# PROTEINS OF THE HOMOGENIZED MILK FAT-GLOBULE MEMBRANE

by

Robert Howard Jackson

AN ABSTRACT

Submitted to the School for Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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ABSTRACT ROBERT HOWARD JACKSON

The fat-globule membranes of milk are materially altered as a result of homogenization. In the literature, one can find analytical, ultracentrifugal, and electrophoretic evidence showing the difference in membrane material before and after homogenization. The principal objective of this research was to isolate, characterize, and identify the proteins of the homogenized milk-fat globule. Also, the effect of heat was studied on the total amount of protein associated with the milk fat after homogenization.

For the isolation of homogenized fat-globule membrane proteins, a scheme developed for the isolation of nonhomogenized fat-globule membrane proteins was modified and extended. Sucrose was used to increase the specific gravity differential of the fat and serum of the homogenized milk. In so doing, greater yields of membrane materials were obtained.

The total amount of protein on the homogenized fat-globule membrane was found to be inversely dependent upon the amount of heat applied to the milk prior to homogenization. At 60°, 70°, and 80° C., the amount of protein associated with 100 grams of homogenized milk fat was 2.27, 1.60, and 1.31 grams, respectively. The 60° C. preparation had a total protein value 5 to 6 times greater per 100 grams of fat than the average protein value reported for the nonhomogenized fat-globule membrane, based on grams of protein per 100 grams of fat. When one considers that homogenization increases the fat surface of milk by a factor of 5 or 6, these data might indicate that the ratio of membrane protein to fat surface is relatively constant.

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Characterization and identification of protein fractions were based on electrophoretic, spectrophometric, and ultracentrifugal analyses. The protein fractions which were isolated from the homogenized milk fat-globule membrane were:

1. Insoluble membrane protein. This protein presented a reddish-brown, mucoidal appearance. After solubilization with peracetic acid, it was electrophoretically homogeneous. The characteristics of this protein were identical to the insoluble membrane protein of the nohomogenized fat-globule membrane.

2. Soluble membrane protein. Investigations indicated that a portion of this protein forms a complex with the alpha-casein adsorbed on the homogenized fat-globule membrane. This complex seemed to form only in the presence of milk-fat. The remainder of the soluble membrane protein was isolated by denaturing the heat-labile, adsorbed proteins at 90° C. for 30 minutes, leaving the soluble membrane protein in a relatively pure form.

3. Casein. A complex seemed to be formed with alpha-casein and a portion of the soluble membrane protein, in the presence of milk-fat. The protein-lipid interaction gave rise to an electrophoretic component with a mobility slightly less than that of alpha-casein. Also, the mobility of alpha-casein was decreased, and the ratio of alpha- to beta- casein was abnormally large.

4. Heat-labile protein(s). Although these proteins were not isolated

and identified, they were considered to be whey proteins.

In general, the fat-globule membrane proteins did not appear to be completely dissociated from the fat globule when the membrane was disrupted by homogenization. The increased surface area created by homogenization was covered, in part, by casein and by unidentified whey proteins.

The results of this research did not indicate that the resurfacing of fat globules subsequent to homogenization was limited to any specific milk proteins, but that a possible lipid-protein complex was formed as a result of the treatment.

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# INTRODUCTION

Homogenization had its inception at the beginning of the twentieth century, but did not receive consumer acceptance for nearly twenty-five years. The homogenization of fluid milk, to produce a smoother, more palatable commodity, has since become a fundamental process in the dairy industry. Essentially, homogenization is a mechanical method of reducing the milk fat-globule in size and increasing it in number.

A large amount of research has been devoted to determining the optimum conditions for producing the best homogenized milk. Unfortunately, there has been very little research directed toward a most important area affected by the process - the fat/serum interface.

Lipolysis and sunlight flavor are enhanced, while copper-induced oxidation is retarded by an increase in fat surface following homogenization. These are but a few of the reactions indicating that the fat/serum interface is an important site in the chemical and physical modification of homogenized milk. Such defects as "free fat" in whole milk powder and "chalky" flavor in fluid homogenized milk might be explained by the presence or absence of adsorbed materials on the homogenized fat-globule membrane. Only when these adsorbed materials are identified and their arrangement on the homogenized fat-globule is established, will we gain an insight into the problems inherent in homogenizing milk.

In this research, the principal objective was to isolate, characterize, and identify the proteins of the homogenized milk-fat globule. Also, the effect of heat was studied on the total amount of protein

associated with the milk fat after homogenization. The results of these investigations are presented and discussed in the body of this manuscript.

## REVIEW OF LITERATURE

### The Proteins of the Nonhomogenized Milk Fat-Globule Membrane

Since Ascherson (1840) first proposed that a membrane surrounds the fat globule of bovine milk, there has been an ever-increasing compilation of evidence to support his original theory. The bulk of this evidence has been submitted within the past fifty years by researchers using techniques ranging from the visual observations of stained fat-globule membranes by Storch (1897) to the electrophoretic studies of Brunner, Lillevik, Trout and Duncan (1953c).

Storch was the first to use a washing technique in isolating fat-globule membrane materials. His fat-globule membrane had a nitrogen content of 14.76% and contained an associated substance capable of reducing Fehling's solution. He believed that this membrane, which he called "albuminoid", differed from any previously recognized albuminoid of milk. Wiegner (1914), calculating from viscosity measurements, reported that the nonhomogenized fat globule adsorbed 2% of the casein of milk. Using a technique in which milk was treated with water which was saturated with chloroform, Hattori (1925) isolated the material surrounding milk fat globules and found it to be principally protein. This protein, which he called "haptein", differed from any known milk protein on the basis of its solubility and chemical composition. "Haptein" was reported to have a low nitrogen content and a cystine content exceeded only by keratin. Palmer and Weise (1933) revealed that

the material most closely adsorbed on the fat globule surface is a mixture of phospholipids and proteins. Furthermore, they contended that this protein had both a hydrophilic and a hydrophobic nature and a phosphorous, nitrogen, and sulfur content unlike any other milk protein. Titus, Sommer, and Hart (1928) believed the membrane protein to be closely related to, if not identical with, casein, based on its nitrogen distribution characteristics, specific rotation, and sulfur, phosphorous, and tryptophane content. Moyer (1940) concluded from electrokinetic studies that the fat-globule membrane protein differed from other known milk proteins, and that much of the confusion of previous research in this area could be attributed to the incomplete removal of other milk plasma proteins from those occurring on the surface of fat globules.

Mulder (1949) postulated that the surface layer of fat globules should not be regarded as having been adsorbed from milk plasma. Instead, he offered the conjecture that it consisted of components of the protoplasm of the cells of the lactiferous glands as well as substances adsorbed from the plasma of milk. Mulder (1957) further postulated that the composition of the surface layer varied from globule to globule, depending upon the disturbance of the layers surrounding the fat globule. In this same publication, Mulder professed that the degree of disturbance of the membrane layers was proportional to the adsorption of material from the serum.

Hare, Schwartz, and Weese (1952), using a microbiological assay technique, showed that the membrane protein contained more phenylalanine, threonine, and glycine and less aspartic acid than any of the major milk proteins. Brunner, Duncan, and Trout (1953b), using a similar technique,

showed the amino acid composition of the nonhomogenized fat-globule membrane to be of the same order. Brunner et al. (1953c) presented the first data on the electrophoretic behavior of isolated, nohomogenized membrane proteins which revealed from one to three electrophoretic components. Brunner, Duncan, Trout, and MacKenzie (1953a) showed the nonhomogenized fat-globule membrane protein to have a sedimentation diagram featuring one principal, homogeneous component with an $S_{20}$ of 7.3. They classified this protein as a globulin in nature.

Herald and Brunner (1957) isolated and characterized the nonhomogenized fat-globule membranes as having soluble and insoluble entities in 0.02 M NaCl and/or water. Furthermore, these researchers found carbohydrate associated with the soluble fraction. The insoluble fraction was classified tentatively as a pseudokeratin, based on it solubility and chemical properties. The soluble fraction was analyzed electrophoretically by Herald and Brunner (1958) in various buffers, ranging in pH values from 2.05 to 8.79, and showed one small and two large migrating boundaries. After treating the insoluble fraction with various protein-solubilizing agents, these workers showed a single, electrophoretic peak with sodium-sulfide-treated samples, but one small and two large migrating boundaries in detergent-treated samples. Sasaki, Koyama, and Nishikawa (1956b) postulated that the fat-globule membrane is electrophoretically similar to whey proteins or whey proteins less beta-lactoglobulin.

Morton (1954) produced electron micrographs showing milk microsomes which were adsorbed to the membrane protein surrounding the fat globule. These microsomes were shown to be brown in color and to contain 22%

lipid, 10% nucleic acid, a haemochromogen, alkaline phosphatase, xanthine oxidase, diaphorase, and DPN cytochrome c reductase. Zittle, DellaMonica, Custer, and Rudd (1956) found only 0.5% nucleic acid in the microsomes of milk and defined them as parcels of enzymes cemented together with nucleic acid and phospholipid. The enzymes given most consideration in this study were alkaline phosphatase and xanthine oxidase. The monograph of King (1955) should be given special recognition as a complete and comprehensive review of research on the milk fat-globule membrane.

## Emulsification of Butterfat in Various Solutions of Milk Origin

Weise and Palmer (1932) emulsified butterfat in buttermilk and solutions of calcium caseinate, lactalbumin, globulin, phospholipid, and globulin plus phospholipid. Using churnability as an index, they maintained that buttermilk most closely replaces the substances adsorbed on the fat globules in cream. By comparing the material which did not wash off the fat globule, after emulsification in sweet rennet-whey, skimmilk, and buttermilk, with the natural fat globule membrane, Rimpila and Palmer (1935) showed the materials to differ in percent and proportion of phospholipids and protein and in their nitrogen distribution characteristics. They also concluded that in the synthesis of milk, the natural fat-globule membrane is not derived from milk plasma. Sasaki and Koyama (1956a) emulsified butterfat in solutions of radioactive casein and radioactive whey proteins. Two washings removed casein and the beta-lactoglobulin of the whey protein, but the remainder of the whey proteins was still associated with the fat after four

washings. These workers then concluded that the fat-globule membrane does not contain casein or beta-lactoglobulin, but some whey proteins plus a possible unknown or specific protein.

## The Proteins of the Homogenized Milk Fat-Globule Membrane

By applying the mathematical formula, $4/3\pi r^2$, Trout, Halloran, and Gould (1935) calculated that homogenization increased the surface of fat globules by a factor of 5 or 6. They also observed that the stability of milk proteins toward alcohol decreased after homogenization. Doan (1938) and Sommer (1946) felt that the degree of destabilization increased with increasing fat contents. Doan (1938) found milk to be more easily coagulated after homogenization. He explained this phenomenon by stating that "a greater amount of casein is adsorbed on the expanded amount of fat globule surface", and since this casein is fixed, it is non-motile, which is the first stage of coagulation. Wiegner (1914) assumed that the adsorbed protein film of homogenized milk had a density of 1.4. Using this value, he calculated from viscosity measurements that is homogenized milk, 25% of the casein was adsorbed by the fat. Sullam (1941) disagreed with the generally-accepted theories. He maintained that homogenization increased the stability of milk proteins. Chambers (1936) suspected that casein or other polar constituents were adsorbed by the fat in proportion to the increased fat surface made available for such adsorption by the homogenization of the milk. Sammis (1914) found that homogenized cream appeared thicker than ordinary cream of the same fat content and was more difficult to churn. He also reported "that homogenization affects the casein in ways

which cannot be fully explained at present".

Sommer (1946) stated that, quantitatively, there may be differences between the adsorbed material of homogenized fat globules as compared with globules as they exist naturally in milk. Weinstein, Lillevik, Duncan, and Trout (1951) reported that the development of activated flavor in homogenized milk may be attributed to the selective rearrangement of the fat-globule membrane following homogenization. The constituents which give rise to activated flavor may be adsorbed on the increased fat surface, which is the reactive site for the development of this off-flavor. Trout (1950) believed that the protein material of plasma was adsorbed on homogenized fat globules and that this adsorbed membrane is different from the membrane of the original fat globules. In other words, the newly created fat globules of homogenized milk are resurfaced.

Brunner et al. (1953b) showed marked differences in the amino acid composition of homogenized and nonhomogenized fat-globule membranes. From this, they concluded that the adsorbed layer on homogenized fat globules appears to be different from the adsorbed layer on normal fat globules. Brunner et al. (1953c) based their interpretations on an electrophoretic comparison of the two membranes and proposed that the membrane of homogenized milk was composed of adsorbed milk-plasma proteins in addition to the fat-membrane protein complex of the original fat globules. Brunner et al. (1953a) compared the sedimentation velocity diagrams of solutions of the homogenized and nonhomogenized fat-membrane proteins. The homogenized membrane showed three, or possibly four peaks, which were identified as casein, Kekwick's whey protein, and an altered lactoglobulin (beta-lactoglobulin or a complex of two or

more constituent proteins).

## The Effect of Heat on the Protein Adsorbed on the Milk Fat-Globule Membrane

Lowenstein and Gould (1954) showed a decreased adsorption of protein on the fat globule with increased heat. When whole milk was heated momentarily to 40° C. and 62° C. for 30 minutes, and 82° C. for 15 minutes, the amount of protein in the membrane material was 21.86, 15.54, and 7.70 percent, respectively.

## Protein-Lipid Interaction in Homogenized Milk

Fox, Caha, Holsinger, and Pallansch (1959) found that conditions existing at the homogenizer valve produced a fat-protein complex, and that the principal protein, if not the only one, involved in the interaction is the casein micelle. Litman (1955) reported a fat-protein complex or "scum" formation in whole milk powder. Of the 34% protein in the complex, 82% to 96% consisted of casein and denatured whey protein. The fat isolated from the complex had a high melting point, which may be due to oxidation, or the fat may be similar to the high-melting triglyceride isolated from the fat-globule membrane. He further stated that the amount of complex was directly related to the pre-heat treatment of processing.

## EXPERIMENTAL PROCEDURE

### Analytical Methods

Electrophoresis. All electrophoretic data were obtained with a Perkin-Elmer Model 38-A Electrophoresis Unit. Protein solutions were dialyzed against standard buffers for at least 36 hours with no less than two 400 milliliter buffer changes. Electrophoretic runs were begun after a temperature equilibrium of the protein solution and the ice-water bath at 1° C. was reached.

Electrophoretic mobilities were calculated using the following equation:

$$\mu\ (cm^2,\ volt^{-1},\ sec^{-1}) = \frac{d\ a\ k}{t\ i\ r\ m}$$

where:

d = distance boundary traveled

a = cross-sectional area of the cell

k = conductivity cell constant = 0.8491

t = time in seconds

i = current in amperes

r = resistance of buffer in ohms, and

m = magnification factor of the optical system

The distance (d) was measured from the initial boundary, and all mobilities were calculated by this method.

Potential gradient or field strength was calculated from the following equation:

$$F = i/aK$$

where:

F = potential gradient

i = current in amperes

a = cross-sectional area of the cell, and

K = specific conductivity of the buffer protein solution

Since all the electrophoretic analyses were conducted under similar conditions, potential gradient was not considered a variable factor.

Relative areas under individual electrophoretic peaks were obtained by: (1) averaging three planimeter readings of the area beneath the peak or (2) drawing a perpendicular line from the lowest point between peaks, cutting-out the area, and weighing the individual segments.

All interpretations of electrophoretic patterns were made on the descending channel.

Ultracentrifugal analyses. Sedimentation velocity data were obtained with a Spinco Model E analytical centrifuge operated under the conditions indicated with the presentation of results. Proteins were carried in Veronal buffer solutions of pH 8.7 and ionic strength of 0.1. These solutions were prepared and analyzed at three concentration levels, varying between 0% and 2%, and their values plotted and extrapolated to zero concentration. The method of least squares was used to obtain a linear plot of the data, according to Federer (1955).

Calculations were made from the following equation:

$$S \text{ (uncorrected)} = \frac{d + m}{40 \cdot t \cdot rps^2 \cdot r}$$

where:

d = distance migrated in centimeters

m = magnification factor of the instrument - 1.7

40 = $4\pi^2$

t = time in seconds

r = distance from center of rotor to a position at the center of a component, which it would be if an exposure were made half-way between the pair of peaks used for a calculation.

5.7 = distance in centimeters from the center of the rotor to the second margin of the counterbalance hole.

The S (uncorrected) was corrected to $S_{20}$ from a table of conversion factors ($n_t^o/n_{20}^o$) given by Svedberg and Pedersen (1940). The sedimentation velocity constants were uncorrected for viscosity.

Paper-partition chromatography. Unidimensional, descending, paper-partition chromatography was used to identify carbohydrate moities associated with the heat-stable fraction isolated by the fractionation scheme used in this research. An amyl alcohol: pyridine: water solvent system in a 4: 3: 2 ratio carried the sugars over unbuffered Whatman #1 cellulosic filter paper for contact periods ranging from 32 to 40 hours.

Protein hydrolyzates were obtained by dissolving 50 milligrams of protein in 10 milliliters of 2 N HCl, placing the solution in a boiling water bath for 3 hours, and filtering-out the extraneous residue.

Chromatograms were developed after spraying with 2% triphenyl-tetrazolium chloride in an equal volume of 1 N NaOH, according to Block, Durrum, and Zweig (1955).

Ultraviolet analyses. Distilled water solutions of proteins with concentrations of 0.05% were examined in the ultraviolet spectrum ranging

from 220 to 340 millimicrons, using a Beckman Model DK-2 Spectrophotometer.

## Procedure for the Isolation of Homogenized Milk Fat-Globule Membrane Proteins

Essentially, the isolation scheme used in this research was similar to the procedure developed by Herald and Brunner (1957) in their work with the nonhomogenized fat-globule membrane proteins. Since additional proteins were present on the homogenized fat-globule membrane, the isolation scheme was extended to provide for their fractionation. The method of fractionation and isolation employed in this study is shown in Figure 1.

### Preparation of Homogenized Milk Fat-Globule Membrane Proteins

Fresh, raw milk, with approximately 3.5% fat and 12% total solids, was the starting material in all of the preparations. The milk was subjected to one of several selected heat treatments, and then homogenized at 2500 p.s.i. and 58° C. in a Manton-Gaulin Model K homogenizer.

The homogenized milk was separated at 60° C. in a Model 518 DeLaval Laboratory Separator after the addition of 3% (w/w) sucrose, which increased the specific gravity differential of the fat and the serum. The homogenized fat, with its newly-formed surfaces, was washed three times with three volumes each of 3% sucrose solution and water, both at 40° C. The washed homogenized cream was churned between 50° C. and 58° C., after chilling below these temperatures overnight. Separation of the fat and sera at 38° C. was completed in a Model 9 DeLaval Laboratory Separator. The milk fat was discarded and the membrane-

containing serum was salted-out when the solution was adjusted to 2.2 M $(NH_4)_2SO_4$. The membrane material was concentrated by centrifuging in a Model SS-1 Servall Centrifuge for 30 minutes at 25,000 x G.

Two hundred milliliters of 35% (v/v) ethanol in ethyl ether at 0° C. to -5° C. were added to each 50 grams of concentrated, homogenized membrane material. The mixture was agitated 15 minutes and filtered in the cold. The residue was washed 5 times with ethyl ether at 0° C. to -5° C. and extracted 3 times with ethyl ether at 25° C. for 10, 5, and 5 minutes. Residual ether was removed overnight under 16 inches of vacuum.

The homogenized membrane-proteins were extracted 4 times with 0.02 M NaCl and centrifugally separated for 30 minutes at 25,000 x G. The residue was the insoluble membrane protein of the nonhomogenized fat-globule membrane, which was designated in this research as the Insoluble Fraction (I). Peracetic acid was used to solubilize this protein. The supernatant contained a number of proteins which were separated by repeatedly adjusting the pH of the solution to 4.6 and centrifuging at 25,000 x G. for 30 minutes. The residue was redissolved in a dilute NaOH solution with an adjusted pH of 9.0. Electrophoretically, the residue appeared to be casein; however, the pattern showed a component with a mobility slightly less than that of alpha-casein. The supernatant was comprised of at least one heat-stable and one heat-labile protein. The heat-labile fraction was removed by centrifugation at 25,000 x G for 30 minutes following a heat treatment at 90° C. for 30 minutes.

## Determination of the Total Amount of Protein Remaining on the Homogenized Milk-Fat Globule

Washed homogenized cream was prepared in exactly the same manner as illustrated in Figure 1, with the exception that 8% (w/w) sucrose was used in the initial separation and the 5 subsequent washings. This amount of sucrose was taken into account in all calculations.

The washed cream was analyzed for fat and total solids by the method of Mojonnier and Troy (1925) and nitrogen by a modification of the method of Menefree and Overman (1940). Since the fat content of the washed cream ranged between 40% and 55%, it was necessary to use 30 milliliters of nitrogen-free $H_2SO_4$ to digest the 5 gram sample. Sixty milliliters of 50% NaOH was used to neutralize the sample prior to distillation.

The milk used in this portion of the research was analyzed for serum protein denaturation by heat according to a slightly modified method of Kuramoto, Coulter, Jenness, and Choi (1959). The modification entailed the use of a 16.67 gram sample (12.00% T. S.) which was equivalent to a 2 gram sample of dry milk.

## Experimentation with the Fraction Precipitated at pH 4.6

The isolation of this fraction has been discussed and the scheme is shown in Figure 1.

## Various Chemical and Physical Treatments Used in the Separation of the Casein-Complex

Heat. A test tube containing 20 milliliters of a 1.0% (w/w) water solution of the Casein-complex Fraction (III) was heated for 30

minutes at 90° C. in a water bath. The solution was centrifuged at 25,000 x G. for 30 minutes, after which the supernatant was dialyzed against buffer in lieu of electrophoretic analysis.

Urea. The Casein-complex Fraction (III) was solubilized in 6.6 urea and agitated for 18 to 24 hours. The molarity of the urea and the pH of the solution was adjusted to 1.0 and 4.6, respectively. After centrifuging at 25,000 x G. for 30 minutes, the residue was washed with distilled water to remove the urea present. After dialyzing against water and then buffer, the protein was analyzed electrophoretically.

The Casein-complex Fraction (III) was further fractionated with urea according to the method of Hipp, Groves, Custer, and McMeekin (1952). The primary purpose of this experiment was the separation of the unidentified electrophoretic component at some level of urea molarity, for subsequent identification.

Ethyl ether. Three grams of the Casein-complex Fraction (III) were agitated with 50 milliliters of ethyl ether at room temperature. After centrifuging to reclaim the protein, the residual ether was removed under vacuum, and the protein was prepared for electrophoretic analysis.

Rennet. Thirty milliliters of a 3% (w/w) water solution of the Casein-complex Fraction (III) was prepared and agitated for 20 minutes with 0.5 milliliters of a solution of commercial rennet and water, mixed in a ratio of 1 to 20. The temperature and pH of the solution was 30° C. and 8.0, respectively. After agitation, a dilute solution of $CaCl_2$ was added drop-wise until flocculation no longer occurred. The precipitate was concentrated by centrifugation at 25,000 x G. for

30 minutes and redispersed with dilute NaOH. The solution was then dialyzed against buffer before being analyzed electrophoretically.

## Reconstruction of the Casein-complex with Selected-Component Systems

This segment of the research necessitated the preparation of the following materials:

1.) Membrane-containing serum: Fresh, raw nonhomogenized milk was fractionated according to the technique of Herald and Brunner (1957) to obtain membrane-containing serum. This serum can also be called washed-cream buttermilk, which is rich in the soluble and insoluble nonhomogenized fat-globule membrane proteins.

2.) Calcium caseinate: Three liters of a 3% (w/w) solution of sodium caseinate, at pH 7.5, was dialyzed against 10 gallons of pasteurized skimmilk for three days. This yielded a casein solution whose protein approached that of casein in its native state.

Homogenization of a mixture of calcium caseinate and membrane-containing serum. Six hundred milliliters of the calcium caseinate solution and 300 milliliters of the membrane-containing serum were mixed and heated for 30 minutes at 60° C. and homogenized at this temperature at 3000 p.s.i. Two-stage homogenization was used throughout this research; pressure at the first and second stages being 2500 and 500 p.s.i., respectively.

After homogenization, the mixture was salted-out with $(NH_4)_2SO_4$, treated with organic solvents, extracted with 0.02 M NaCl and adjusted to pH 4.6 according to the isolation scheme employed in this research to obtain the Casein-complex Fraction (III). The mixture was analyzed

electrophoretically after sufficient dialysis against Veronal buffer of pH 8.6 and ionic strength of 0.1.

Homogenization of a mixture of calcium caseinate, membrane-containing serum, and butteroil. One thousand milliliters of calcium caseinate solution was mixed with 1000 milliliters of membrane-containing serum and 100 milliliters of fresh butteroil. The mixture was heated to 60° C., for 30 minutes and homogenized at 3000 p.s.i. After homogenization, the isolation scheme shown in Figure 1 was followed to obtain the material which precipitated at pH 4.6.

Homogenization of a mixture of alpha-casein, soluble membrane protein, and butteroil. Two hundred milliliters of a solution of soluble membrane protein was mixed with a solution of 600 milliliters of alpha-casein and 40 milliliters of fresh butteroil. The total solids of the soluble membrane solution was 0.93% and that of the alpha-casein solution was 0.68%. The mixture was heated to 60° C. and held for 30 minutes, after which it was homogenized 3 times at 3000 p.s.i. at 60° C. The solution was cooled, adjusted to pH 4.6, and centrifuged at 25,000 x G. for 30 minutes.

Lipids were extracted in the cold according to the isolation scheme. The residual ether was removed under vacuum, and a sample of the protein was prepared for electrophoretic analysis.

Homogenization of a mixture of alpha-casein and butteroil. The same procedure was followed as in the preceding experiment, with the exception that no soluble membrane protein was included.

## Various Chemical and Physical Treatments of Casein and the Soluble Membrane Protein

Ethanol-ethyl ether treatment of casein. Isoelectrically precipitated casein was prepared and divided into two parts. The first was analyzed electrophoretically as a control, and the second was treated with organic solvents as indicated by Herald and Brunner (1957). Five grams of casein were added to 10 milliliters of 35% ethanol in ethyl ether at 0° C. to -5° C. and agitated for fifteen minutes. The mixture was filtered and washed 5 times with ethyl ether at approximately 0° C. and extracted 3 times with ethyl ether at room temperature for 10, 5, and 5 minutes. Residual ether was removed under vacuum, and the casein prepared for and analyzed electrophoretically.

Peracetic acid treatment of casein. Enough isoelectrically precipitated casein was added to a 3% (v/v) solution of peracetic acid to make a protein solution slightly greater than 1% (w/v) in concentration. The mixture was agitated for 18 to 24 hours, until all the protein particles were dissolved. Centrifugation at 14,000 x G. for 15 minutes yielded a residue which was transferred to filter paper and washed 3 times with cool distilled water. The residue was put into solution with the aid of a few drops of dilute $NH_4OH$. The solution was prepared for electrophoresis by dialysis against distilled water and buffer.

Homogenization of isolated casein and isolated whey proteins. Casein was isoelectrically precipitated from samples of the same lot of raw skimmilk before and after homogenization. The milk was homogenized at 2000 p.s.i. first stage and 500 p.s.i. second stage at 55° C. These two casein samples, together with their supernatants which contained

whey proteins, were analyzed electrophoretically. This investigation provided a comparison of the electrophoretic patterns of casein and whey proteins before and after homogenization.

Precipitation of the soluble membrane protein at pH 4.6. The pH of a 0.95% solution of soluble membrane protein was adjusted to 4.6 with dilute HCl and centrifuged at 9200 x G. for 10 minutes. The percentage total solids of the supernatant was determined.

Precipitation of the soluble membrane protein and alpha-casein at pH 4.6. Equal volumes of solutions of 0.95% soluble membrane protein and 0.98% alpha-casein were mixed. The pH of the solution mixture was adjusted to 4.6 with dilute HCl and centrifuged at 9200 x G. for 10 minutes. The total solids of the supernatant was determined.

Electrophoretic analysis of casein isolated from homogenized milk. Casein was isoelectrically precipitated from homogenized milk with dilute HCl at pH 4.6. Redispersion with NaCH and reprecipitation with acid was completed 3 times. The protein was dialyzed against buffer and analyzed electrophoretically.

FRESH RAW MILK

1. Heated to 60°, 70° or 80° C. for 30 min.
2. Homogenized at 2500 p.s.i. at 60° C.

HOMOGENIZED MILK

1. Separated at 60° C. after addition of 3% sucrose solution.

HOMOGENIZED CREAM

1. Washed 3X with volumes of 3% sucrose solution.
2. Washed 3X with 3 volumes of water at 40° C.

WASHED HOMOGENIZED CREAM

1. Churned after chilling overnight.

HOMOGENIZED FAT AND SERA

1. Separated at 38° C.

MILK FAT

Discard

MEMBRANE-CONTAINING SERUM

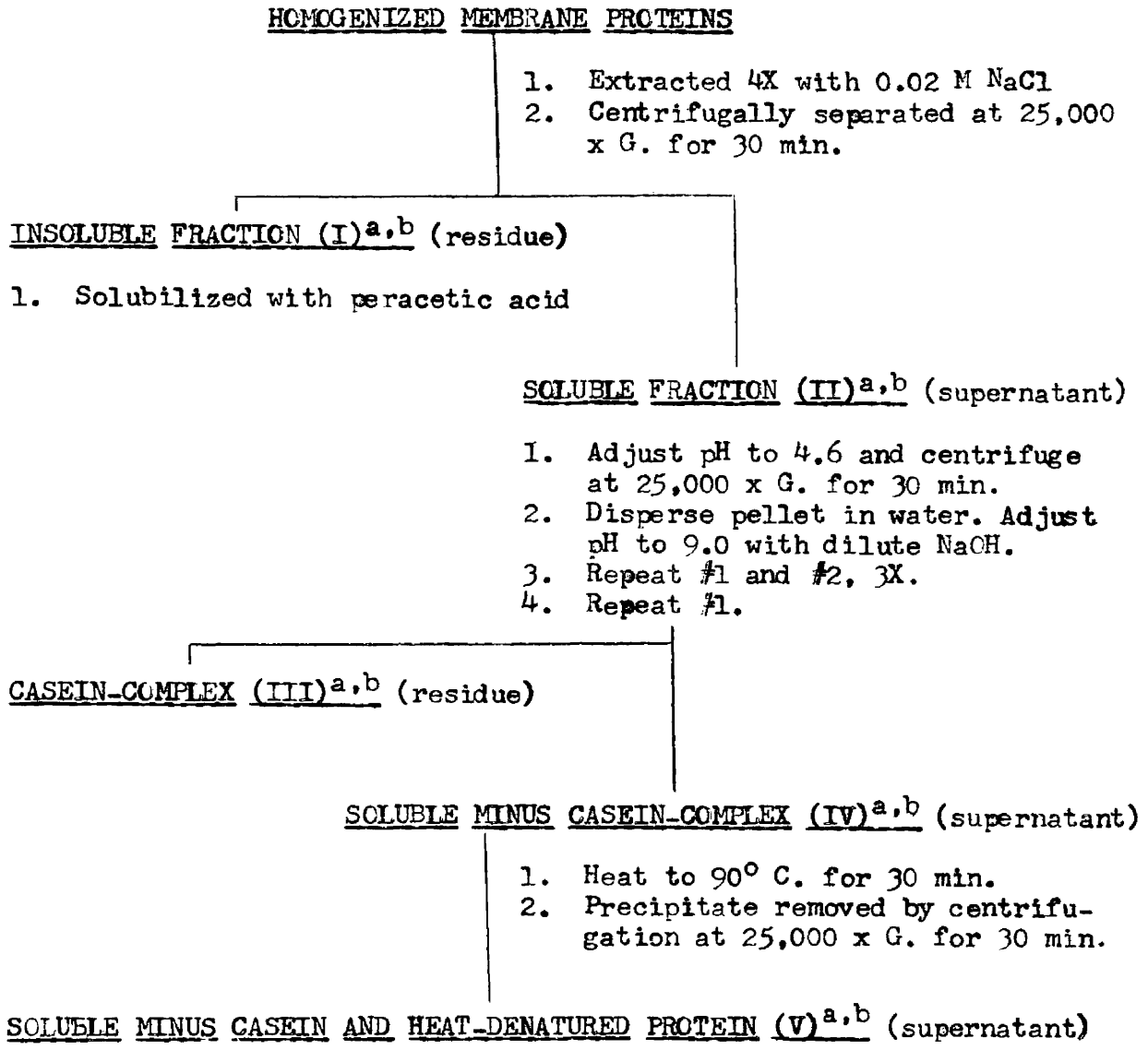
1. Salt out in 2.2 M $(NH_4)_2SO_4$
2. Concentrate by centrifugation at 25,000 x G.

HOMOGENIZED MEMBRANE (concentrated)

Lipid extraction:

1. 200 ml. of 35% EtOH in $Et_2O$ at 0° to -5° C. added per 50 g. membrane.
2. Agitated 15 min. and filtered in the cold.
3. Washed 5X with $Et_2O$ at 0° to -5° C.
4. Extracted 3X for 10, 5, and 5 min. with $Et_2O$ at 30° C.
5. Residual ether removed under vacuum.

(continued)




[a] See Glossary for description of isolated fractions
[b] See Figure II for electrophoretic patterns of isolated fractions.

FIGURE 1. Procedure for isolating the proteins associated with the homogenized milk-fat globule.

## EXPERIMENTAL RESULTS

### Effect of Heat on the Total Amount of Protein Remaining on the Homogenized Milk-Fat Globule

The data in Table 1 show that the total amount of protein remaining with the homogenized milk-fat globule decreased as the temperature was increased. At 60°, 70°, and 80° C. for 30 minutes, the amount of protein associated with 100 grams of homogenized fat was 2.27, 1.60, and 1.31 grams, respectively. As might be expected, the whey protein nitrogen values for the milk decreased with increasing heat treatment. (Table 1).

### Experimentation with the Fraction Precipitated at pH 4.6

#### The Effect of Various Chemical and Physical Treatments in the Separation of the Casein-complex

Heat. Heating the Casein-complex Fraction (III) for 30 minutes at 90° C. did not markedly affect any of the electrophoretic components, as shown in Figure 3. The only apparent affect was the decreased mobilities of the components of the complex after heating. The electrophoretic mobilities and relative areas of the peaks are listed in Table 2.

Urea. After solubilizing the Casein-complex Fraction (III) in 6.6 M urea, the molarity of the solution was adjusted to 1.0, and the aggregated protein was concentrated by centrifugation. The electro-

phoretic characteristics of the protein were not affected by the treatment. Electrophoretic patterns of the complex before and after urea treatment are shown in Figure 3. The electrophoretic mobilities and relative peak areas are given in Table 2.

Using the urea fractionation technique of Hipp et al. (1952), the protein isolated at 4.7 M urea is shown in Figure 6. Based on electrophoretic mobilities, the two observed components were the first two of the Casein-complex Fraction (III), in order of decreasing mobility. Mobilities and relative peak areas are listed in Table 2.

Ethyl ether. The electrophoretic patterns of the Casein-complex Fraction (III) before and after being treated with ethyl ether at room temperature are shown in Figure 4. Component 2 was reduced in relative area by 22.6% (Table 2). The electrophoretic mobilities of the components, listed in Table 2, of the ether-treated sample were somewhat less than the same components in the untreated sample.

Rennet. Addition of rennet to the Casein-complex Fraction (III) did not affect the typical electrophoretic pattern. A comparison of the patterns before and after rennet treatment are shown in Figure 4. Calculated electrophoretic mobilities and relative peak areas are given in Table 2.

## Reconstruction of the Casein-complex with Selected-Component Systems

Homogenization of a mixture of calcium caseinate and membrane-containing serum. This experiment was conducted in anticipation of this selected-component system producing an electrophoretic pattern similar to that of a typical Casein-complex Fraction (III). The pattern

shown in Figure 5 is a typical casein pattern, whose only deviation from a normal pattern was manifested by an increase of the peak area in the position normally occupied by gamma casein. The relative concentration of alpha- to beta-casein was in order (Table 2).

Homogenization of a mixture of calcium caseinate, membrane-containing serum, and butteroil. The selected-component system employed in this experiment differed from the preceding by the incorporation of butteroil. The relative concentration of alpha- to beta-casein is higher than in normal casein. The electrophoretic pattern of this homogenized mixture is shown in Figure 5, and the mobilities and relative peak areas are listed in Table 2.

Homogenization of a mixture of alpha-casein, soluble membrane protein and butteroil. The electrophoretic pattern of the precipitate of the solution mixture at pH 4.6 did not show the ratio of peak areas that was expected. Before homogenization, the ratio of alpha-casein to soluble membrane protein was much lower than the same ratio after homogenization, as determined from areas under electrophoretic peaks. The supernatant, at pH 4.6, had a total solids content of 0.106%, of which 0.025% was fat. In the precipitate, the leading electrophoretic peak, which was alpha-casein, had a descending mobility of 5.8. This value is considerably lower than the corresponding value of 6.5 for the same alpha-casein used as a control under identical conditions. The electrophoretic pattern of this complex is shown in Figure 5.

Homogenization of a mixture of alpha-casein and butteroil. The alpha-casein precipitated at pH 4.6 and treated with organic solvents showed a characteristic lipid spike in its electrophoretic pattern

(Figure 5). However, its electrophoretic mobility was not decreased, as in the preceding experiment (Table 2). The same alpha-casein was used in this and the preceding experiment.

## Analyses of Fractions

Ultracentrifugal characteristics. Sedimentation velocity studies were made on two fractions: the Soluble Minus Casein Fraction (IV) and the Soluble Minus Casein and Heat-denatured Protein Fraction (V).

A diagram of the Soluble Minus Casein Fraction (IV) shown in Figure 8, demonstrates the presence of three molecular species. Figure 9 shows the extrapolation of $S_{20}$ sedimentation values at three concentration levels to obtain an $S_{20}$ for each component at zero concentration.

Also shown in Figure 8 are sedimentation velocity diagrams of the Soluble Minus Casein and Heat-denatured Protein Fraction (V). There were two components in this fraction. Figure 10 shows the $S_{20}$ values extrapolated to zero concentration for both components. Table 4 lists the sedimentation velocity constants for all the components in both fractions.

Paper-partition chromatography. The Soluble Minus Casein and Heat-denatured Protein Fraction (V) showed three definite carbohydrate moities. A fourth carbohydrate spot appeared frequently but could not be accounted for in all the chromatograms. Identification of chondrosamine, glucosamine, and mannose was made by comparing the Rf values of known carbohydrates on the same chromatogram. The known sugars were spotted alone and in mixtures. When discernible, the fourth sugar had an Rf value identical to that of glucose.

Ultraviolet analyses. Figure 11 shows the spectrophotometric pattern of the Soluble Minus Casein and Heat-denatured Protein Fraction (V) in the ultraviolet region of the spectrum. In the same figure, a pattern of the soluble membrane protein is shown for comparison. The soluble membrane protein was heated for 30 minutes at 90° C. prior to being analyzed. The coincidence in the positions of the small absorption peaks should be noted.

## The Effect of Various Chemical and Physical Treatments of Casein and the Soluble Membrane Protein

Effect of an ethanol-ethyl ether treatment on the electrophoretic characteristics of casein. The electrophoretic pattern of whole casein after treatment with ethanol and ethyl ether is shown in Figure 6. No major alteration of the components was electrophoretically evident. The mobilities of the peaks appearing in the treated sample were slower than those in the control sample. Table 2 lists the mobilities and relative areas of the electrophoretic peaks.

Effect of a peracetic acid treatment on the electrophoretic characteristics of casein. Treating whole casein with peracetic acid did not appreciably affect its electrophoretic pattern, although it appears slightly atypical in the relative concentration of alpha- to beta-casein. (Figure 6, Table 2)

Effect of homogenization on the electrophoretic characteristics of isolated casein and isolated whey proteins. There was no change in the electrophoretic patterns of either casein or whey proteins before and after homogenization. (Figure 7, Table 2)

Precipitation of the soluble membrane protein at pH 4.6. The super-

natant of the solution adjusted to pH 4.6 had a total solids value of 0.71%. With the original solution having a total solids content of 0.95%, calculations show that 25.2% of the soluble membrane protein will precipitate at pH 4.6.

Precipitation of the soluble membrane protein and alpha-casein at pH 4.6. The total solids of the supernatant of the solution adjusted to pH 4.6 was 0.35%. Calculations made from this value show that 63.8% of the total protein was precipitated. Assuming complete precipitation of the alpha-casein and 25.2% precipitation of the soluble membrane protein, the 36.2% protein remaining in solution was completely assigned to the soluble membrane protein. That is, the 74.8% soluble membrane protein, not precipitated at pH 4.6 and diluted by a factor of 2, gave 37.4% total solids in the supernatant.

Electrophoretic analysis of casein isolated from homogenized milk. The electrophoretic pattern of casein isoelectrically precipitated from homogenized milk does not indicate clearly the presence or absence of a complex formation in this preparation (Figure 6). The electrophoretic mobilities of the components, listed in Table 2, are in order with those of whole casein from nonhomogenized milk and do not seem to be deterred by a complex formation. On the other hand, the concentration ratio of the alpha- to beta- casein components, in Table 2, is too large and the small component behind the alpha-casein peak may not be merely a contaminant of the preparation.

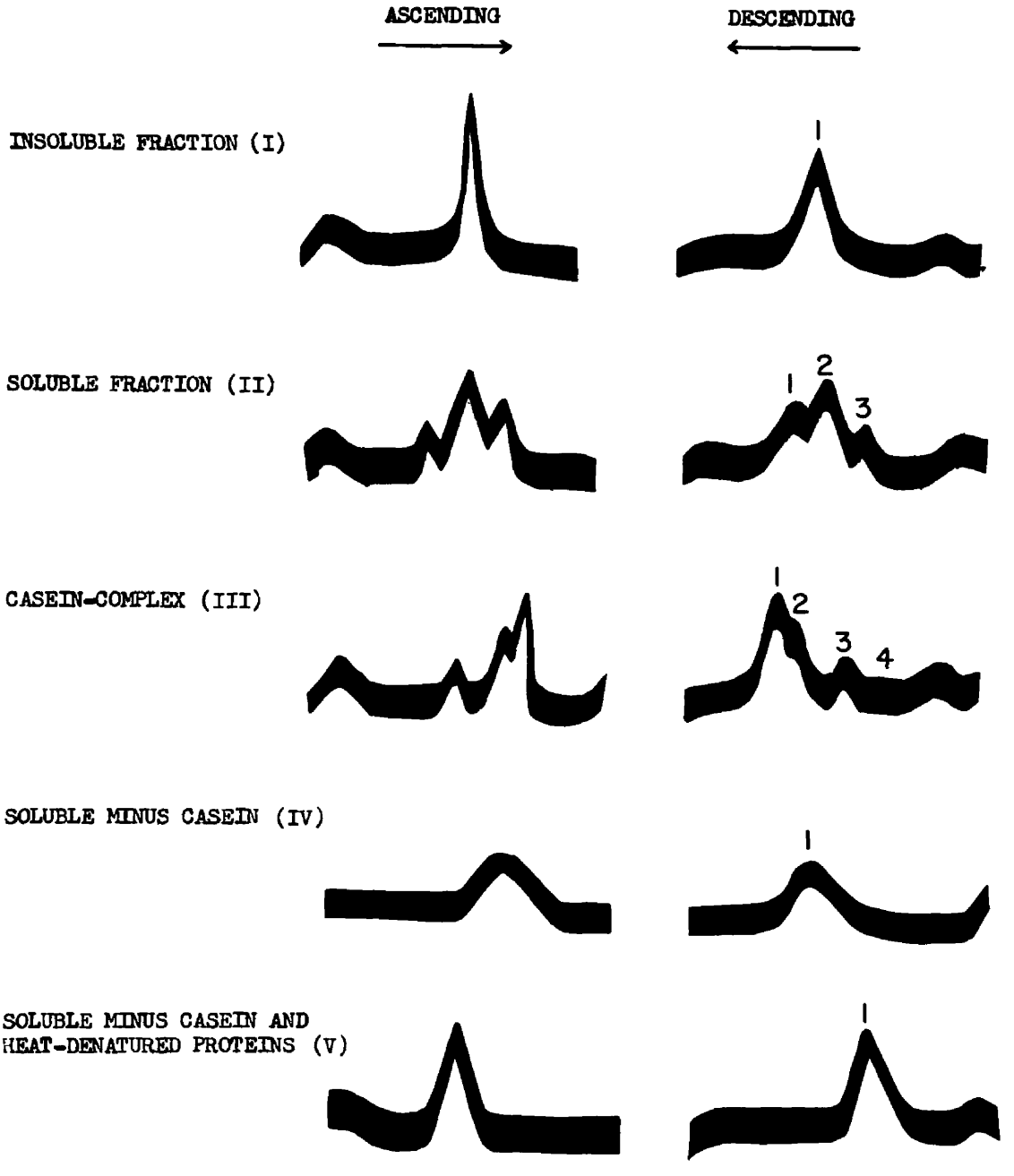



FIGURE 2. Electrophoretic patterns which are representative of the various fractions isolated by the fractionation scheme shown in Figure 1.

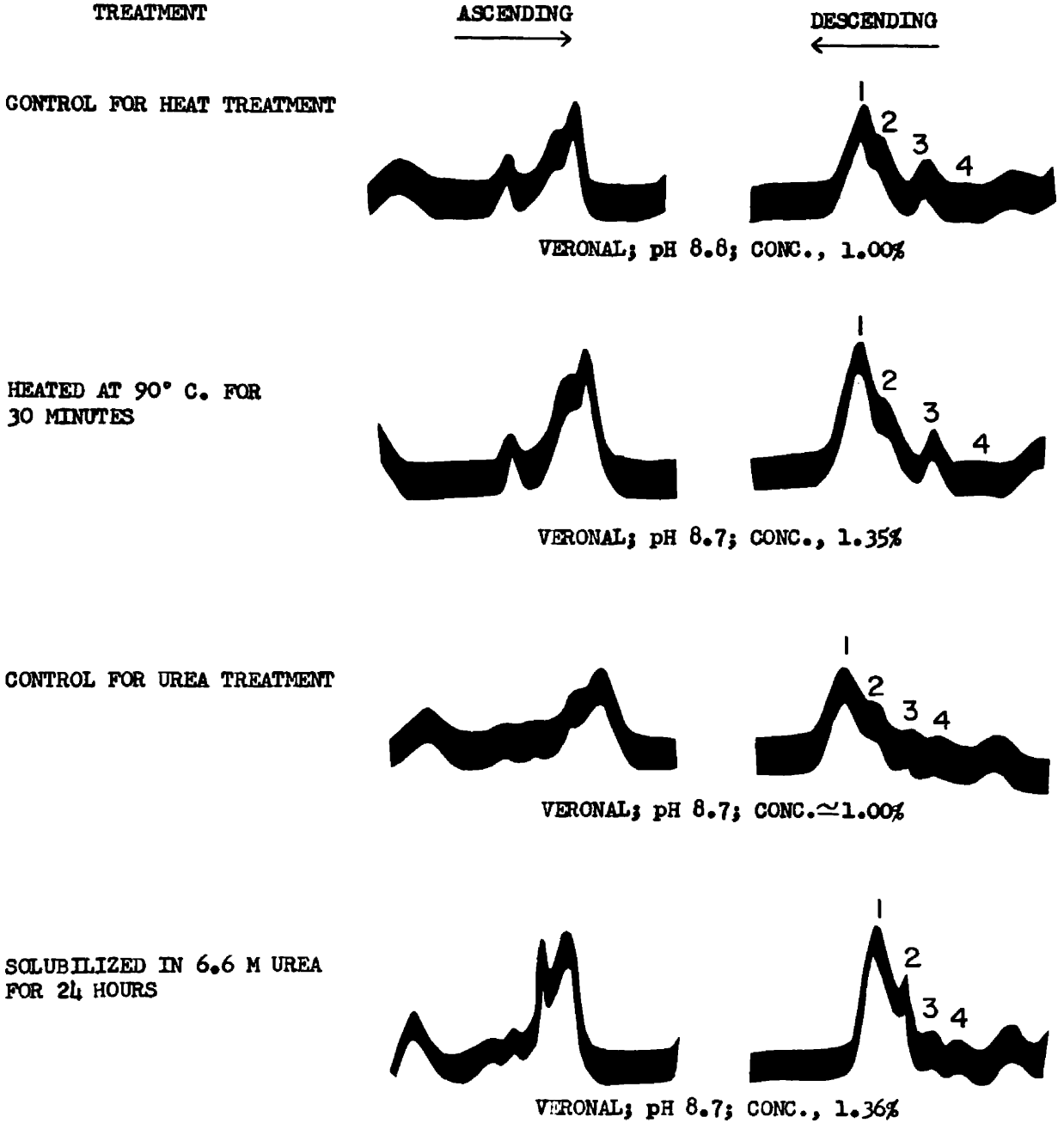



FIGURE 3. The effect of heat and urea on the Casein-complex Fraction (III).

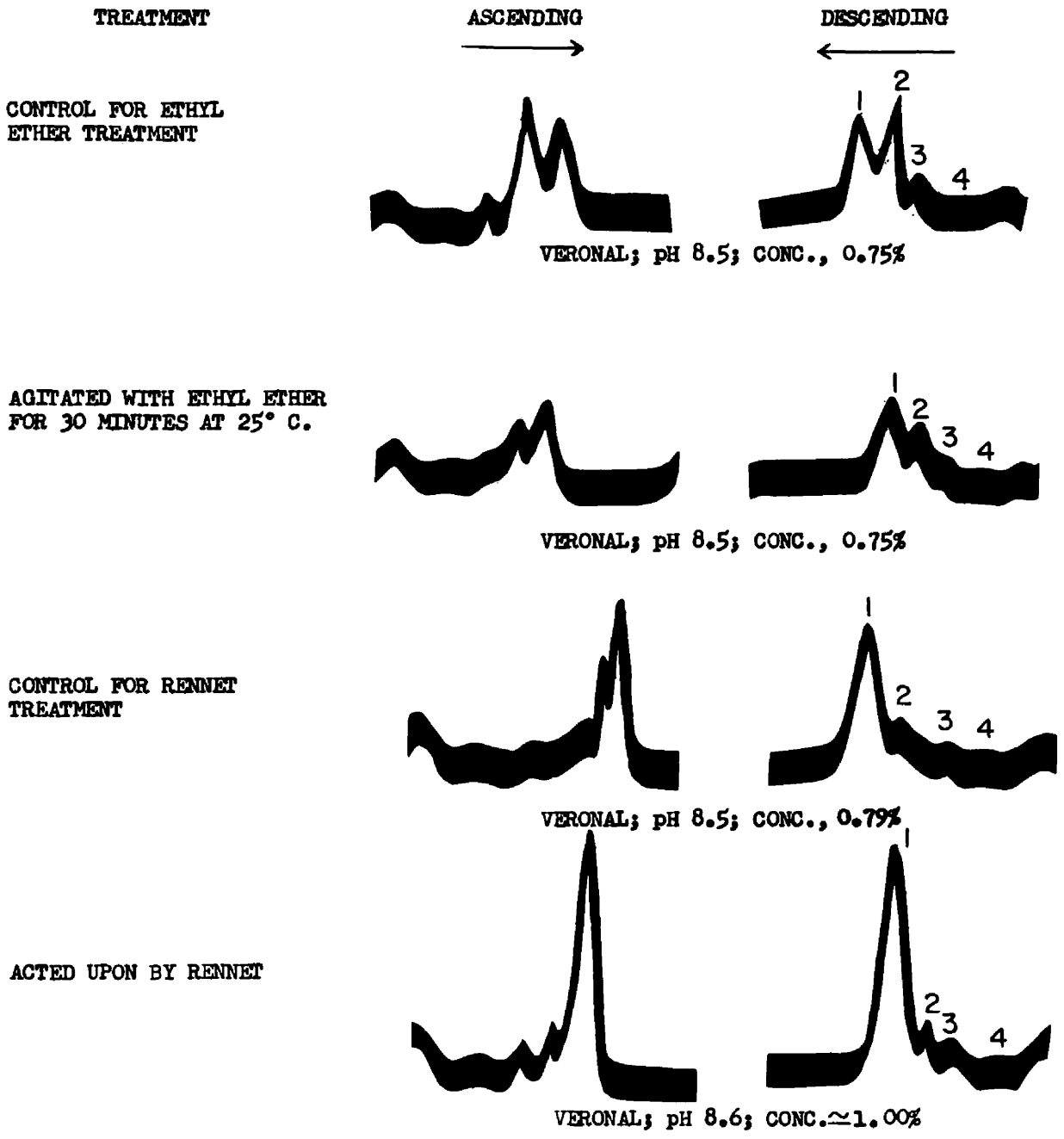



FIGURE 4. The effect of ethyl ether and rennet on the Casein-complex Fraction (III).

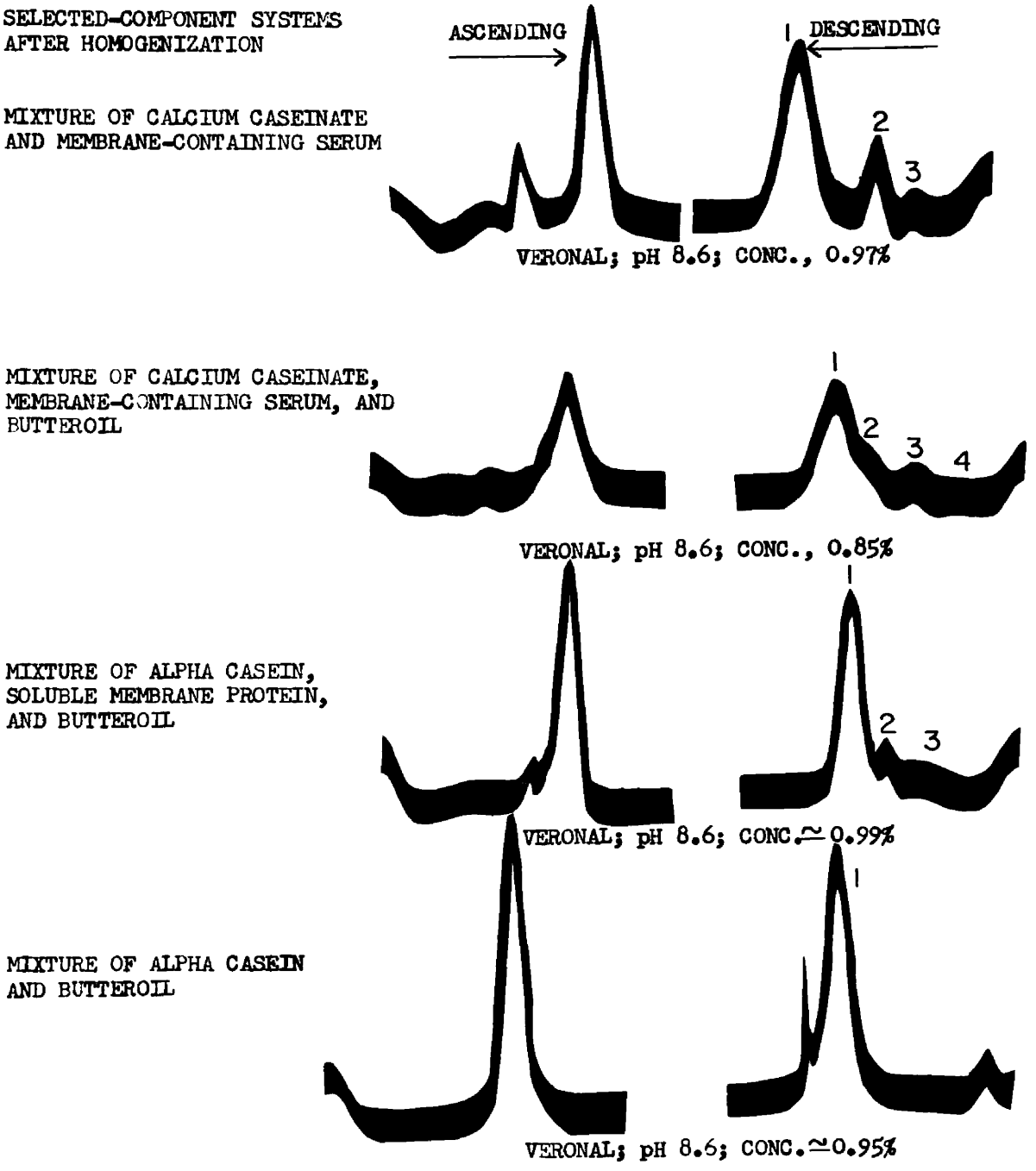



FIGURE 5. Electrophoretic patterns of selected-component systems.

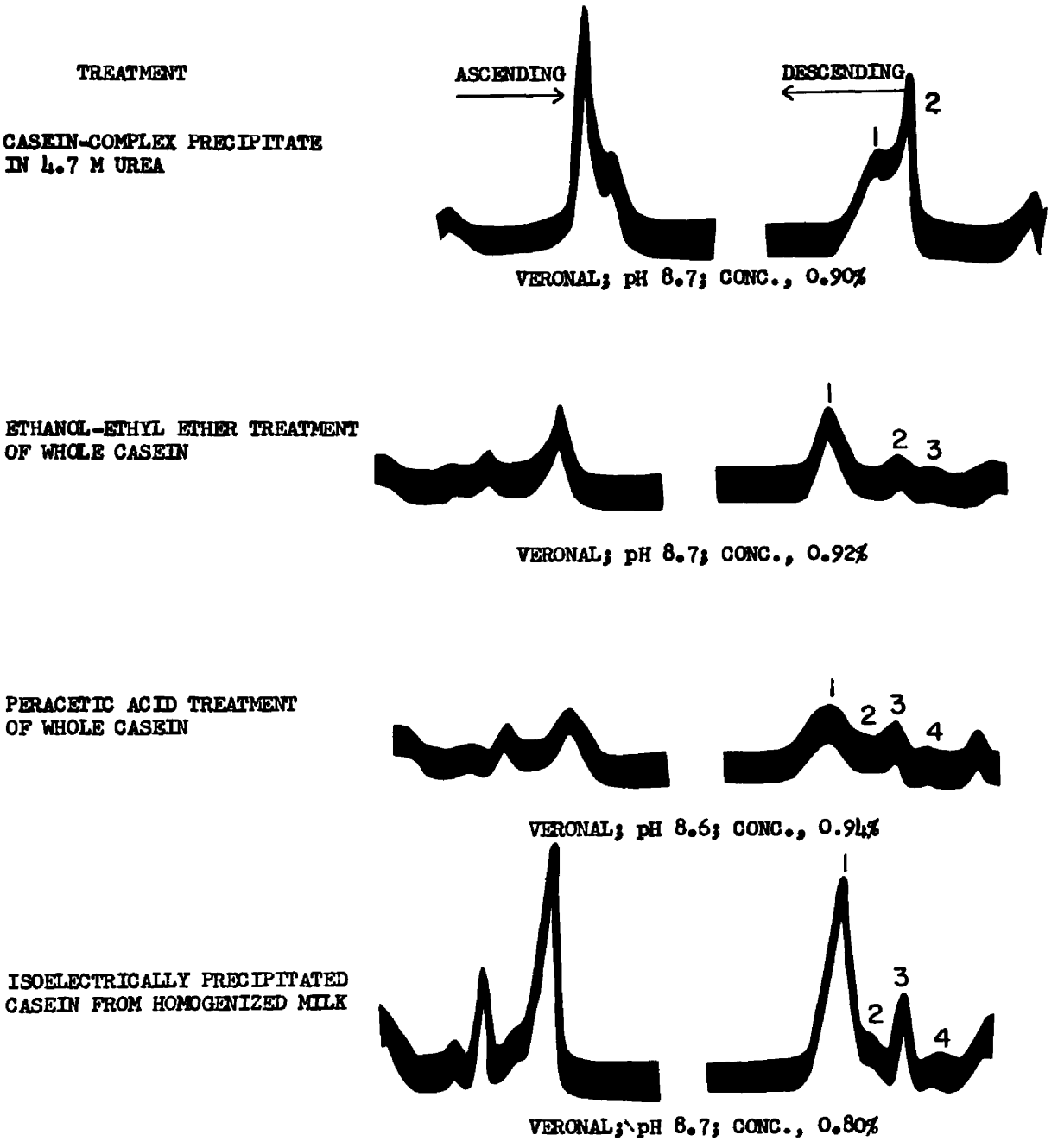



FIGURE 6. The effect of various chemical agents and homogenization in milk on the electrophoretic characteristics of casein.

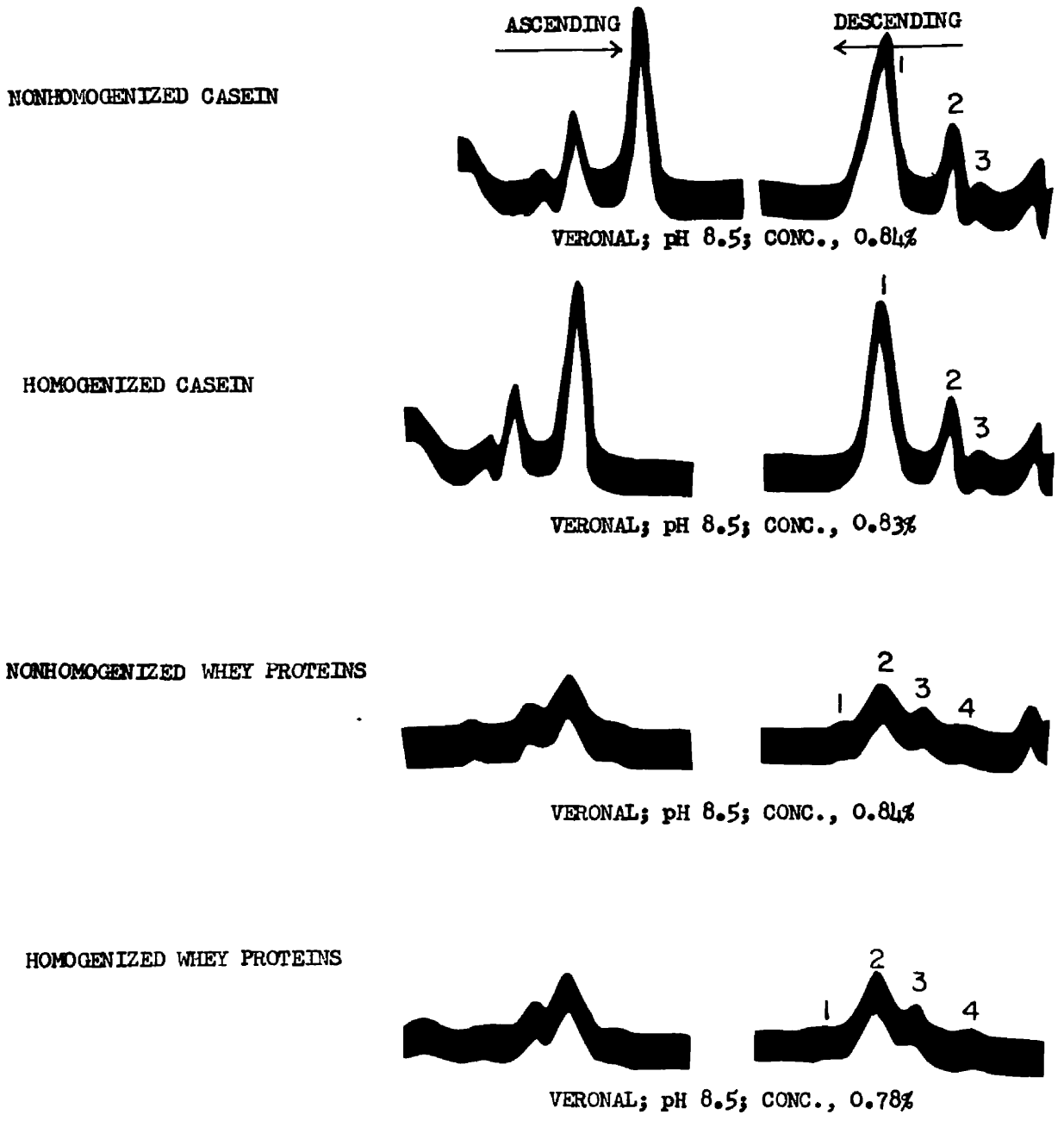



FIGURE 7. Electrophoretic patterns of isolated casein and isolated whey proteins before and after homogenization.

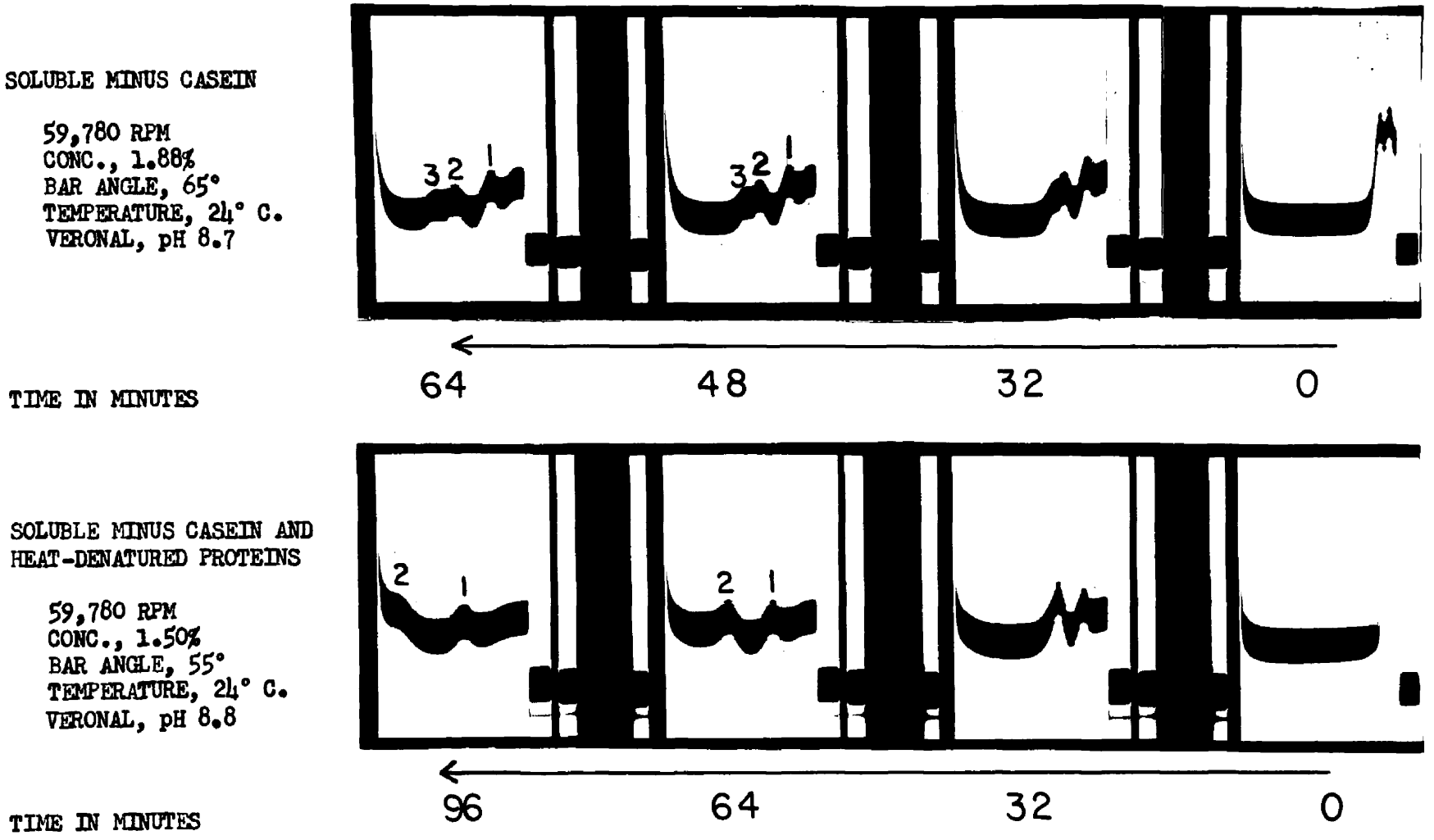



FIGURE 8. Sedimentation velocity diagrams of the Soluble Minus Casein Fraction (IV) and the Soluble Minus Casein and Heat-denatured Protein Fraction (V).

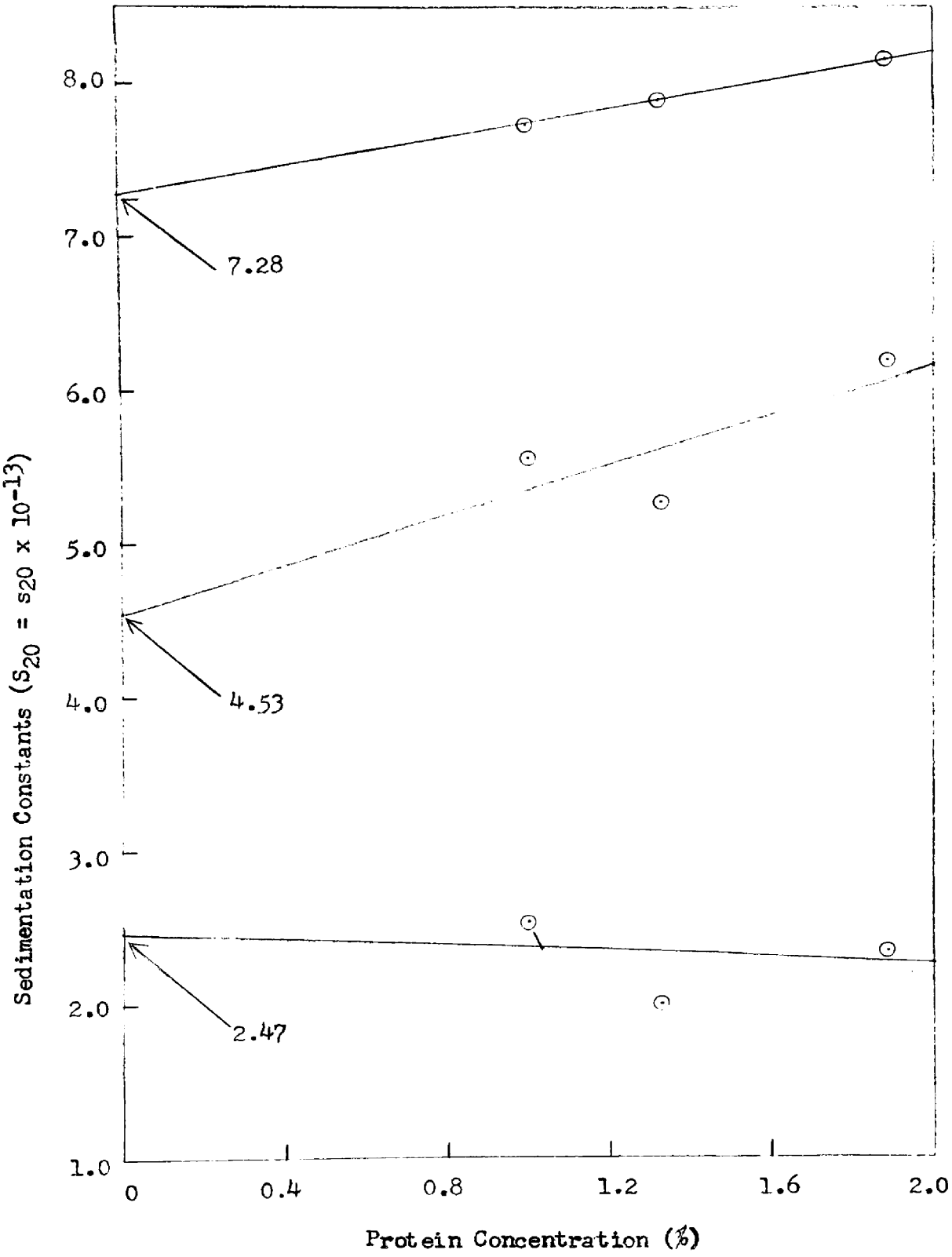



FIGURE 9. Extrapolation of sedimentation velocity constants to zero concentration for the Soluble Minus Casein Fraction (IV).

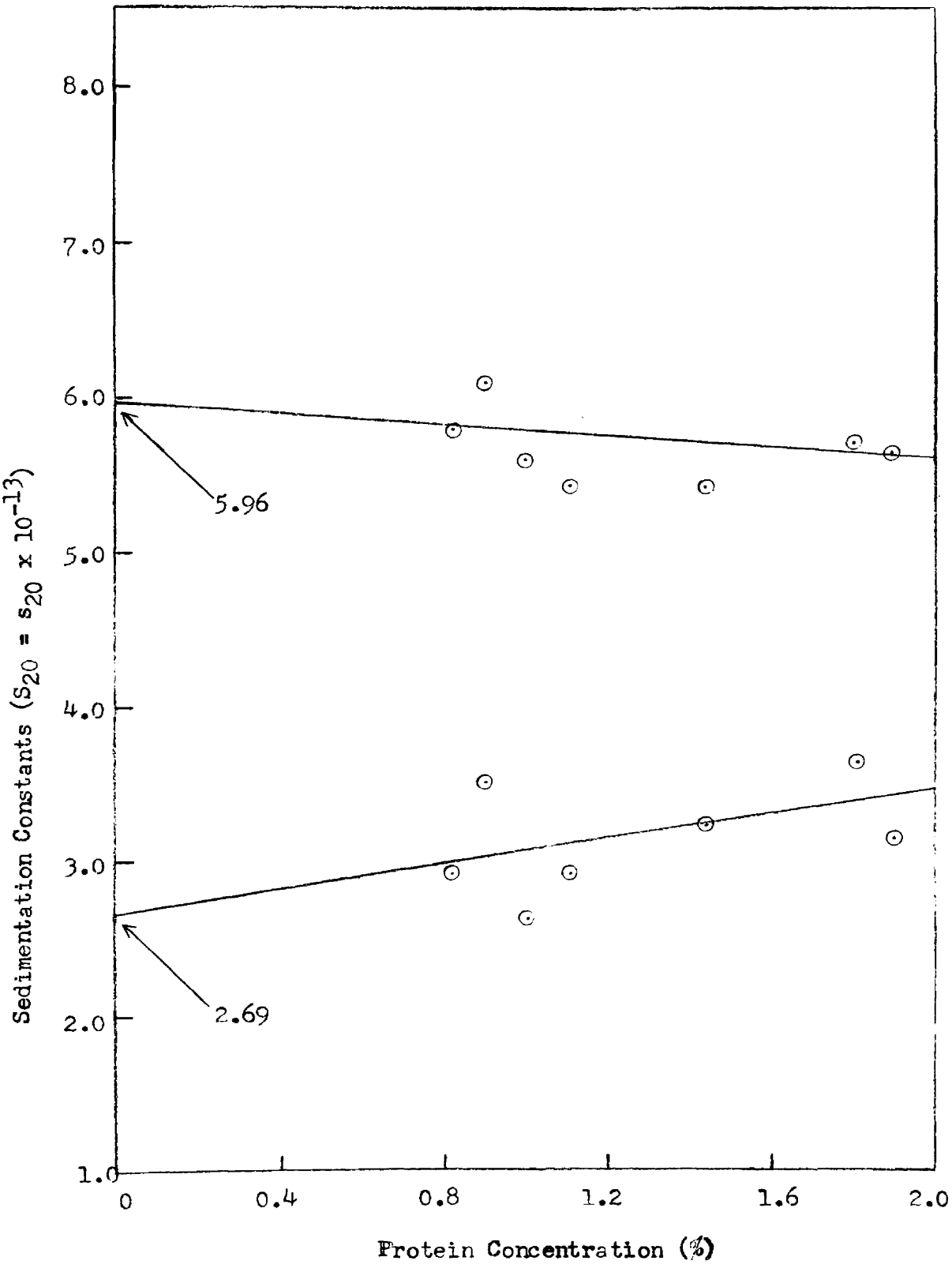



FIGURE 10. Extrapolation of sedimentation velocity constants to zero concentration for the Soluble Minus Casein and Heat-denatured Protein Fraction (V).

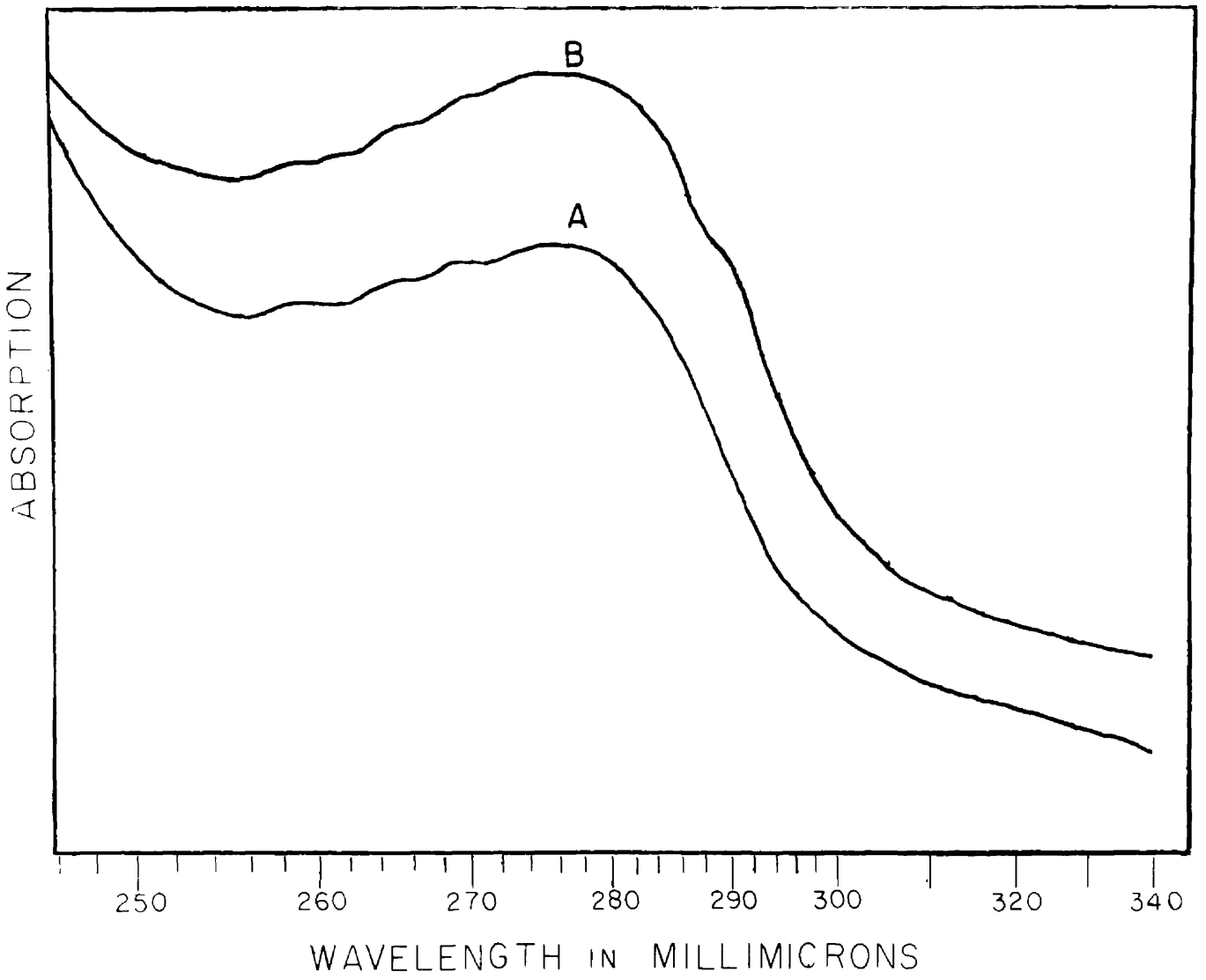



FIGURE 11. Ultraviolet spectrogram of the Soluble Minus Casein and Heat-denatured Protein Fraction (V) and the soluble membrane protein after 30 minutes at 90° C.

TABLE 1

TOTAL PROTEIN REMAINING WITH THE HOMOGENIZED MILK FAT-GLOBULE WITH INCREASING HEAT TREATMENT

| Observations | Heat treatment | | |
|---|---|---|---|
| | 60° C. (30 min.) | 70° C. (30 min.) | 80° C. (30 min.) |
| Index of heat treatment | | | |
| Whey protein nitrogen (mg/g total solids) | 8.1 | 6.7 | 4.1 |
| Composition and yields | | | |
| Volume of washed homogenized cream (ml)[a] | 420 | 610 | 730 |
| Fat in washed homogenized cream (%) | 46.4 | 49.9 | 55.1 |
| Yield of fat (g) | 194.9 | 304.4 | 402.2 |
| Total solids in washed homogenized cream (%)[b] | 52.2 | 55.1 | 59.8 |
| Yield of total solids (g) | 219.2 | 336.1 | 436.5 |
| Kjeldahl nitrogen in cream (mg %) | 165 | 126 | 116 |
| Total solids due to protein (%) (% N x 6.38) | 1.4 | 0.6 | 0.2 |
| Grams of protein/100 grams fat | 2.27 | 1.60 | 1.31 |

[a] Prepared from 10 gallons of raw milk

[b] Assume 4.53% total solids in sucrose

TABLE 2

MOBILITIES AND AREAS OF THE ELECTROPHORETIC COMPONENTS
SHOWN IN FIGURES 2 THROUGH 7

| Electrophoretic patterns | | Mobilities ($\mu$=cm$^2$, volt$^{-1}$, sec$^{-1}$ x 10$^{-5}$) of: Peak No. | | | | Relative areas (%) of: Peak No. | | | |
|---|---|---|---|---|---|---|---|---|---|
| Figure | Row | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 2 | 1 | 4.61 | .... | .... | .... | 100.0 | .... | .... | .... |
| | 2 | 4.47 | 3.74 | 2.52 | .... | 31.8 | 56.1 | 12.1 | .... |
| | 3 | 5.10 | 4.49 | 3.23 | 1.74 | 63.5 | 23.5 | 12.0 | 1.0 |
| | 4 | 3.55 | .... | .... | .... | 100.0 | .... | .... | .... |
| | 5 | 4.02 | .... | .... | .... | 100.0 | .... | .... | .... |
| 3 | 1 | 5.10 | 4.49 | 3.23 | 1.74 | 63.5 | 23.5 | 12.0 | 1.0 |
| | 2 | 4.68 | 3.96 | 2.73 | 2.03 | 59.6 | 24.0 | 14.7 | 1.7 |
| | 3 | 5.53 | 4.57 | 3.59 | 2.77 | 59.1 | 29.2 | 10.4 | 1.3 |
| | 4 | 5.38 | 4.27 | 3.33 | 2.50 | 65.4 | 24.3 | 5.6 | 4.7 |
| 4 | 1 | 5.94 | 4.64 | 3.43 | 1.76 | 53.2 | 37.6 | 8.1 | 1.1 |
| | 2 | 5.43 | 4.18 | 3.07 | 1.52 | 60.9 | 29.1 | 9.1 | 0.9 |
| | 3 | 6.08 | 4.86 | 3.26 | 1.86 | 75.6 | 19.3 | 4.5 | 0.6 |
| | 4 | 5.96 | 4.30 | 3.31 | 1.80 | 75.0 | 12.1 | 10.0 | 2.9 |

TABLE 2 (continued)

MOBILITIES AND AREAS OF THE ELECTROPHORETIC COMPONENTS SHOWN IN FIGURES 2 THROUGH 7

| Electrophoretic patterns | | Mobilities ($\mu$=cm$^2$, volt$^{-1}$, sec$^{-1}$ x 10$^{-5}$) of: Peak No. | | | | Relative areas (%) of: Peak No. | | | |
|---|---|---|---|---|---|---|---|---|---|
| Figure | Row | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| 5 | 1 | 6.00 | 3.28 | 2.08 | .... | 80.0 | 15.8 | 4.2 | .... |
| | 2 | 5.93 | 4.75 | 3.13 | 1.71 | 61.1 | 27.8 | 10.0 | 1.1 |
| | 3 | 5.85 | 4.16 | 2.99 | .... | 83.7 | 11.5 | 4.8 | .... |
| | 4 | 6.38 | .... | .... | .... | 100.0 | .... | .... | .... |
| 6 | 1 | 5.54 | 4.46 | .... | .... | 54.5 | 45.5 | .... | .... |
| | 2 | 5.15 | 2.90 | 1.91 | .... | 75.9 | 21.2 | 2.9 | .... |
| | 3 | 5.80 | 5.10 | 3.32 | 2.14 | 64.8 | 14.3 | 19.0 | 1.9 |
| | 4 | 5.76 | 4.44 | 3.19 | 1.92 | 74.9 | 9.2 | 13.7 | 2.2 |
| 7 | 1 | 5.90 | 3.38 | 2.31 | .... | 79.0 | 17.7 | 3.3 | .... |
| | 2 | 5.92 | 3.42 | 2.27 | .... | 76.1 | 22.1 | 1.8 | .... |
| | 3 | 6.76 | 5.26 | 3.88 | 2.42 | 8.7 | 58.2 | 27.2 | 5.9 |
| | 4 | 6.80 | 5.36 | 3.94 | 2.40 | 9.9 | 57.2 | 25.2 | 7.7 |

TABLE 3

SEDIMENTATION VELOCITY CONSTANTS FOR THE SOLUBLE MINUS CASEIN FRACTION (IV) AND THE SOLUBLE MINUS CASEIN AND HEAT-DENATURED PROTEIN FRACTION (V), CORRECTED TO 20° C.[a]

| Protein fraction | Concentration (%) | Sedimentation velocity constants ($S_{20}=s_{20} \times 10^{-13}$) of: Peak Number 1 | 2 | 3 |
|---|---|---|---|---|
| Soluble Minus Casein and Heat-denatured Protein Fraction V. | 1.90 | 5.62 | 3.13 | .... |
| | 1.81 | 5.69 | 3.62 | .... |
| | 1.44 | 5.40 | 3.20 | .... |
| | 1.11 | 5.43 | 2.88 | .... |
| | 1.01 | 5.58 | 2.60 | .... |
| | 0.90 | 6.08 | 3.48 | .... |
| | 0.83 | 5.77 | 2.90 | .... |
| Soluble Minus Casein Fraction (IV). | 1.88 | 8.17 | 6.20 | 2.35 |
| | 1.33 | 7.90 | 5.28 | 2.00 |
| | 1.00 | 7.76 | 5.58 | 2.54 |

[a] All analyses were made in Veronal buffer; pH 8.8; ionic strength; 0.1.

## DISCUSSION

With the isolation scheme used in this research, comparable protein fractions were nearly identical from preparation to preparation. In a biological system as complex as milk, this reproducibility was gratifying. However, some doubt remains as to the degree of denaturation imposed by the techniques of isolation. The use of organic solvents to extract lipid materials from the proteins would probably draw the most criticism. The effect of the cold ethanol-ethyl ether treatment on the electrophoretic characteristics of casein was determined. Seemingly, the only effect of the solvents on the casein was the decreased electrophoretic mobilities of the components. No investigations of the effect of solvents on the other milk proteins were made, but it seems likely that the chemical and physical characteristics of some of the more labile species would be altered.

### Effect of Heat on the Total Amount of Protein Remaining on the Homogenized Milk-Fat Globule

By increasing the heat treatment of the milk prior to homogenization, the total amount of protein remaining with the homogenized fat was decreased. At 60° C. for 30 minutes, the time and temperature used in all isolations, not much protein denaturation would be expected. At 70° C. for 30 minutes and especially 80° C. for 30 minutes, the whey proteins undergo extensive heat denaturation. Although the latter two temperature treatments are drastic compared to normal processing condi-

tions, they serve to illustrate the effect of heat on the resurfacing of the homogenized fat globule. Possibly, a large portion of the denatured protein was removed in the washing procedure, as evidenced by an increasing amount of separator slime with increasing heat treatments. The results of the serum protein denaturation test of Kuramoto et al. (1959) showed that more serum protein was denatured with successively higher heat treatments. Perhaps, as the coiled structure of the whey proteins unfold with heat, their ability to remain electrostatically attracted to the fat is lessened.

A dramatic demonstration of the effect of heat on the resurfacing of homogenized fat was experienced in this phase of the research. One hundred and twenty-five milliliter samples of washed homogenized creams, which had been heated to 60° C., 70° C., and 80° C. for 30 minutes prior to homogenization, were heated to 38° C. and allowed to stand for 30 minutes. The three samples of cream had nearly the same fat percentage. Approximately fifteen milliliters of free-fat rose to the surface in the 80° C. preparation, whereas the amount of free-fat on the surface of the 70° C. cream was less than 3 milliliters. There was no free-fat apparent on the surface of the 60° C. preparation. This observation indicates poor fat emulsification, which may be due, in part, to a poor resurfacing of the homogenized fat globule by proteins, or of heat-induced liberation of adsorbed protein from the fat surface.

Jenness and Palmer (1945) reported that the nonhomogenized fat globules membrane consisted of 0.46 to 0.86 grams of protein per 100 grams of fat, while Herald (1956) estimated the membrane to consist of 0.51 grams of protein per 100 grams of fat. In this study, the 60° C.

homogenized preparations yielded 2.27 grams of protein per 100 grams of fat, or 5 to 6 times more protein per 100 grams of fat than the average value reported by the workers previously mentioned. This fact is noteworthy when one considers that homogenization increases the fat surface of milk by a factor of 5 or 6. In other words, the amount of protein which normally associates with milk fat may be relatively constant.

## Proteins of the Homogenized Milk Fat-Globule Membrane

The proteins of the homogenized fat globule will be discussed in the order in which they were isolated. (See Figures 1 and 2.)

### Insoluble Fraction (I)

This is the insoluble membrane protein which was isolated and characterized by Herald (1956). Its reddish-brown color and mucoidal appearance made it easy to recognize. Very little was done with this protein after it was isolated, except to solubilize it with peracetic acid and examine its electrophoretic characteristics. Herald (1956) provisionally classified this protein as pseudo-keratin in nature. Peracetic acid was used as a solubilizing agent because of its ability to disrupt disulfide linkages. Some doubt remains as to the applicability of these data, accumulated after harsh solubilizing treatments, to the characteristics of the protein as it exists on the fat-globule membrane in milk. Electrophoretically, the insoluble membrane protein showed one single homogeneous peak with an electrophoretic mobility of 4.61, in Veronal buffer of pH 8.7 and ionic strength of 0.1.

## Soluble Fraction (II)

This fraction was so designated because of its solubility in 0.02 M NaCl solutions or water. There are several proteins in this fraction. Among them is the soluble membrane protein isolated and characterized by Herald (1956). The soluble membrane protein is part of the soluble fraction and the two should not be confused. The pattern presented by the soluble fraction is too gross to be of much value in identifying individual proteins. However, it is very similar to the electrophoretic pattern of homogenized fat-globule membrane proteins as presented by Brunner *et al.* (1953c).

## Casein-complex Fraction (III)

When the pH of a solution of the Soluble Fraction (III) was adjusted to 4.6, it was anticipated that any casein present would be isoelectrically precipitated. There was a protein precipitate, but the electrophoretic characteristics differed from those of normal whole casein. The major difference was the appearance of a component with an electrophoretic mobility close to that of alpha casein. Other noticeable differences were the abnormally large ratio of alpha- to beta-casein, and the abnormally low mobility of alpha-casein.

At first, the associated material was thought to be a whey protein contaminant. To remove the contaminant, the protein was reprecipitated four extra times at pH 4.6 with dilute HCl. The material following the alpha peak was unchanged in its electrophoretic mobility and peak area.

At this point, it should be stated that due consideration was given

to the fact that this associated material may have been beta-lactoglobulin, which had been heat-complexed with kappa-casein. If so, one might expect to notice some differences at the various temperature levels. The possibility of a heat complex appeared slight because a typical casein-complex fraction was obtained at all levels of temperature treatment prior to isolating the membrane material.

## The Effect of Various Chemical and Physical Treatments in the Separation of the Casein-Complex

Heat. Since casein is not heat-labile at 90° C. for 30 minutes, the fraction was subjected to this treatment to determine whether or not the associated material would be selectively denatured. Electrophoretically, it was unaltered. This suggests that the associated material is heat-stable.

Urea. The possibility of a complex formation was tested by the action of a strong urea solution on the Casein-complex Fraction (III). If a complex existed through the mechanism of hydrogen-bonding, urea could release the complexed components. The use of urea had no effect on the electrophoretic characteristics of the Casein-complex Fraction (III).

Employing the urea-fractionation technique of Hipp *et al.* for casein, the associated material was precipitated with alpha-casein in 4.7 M urea. This indicated that either the associated material was firmly complexed with alpha-casein or 4.7 M urea was a critical molarity for its aggregation as well as for alpha-casein. The latter argument seemed unlikely.

Ethyl ether. The Casein-complex Fraction (III) was agitated with

ethyl ether at room temperature for 30 minutes to remove lipids and/or lipid-bound proteins. The relative concentration of the associated material was decreased by nearly 25%, with a corresponding increase of 14.5% in the concentration of the alpha-casein peak. These values can be determined from Table 2. This prompted the determination of the lipid content of the Casein-complex Fraction (III). A value of 8.5% was obtained. At this point, the speculation that the associated material was involved in a protein-lipid interaction with casein, seemed logical.

Rennet. Since treatment of the complex with rennet did not alter the typical electrophoretic pattern of the Casein-complex Fraction (III), the possibility of the co-precipitation of the associated material with the casein was discounted. If the associated material were not complexed, it is unlikely that it would precipitate with the calcium-sensitive casein after the complex was acted upon by rennet.

## Reconstruction of the Casein-Complex with Selected-Component Systems

Homogenization of a mixture of calcium caseinate and membrane-containing serum. The reproduction of the Casein-complex Fraction (III) was not achieved in this system. There was no peak following alpha-casein. The mobility of alpha-casein was reported at 6.00, which is fairly fast when compared with similar mobilities of the alpha-casein isolated from the homogenized membrane preparations. Also, the relative concentration of beta-casein was nearly normal.

Homogenization of a mixture of calcium caseinate, membrane-containing serum, and butteroil. This system differs from the first only by the

incorporation of butteroil. A closer approximatation of the Casein-complex Fraction (III) was obtained. Most notable was the appearance of an electrophoretically discernible component whose mobility was slightly lower than that of alpha-casein. The concentration ratio of alpha- to beta-casein was only slightly lower than one might expect. In this case, the significance of this ratio was relatively small due to the apparent variation in the alpha- to beta-casein ratio in normal casein preparations. The ratio of alpha- to gamma-casein was decreased by a factor of approximately 3, when compared with similar ratios in the previous system. This comparison seems legitimate, in that the components used in both systems were from the same preparation. Consideration has been given to the ratios of alpha- to beta- and gamma-casein, because not all of the area under the electrophoretic peak was considered to be alpha-casein.

Homogenization of a mixture of alpha-casein, soluble membrane protein, and butteroil. This system presented the most interesting evidence in support of the nature of the Casein-complex Fraction (III). The concentrations of alpha-casein, soluble membrane protein, and butteroil in the system was determined before homogenization. After homogenization, the pH of the solution was adjusted to 4.6 and the resulting precipitate was examined electrophoretically. The total concentration of the soluble membrane protein added to the system could not be accounted for by the total solids in the supernatant of the pH 4.6 solution or by the area of the peaks in the electrophoretic pattern. Apparently, the soluble membrane protein interacts with the alpha-casein in the presence of butteroil and contributes to the electrophoretic area

of the alpha-casein peak. The electrophoretic mobility of the alpha-casein peak was decreased from a normal value of 6.5 to 5.8, which was considered to be a significant reduction.

Homogenization of a mixture of alpha-casein and butteroil. Apparently, no interaction exists between alpha-casein and the lipids of butteroil, in the absence of the soluble membrane protein. The electrophoretic mobility of alpha-casein in this system was reduced from 6.5 to 6.4 after homogenization. The difference between these two mobilities was not considered significant. A lipid spike appeared in the descending electrophoretic pattern, as shown in Figure 5.

The Casein-complex Fraction (III) isolated from the homogenized fat-globule membrane did not seem to be present in the serum of the milk. At any rate, casein precipitated from commercially-processed homogenized milk did not exhibit all the characteristics of the Casein-complex Fraction (III) isolated by the techniques employed in this study. A higher homogenization pressure may be necessary to provide enough casein-complex to be detected in the serum of homogenized milk. This was suggested by Fox et al. On the other hand, the casein-complex may exist only at the fat/serum interface of homogenized milk.

## Soluble Minus Casein Fraction (IV)

The electrophoretic pattern of the soluble fraction, following precipitation of the Casein-complex Fraction (III), showed a broad peak, presenting very little information of a qualitative nature. This fraction was suspected of containing whey proteins associated with the homogenized fat globule. Using a gravimetric technique, it was determined

that 52% of this fraction was precipitated by heating to 90° C. for 30 minutes. A sample from a low temperature preparation was prepared for the ultracentrifuge, and the sedimentation velocity diagrams showed three molecular species, as shown in Figure 8. From all indications, there were at least two, and possibly three, proteins in this fraction. The Soluble Minus Casein and Heat-denatured Protein Fraction (V) was characterized and is discussed in the following section. No investigation was conducted on the heat-labile proteins of the Soluble Minus Casein Fraction (IV).

Soluble Minus Casein and Heat-denatured Protein Fraction (V)

Experimentation suggested that this fraction is composed largely of the soluble membrane protein. This fraction is heat-stable, as is the soluble membrane protein, and showed two molecular species in the ultracentrifuge. The sedimentation velocity diagrams of this fraction are shown in Figure 8.

Although not directly applicable for comparison, the data of Thompson and Brunner (1959) support the contention that, based on the associated carbohydrates, this fraction may be the soluble membrane protein, proteose-peptone, or both.

A comparison was made of the absorption patterns of the Soluble Minus Casein and Heat-denatured Protein Fraction (V) and the soluble membrane protein in the ultraviolet region of the spectrum. Little similarity existed between the two proteins. After the soluble membrane protein was heated for 30 minutes at 90° C., as is the Soluble Minus Casein and Heat-denatured Protein Fraction (V), it rearranged to give

the absorption pattern presented in Figure 11. The spectrogram showed small absorption peaks at 268, 264, and 258 millimicrons and one large absorption peak at 276 millimicrons for both proteins. The coincidence of these small absorption peaks suggests that the Soluble Minus Casein and Heat-denatured Protein Fraction (V) is the soluble membrane protein.

## SUMMARY

Homogenization mechanically reduces the size of the fat globule and increases its number. The membrane surrounding the nonhomogenized fat globule is disrupted by homogenization. Among other materials, the membrane is composed of proteins, phospholipids, and a high-melting triglyceride. Seemingly, the increased surface area created by homogenization cannot be adequately covered by the existing membrane material.

The purpose of this research was to identify the proteins which contribute to the structure of the homogenized milk fat-globule membrane. The effect of heat on the total amount of protein associated with the homogenized fat globule was also studied.

The techniques of isolation were similar to a procedure developed to isolate the membrane proteins of nonhomogenized fat globules. This technique was modified and extended to apply to the homogenized fat-globule membrane proteins. Greater yields were obtained by using sucrose to increase the specific gravity differential of the fat and serum of the homogenized milk.

The total amount of protein on the homogenized fat globule was found to be inversely dependent upon the amount of heat applied to the milk prior to homogenization. At 60°, 70°, and 80° C., the amount of protein associated with 100 grams of homogenized milk-fat was 2.27, 1.60, and 1.31, grams, respectively. The 60° C. preparation had a protein value 5 to 6 times greater per 100 grams of fat than the average protein value reported for the nonhomogenized milk fat-globule membrane,

based on grams of protein per 100 grams of fat. Since homogenization increases the fat surface of milk by a factor of 5 or 6, the possibility of a relatively constant ratio of membrane protein to fat surface should be considered. Electrophoretic, ultracentrifugal, and spectrophotometric analyses were used to characterize and identify the protein fractions isolated from homogenized milk fat-globule membranes.

The insoluble membrane protein of the nonhomogenized fat-globule membrane was isolated from the homogenized fat-globule membrane. This protein remained, at least in part, with the fat globule after homogenization.

The soluble membrane protein of the nonhomogenized fat-globule membrane also remained with the fat globule after homogenization. A portion of this protein seemed to interact with the alpha-casein adsorbed on the homogenized fat-globule membrane. Milk fat must be present for this complex to occur. Another portion of the soluble membrane protein was isolated by denaturing the heat-labile, adsorbed proteins at 90° C. for 30 minutes, leaving the soluble membrane protein in a relatively pure form.

Casein was found adsorbed on the homogenized fat-globule surface. Its alpha-component seemed to interact with the soluble membrane protein in the presence of milk fat. In the electrophoretic pattern of this casein, a component appeared with a mobility slightly less than that of alpha-casein, the mobility of alpha-casein was decreased, and the ratio of alpha- to beta-casein was abnormally large. Some of the proteins isolated from the homogenized fat-globule membrane were not identified. These were probably heat-labile whey proteins which were heat-denatured

in the isolation of the heat-stable fraction.

The fat-globule membrane proteins did not appear to completely dissociate from the fat globule after homogenization. The increased fat surface created by homogenization was covered, in part, by casein complexed with some of the soluble membrane protein and by unidentified whey proteins.

# CONCLUSIONS

The total amount of protein surrounding a given quantity of homogenized milk fat decreased as the temperature to which the milk was heated prior to homogenization was increased.

The specific proteins which were isolated from the homogenized milk fat-globule membrane were:

1. Insoluble membrane protein
2. Soluble membrane protein
3. Casein. A complex seemed to be formed with alpha casein and the soluble membrane protein, in the presence of milk fat. The product of the protein-lipid interaction gave rise to an electrophoretic component with a mobility slightly less than that of alpha casein.
4. Heat-labile protein(s). Although they were not isolated and identified, these proteins were thought to be whey proteins.

The fat-globule membrane proteins did not appear to be completely dissociated from the fat globule when the membrane was disrupted by homogenization. The increased surface area created by homogenization was covered, in part, by casein and, probably, by whey proteins.

The results of this research did not indicate that the resurfacing of fat globules subsequent to homogenization was limited to any specific milk proteins, but that a possible lipid-protein complex was formed as a result of the treatment.

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## GLOSSARY OF TERMS

A description of the protein fractions isolated during the course of this research has been prepared to aid in the presentation and discussion of data. The following designations are used throughout this manuscript:

INSOLUBLE FRACTION (I). The homogenized fat-globule membrane proteins, in 0.02 M NaCl, were centrifuged for 30 minutes at 25,000 x G. The protein residue was designated as the Insoluble Fraction (I).

SOLUBLE FRACTION (II). The proteins in the supernatant of the solution from which the Insoluble Fraction (I) was separated, were designated as the Soluble Fraction (II).

CASEIN-COMPLEX FRACTION (III). When the pH of a solution of the Soluble Fraction (II) was adjusted to 4.6, the proteins that precipitated were designated as the Casein-complex Fraction (III).

SOLUBLE MINUS CASEIN FRACTION (IV). The proteins in the supernatant of the solution, from which the Casein-complex Fraction (III) was precipitated, were designated as the Soluble Minus Casein Fraction (IV).

SOLUBLE MINUS CASEIN AND HEAT-DENATURED PROTEIN FRACTION (V). A solution of the Soluble Minus Casein Fraction (IV) was heated for 30 minutes at 90° C. The heat-stable proteins remaining in solution were designated as the Soluble Minus Casein and Heat-denatured Protein Fraction (V).

Two additional proteins are referred to throughout this manuscript. These are the soluble membrane protein and the insoluble membrane protein

of the nonhomogenized fat-globule membrane as isolated and characterized by Herald and Brunner (1957).