The creaming abilities of recombined milk and model systems were studied at various electrolyte concentrations. The results are shown in Table 21.

It was found that the dilution of the skim milk or the milk salt solution improves the creaming properties of recombined milk and model system. For both systems, a maximum cream layer was obtained at 50% dilution with deionized water. This observation supports the early explanation for the improved creaming of diluted milk (Orla-Jensen, 1928; Dunkley and Sommer, 1944). They conceded that the salts in milk hinder the action of agglutinin and dilution favors fat clustering by providing more favorable conditions for the action of agglutinin even with its decreased

TABLE 20. The effects of temperatures on the creaming properties[a]

Sample	Temperature (° C)	Cluster Index	Cream Volume (%)	
			1 Hr	4 Hr
Normal Milk				
	3 - 4	8.5	10.2	8.3
	12 - 15	8.6	9.8	8.3
	23 - 25	7.2	8.1	7.0
	32 - 35	3.2	5.3	4.8
	42 - 45	1.3	3.1	2.2
Recombined Milk[b]				
	3 - 4	7.7	8.3	7.4
	12 - 15	6.9	7.8	7.0
	23 - 25	5.1	6.3	5.7
	32 - 35	2.4	3.7	2.9
	42 - 45	0.7	2.4	1.9
Model System[c]				
	3 - 4	4.3	4.4	3.6
	12 - 15	4.0	4.2	3.5
	23 - 25	3.1	3.3	2.9
	32 - 35	1.9	2.4	1.6
	42 - 45	0.3	1.7	1.2

[a]After the milk samples were warmed as described in the experimental section, the creaming cells and the graduated cylinders containing warmed samples were placed in water baths maintaining the specified temperatures instead of placing them in a 4° C bath.

[b]Five-time washed cream was redispersed in thrice-separated skim milk at 3.6% fat concentrations (w/v).

[c]The concentrations of euglobulin and fat were 0.01 and 3.6%, respectively, in milk salt solution (w/v).

TABLE 21. The effects of electrolyte concentrations on creaming phenomenon

Sample	Dilution[a] (%)	Cluster Index	Cream Volume (%)	
			1 Hr	4 Hr
Recombined Milk				
	None	7.7	8.3	7.4
	25	11.3	8.8	8.1
	50	20.8	9.3	8.9
	75	20.2	9.0	8.5
	100[b]	0.0	0.3	0.7
Model System				
	None	4.3	4.4	3.6
	25	4.8	4.7	4.0
	50	5.8	5.0	4.0
	75	5.5	3.8	3.0
	100[c]	0.0	0.3	0.9

[a]The electrolyte concentrations were adjusted by diluting the whey or milk salt solution with deionized water (v/v).

[b]Cream was suspended in deionized water at 3.6% fat concentration (w/v), and thus no euglobulin was contained in this system.

[c]Cream and euglobulin were suspended in deionized water at the concentrations of 3.6 and 0.01%, respectively, (w/v).

concentration. However, it was not known exactly what conditions would be changed favorably for creaming by dilution of the milk.

When salt ions were completely removed from the system, no detectable creaming was observed in either system. It appears that an optimum combination of salts in an optimum concentration is required to enhance euglobulin-fat globule interactions. As discussed in a previous section of this thesis, both the sedimentation coefficient and the molecular weight of euglobulin decreased as the ionic strength of its solvent was decreased. This behavior was explained as a possible dissociation of euglobulin, affected by the low electrolyte concentrations. It should be pointed out, however, that the concentration range of euglobulin used in the ultracentrifuge studies was much higher, about five-fold or more, than that used in the creaming studies. If the creaming studies were carried out at sufficiently high euglobulin concentration range, comparable to that of centrifuge studies, it might be possible to observe slight decrease in the creaming ability at lower electrolyte concentrations. On the other hand, it might also be possible that the effect of slight denaturation of euglobulin in low salt systems could have been overcome by the more favorable conditions provided for the activity of euglobulin.

The Effects of Hydrogen Ion Concentrations on Creaming

Table 22 contains the results of creaming studies performed with normal and recombined milk as well as model systems at various hydrogen ion concentrations. As the hydrogen ion concentration of the systems departed from the natural value of milk, about pH 6.6-6.8, the creaming abilities of these milk samples decreased. The creaming ability was affected more adversely at low hydrogen ion concentrations than at the higher levels. The extremely poor creaming observed in the model system at low hydrogen ion concentrations may be related to the denaturation or dissociation of euglobulin molecules previously observed in the sedimentation studies.

Interpretation of The Creaming Phenomenon in Relation to the Physical Properties of Euglobulin

That euglobulin is required in creaming of cow's milk was demonstrated in the experiments with model systems. No creaming was observed when euglobulin was completely absent in the system, whereas creaming gradually improved as the concentration of euglobulin was increased.

When the euglobulin concentration was kept at a constant level of 0.01%, optimum creaming was observed at temperatures ranging from 3 to 5° C in milk salt solution. Interestingly, euglobulin itself undergoes significant changes at 5° C, that is, the weight average molecular weight in milk salt solution was approximately twice the

TABLE 22. The effects of hydrogen ion concentrations on the creaming properties

Sample	pH[a]	Cluster Index	Cream Volume (%)	
			1 Hr	4 Hr
Normal Milk[b]				
	5.0	---	3.7	3.4
	6.6	8.3	10.0	8.1
	9.0	6.9	7.2	6.4
Recombined Milk[b]				
	5.0	---	3.3	2.7
	6.6	7.5	8.0	7.1
	9.0	6.2	6.5	5.2
Model System[c]				
	3.0	0.9	0.6	0.9
	5.0	2.4	2.9	2.1
	6.6	4.3	4.6	3.9
	9.0	3.9	3.2	2.4

[a]pH of the skim milk or milk salt solution was adjusted with 0.1 N HCl or 0.1 N NaOH.

[b]Satisfactory determinations of both cluster index and cream volume were not possible at pH values lower than 5.0 since casein micelles in the normal and skim milk started to aggregate.

[c]Euglobulin concentration of 0.01% (w/v).

value observed at 20° C in veronal buffer. Therefore, it is conceivable that the enhanced polymerization of euglobulin in milk salt solution at low temperatures could enhance or even initiate the creaming of milk.

On the basis of the experimental results obtained in this investigation and the available information regarding the subject, it is concluded that the creaming phenomenon is favored by; (1) a high concentration of euglobulin available for the interaction with fat globules, (2) a creaming temperature low enough to enhance the polymerization of euglobulin, and (3) optimum concentrations of hydrogen ion and electrolytes to promote the association of euglobulin molecules.

In the foregoing discussions, various chemical and physical aspects of euglobulin and pseudoglobulin fractions of cow's milk were reviewed with particular emphasis on the association-dissociation phenomenon of euglobulin under various conditions. Specially designed experiments were also performed to assess the role of the euglobulin fraction in creaming of normal cow's milk.

Both euglobulin and pseudoglobulin fractions must be considered as highly heterogeneous molecules, containing more than one molecular species in variable amounts. Under specific conditions, these molecular species undergo substantial changes, i.e., association, dissociation, or aggregation. A typical example is the possible interaction of euglobulin with fat globules to enhance fat globule

clustering and subsequent creaming. The precise operational function of these proteins and the mechanism of euglobulin interaction with fat globules have not been elucidated in specific terms, and thus further extensive research is needed to unveil the mystery surrounding the physical and chemical behavior of this interacting protein.

SUMMARY

Sepharose 6B gel filtration and DEAE-cellulose anion exchange column chromatographic techniques in conjunction with ammonium sulfate precipitation method were employed to isolate euglobulin and pseudoglobulin from cow's milk in electrophoretically pure form.

These two immunoglobulins were nearly identical in chemical composition. The nitrogen content of euglobulin was 13.86% and that of pseudoglobulin was 14.14%. Euglobulin contained 11.24% carbohydrates and pseudoglobulin 10.64%. Both proteins contained fucose, galactose, galactosamine, glucosamine, mannose and sialic acid. Both contained less glutamic acid and proline and more serine and threonine than other milk proteins. Neither protein contained phosphorus.

The electrophoretic mobilities of euglobulin and pseudoglobulin in veronal buffer at pH 8.6 were -1.82 and -2.02 Tiselius units, respectively. Corresponding isoelectric points were pH 6.03 and pH 5.54. The partial specific volumes of euglobulin and pseudoglobulin were calculated from ultracentrifuge data and were reported as 0.712 and 0.710 ml/g, respectively.

The diffusion coefficients of euglobulin in veronal buffer, a milk salt solution and a guanidine hydrochloride-

mercaptoethanol dissociating system, as determined from ultracentrifugal data, were reported as 3.20, 3.20, and 37.90 Ficks, respectively. Corresponding values for pseudoglobulin were 4.20, 3.62, and 30.00 Ficks. The diffusion coefficient of euglobulin decreased with decreasing temperatures, but increased with decreasing hydrogen ion or electrolyte concentrations.

The sedimentation coefficients of euglobulin in veronal buffer, milk salt solution and the dissociating system were 6.24S, 6.15S and 4.63S, respectively, for the slow sedimenting component and 19.04S, 19.16S and 13.66S for the fast moving component. The sedimentation values of these two components increased with decreases in temperature and decreased as the hydrogen ion or electrolyte concentrations were lowered. Pseudoglobulin exhibited sedimentation coefficients of 6.00S, 5.91S and 4.61S, respectively, in the above buffer systems.

The weight average molecular weights of euglobulin and pseudoglobulin, determined by a low-speed equilibrium technique, in veronal buffer, milk salt solution and the dissociating system were 175,000; 244,000 and 89,000, respectively, for euglobulin and 167,000; 174,000 and 86,000 for pseudoglobulin. As the temperature was decreased, the weight average values of euglobulin increased, but these values decreased as the hydrogen ion or electrolyte concentrations were lowered. The weight average molecular weights of euglobulin in veronal buffer

and milk salt solution at 5° C were approximately 276,000 and 388,000, respectively.

The requirement for euglobulin as a component in the creaming phenomenon was evident from the experiments with model systems of washed fat globule, milk salts and euglobulin. No creaming occurred when euglobulin was absent in the system, whereas the creaming ability was improved as the euglobulin concentration was increased. At constant euglobulin concentration, approximately 0.01% by weight, temperature affected the creaming property significantly. The best creaming occurred in the temperature range of 3-5° C. Ionic strength had little effect on the creaming properties of both recombined milk and model system. Approximately 50% dilution of the whey resulted in the best creaming. The creaming ability decreased as the hydrogen ion concentration departed from pH 6.0 to pH 7.0.

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APPENDIX

APPENDIX

Composition of the Buffers Employed for Isoelectric Point Determinations

The following quantities were made to two liters with deionized water for use solutions.

1. Sodium Acetate, pH 4.0, $\mu = 0.2$

   72.0 ml of 5.0 M NaCl
   20.0 ml of 2.0 M sodium acetate
   33.7 ml of 3.5 M acetic acid

2. Sodium Acetate, pH 5.0, $\mu = 0.2$

   72.0 ml of 5.0 M NaCl
   20.0 ml of 2.0 M sodium acetate
   3.7 ml of 3.5 M acetic acid

3. Sodium Phosphate, pH 6.0, $\mu = 0.2$

   72.0 ml of 5.0 M NaCl
   9.2 ml of 0.5 M Na_2HPO_4
   6.6 ml of 4.0 M NaH_2PO_4

4. Sodium Phosphate, pH 7.0, $\mu = 0.2$

   72.0 ml of 5.0 M NaCl
   22.7 ml of 0.5 M Na_2HPO_4
   1.6 ml of 4.0 M NaH_2PO_4

5. Sodium Veronal, pH 8.6, $\mu = 0.2$

   72.0 ml of 5.0 M NaCl
   3.5 ml of 2.0 M HCl
   80.0 ml of 0.5 M sodium veronal

Composition of Jenness and Koops' Milk Salt Solution

Solution I

The following quantities of salts were made to 900 ml with deionized water:

KH_2PO_4	1.58 g
K_3 citrate$\cdot H_2O$	0.51 g
Na_3 citrate$\cdot 5H_2O$	2.12 g
K_2SO_4	0.18 g
Mg_3 citrate$\cdot H_2O$	0.50 g
K_2CO_3	0.30 g
KCl	1.08 g

Solution II

A 1.32 g of $CaCl_2\cdot 2H_2O$ was dissolved in 50 ml of deionized water.

Solution II was added very slowly to Solution I while stirring the mixture vigorously, and the content made up to one liter with deionized water.

Densities and Relative Viscosities of the Buffers Used

APPENDIX TABLE 1. Densities and relative viscosities of the buffers used[a]

Buffer	Relative Viscosity	Density
Veronal, pH 8.6, μ = 0.1	1.055	1.0039
Sodium Acetate, pH 4.0, μ = 0.1	1.058	1.0041
Jenness and Koops' Milk Salt Solution, pH 6.6	1.074	1.0073
6 M Guanidine Hydrochloride plus 0.02 M 2-Mercapto-ethanol	1.443	1.1205

[a]The densities were determined in 25 ml pycnometers and the viscosities in a Cannon-Ubelohde semi-micro dilution viscometer at 20 ± 0.01° C.