CHANGES OBSERVED IN SOME OF THE CHARACTERISTICS OF THE MILK FAT MEMBRANE MATERIAL DURING ITS PREPARATION

Thesis for the Degree of M. S.
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By

Sherman Rosenberg

AN ABSTRACT

Submitted to the College of Agriculture of
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ABSTRACT SHERMAN ROSENBERG

The primary reason for this study was to observe some of the changes which occur to the fat globule membrane during the isolation procedure. Comments were given concerning yield and general composition.

Yields of 1.11 - 1.44 grams of total membrane material per 100 grams of fat and 0.59 gram of membrane protein per 100 grams of fat were obtained. The soluble protein fraction composed 46 percent and the insoluble fraction 54 percent of the membrane proteins.

A distribution curve for the nitrogen in successive washed creams showed that less than the theoretical amount was lost as a result of repeated dilutions and separations of the original cream. This is due to the fact that membrane as well as plasma nitrogen was measured.

Washing with a sugar solution rather than plain water resulted in an increase in the separation efficiency and consequently an increase in the yield of membrane material. Separation and washing at 38° C. resulted in a higher recovery of lipoprotein complex than did working at 4° C.

Ash determinations on the membrane proteins yielded lower results than those obtained by other researchers.

Enzyme activities were reduced quite drastically during washing. Warm separated and washed creams retained more activity than the cold counterparts. Loss of activity after

salting-out the membrane material with ammonium sulfate was quite pronounced. Use of organic solvents also inhibited activity or destroyed the enzymes.

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INTRODUCTION

The fact that milk fat exists in the form of tiny globules three to six microns in diameter has been known for approximately 250 years. But not until Ascherson (1840) did his classical work on olive oil in contact with egg white was it postulated that these globules are surrounded by a "membrane" complex. Present knowledge classifies this material as a protein-phospholipid complex.

The origin of the membrane is obscure. Mulder (1947) believed that when a fat globule is detached from the secreting cell, it drags along with it a thin film of material representing a complicated system of substances such as proteins, enzymes, phospholipids, carbohydrates, and other biological substances.

Sommer (1952) postulated that the triglyceride molecule exists independently in the secreting cell. However the lipid cannot remain by itself in solution, and thus it joins with others to form fat globules. As this progresses, phospholipids attach to the globule, but since the former are both hydrophilic and lipophilic, they cannot be absorbed completely by the fat. Therefore the phospholipids project out from the globule surface. The proteinaceous material may become associated with the globule at this same time. Eventually the fat globule will "grow" to a size such that

it is just large enough for the phospholipid and protein material to completely envelope it in a single layer, thus effectively sealing the globule from further growth.

Obviously the origin of the fat globule membrane may be explained by the physiologist as well as the chemist. But since the membrane has been shown to be closely associated with flavor and palatability problems in milk (Palmer, 1944), knowledge of its chemical and physical characteristics is needed.

This manuscript deals in particular with the protein materials of the membrane. Researchers believed for some time that only a single protein existed in the membrane-complex, or else that it was homogeneous. However Herald (1956) was able to classify this material into two portions, differentiated by their solubility in a dilute salt solution. Electrophoretic studies showed each of these fractions to be heterogeneous, patterns of both consisting of two or more peaks.

This present study deals with the detailed preparation of the membrane proteins. The procedure employed is given in some detail in the body of this thesis. The biological material is characterized by its chemical composition at various steps in its preparation. Also, compositional differences found when isolating membrane material from warm and cold separated cream were observed.

REVIEW OF LITERATURE

Two distinct approaches have been used for separating membrane material from the plasma phase and two for removing membrane from the lipid phase of milk. Because of the nature of the work involved, these will be discussed concurrently.

Eliminating the aqueous or plasma phase involves washing the cream with water. Völtz (1904) and Abderhalden and Völtz (1909) used extensively a method whereby cream was allowed to rise through a column of water. These workers used a 60 centimeter column and removed the washed fat globules after 12, 24, 48, 72, and 96 hours. Although this is quite effective, it has the disadvantage of carrying particles from the plasma along with the cream. The product was filtered at 50 - 70° C., after which the dried residue was extracted by means of boiling ether.

Titus, Sommer, and Hart (1928) employed a similar technique except that their water column was twice as high; before filtration they mixed the washed cream with one-third its volume of 95 percent ethanol; and their method of filter residue purification was more involved.

The second method of washing cream was devised and used by Storch (1897). Even though it came chronologically before that of Völtz (1904), this technique has proven to be the

more popular means of removing milk plasma from around the fat globule. Cream, after being separated from the skim milk, was diluted back to its original volume of whole milk and separated again. This was repeated four or five times. Removing fat from the membrane was achieved by organic solvent extraction according to Völtz and Titus et al. (1928). The washed cream was shaken in strong alcohol followed by the addition of ether and benzene. A gelatinous precipitate was filtered from the aqueous phase, washed with strong alcohol and ether, and dried in air at room temperature. Again there was the problem of obtaining a product the composition of which had been altered from that of the original membrane. In this case the procedure involved the loss of membrane phospholipids along with the milk fat.

Palmer and Samuelsson (1924) used this method of repeated dilutions and separations for washing the fat globules. Then, in order to keep mechanical loss of membrane lipids to a minimum, these researchers churned the washed cream, the agitation from this procedure being sufficient to rupture the membrane. The lipoprotein complex was recovered from the buttermilk and the melted and separated butter.

Jenness and Palmer (1945) published an elaborate procedure for membrane protein isolation. Included was their mode of dialyzing the membrane-containing serum in order to

concentrate said membrane. The final step consisted of extracting phospholipids and a high-melting triglyceride fraction from the membrane material by means of ethanol and ethyl ether, leaving essentially nothing but protein.

A modification of this technique was employed by Hare, Schwartz, and Weese (1952). They mixed the cream washings and buttermilk after churning and used acetone to precipitate the membrane material. The dry precipitate (moisture removed by centrifugation) was washed with absolute alcohol, a 1:4 alcohol-ether mixture, and finally anhydrous ether to remove the lipid portion of the membrane.

Brunner, Duncan, and Trout (1953a) used a method similar to that of Hardy and Gardiner (1910) for extracting the lipoidal materials. They first treated the membrane with a cold ethanol-ether mixture to separate the lipids from protein and then extracted the former with 40° C. ether.

Varying amounts of membrane material have been isolated by different workers. Palmer and Wiese (1933) found 0.66 - 0.89 gram per 100 grams of fat. Jenness and Palmer (1945) reported 0.71 - 1.20 grams of membrane substance per 100 grams of fat from different breeds and at various stages of lactation. Herald (1956) prepared 1.27 grams per 100 grams of milk fat. Storch (1897) estimated 38 grams per 100 grams of fat, a value which is obviously high. The large error was due possibly to his observations of membrane material

under the microscope. In another section of his article, Storch claims that butter churned from fresh cream contains 7 percent membrane by weight. At the other extreme Schwarz and Fischer (1937) reported only 0.12 gram per 100 grams of fat. This low figure is attributed to the use of physiological saline for washing the cream, a procedure which would have removed some globulin protein. The milk fat was separated from the membrane by means of ether extraction; thus much of the lipid portion would have been removed.

Again we find varying reports for the amount of protein in the membrane. Rimpila and Palmer (1935) found 0.46 - 0.71 gram per 100 grams of fat. Jenness and Palmer (1945) reported 0.38 - 0.86 gram per 100 grams of fat. Herald (1956) obtained a value of 0.51 gram of membrane protein per 100 grams of milk fat. Schwarz and Fischer (1937) reported 0.12 gram per 100 grams of fat, a figure not only decidedly low when compared to other published quantities of membrane protein but also incompatible with their report of 0.12 gram of membrane material per 100 grams of fat. Their values are valid only if one considers the fat globule membrane to be composed exclusively of protein, a fact which is strongly disputed by the literature on this subject. Roland (1956) found 0.44 - 2.22 grams of protein per 100 grams of fat, basing his figures on a formula devised by himself.

Herald (1956) also reported a procedure for fractionating the membrane protein into soluble and insoluble fractions. As this technique was followed rather closely in the work reported in this manuscript, the details will be given under the experimental procedure. Suffice it to say that a different means of concentrating the membrane material was used (by Herald). Instead of dialyzing the membrane-containing serum, saturated ammonium sulfate was added to a final concentration of 55 percent. This "salted out" the entire membrane substance, not the protein portion alone, because of the strong physical attraction and chemical bonds between components of the membrane. Herald estimated that the soluble fraction contained 44 percent and the insoluble fraction 56 percent of the original membrane protein.

King (1955) wrote a brilliant, comprehensive review of this field of research which cites all but the most recently published work in the area.

EXPERIMENTAL PROCEDURE

Isolation of Membrane Proteins

Fifty gallon lots of relatively fresh, raw, mixed herd cow's milk were separated at 4° C. and 38° C. using a De-Laval Model No. 390 Standardizing-Clarifier. The cream from each separation was diluted back to its original volume with tap water of the same temperature. The wash water was removed from the cream by means of the separator used above. This washing-separation process was repeated for a total of three to five times. The final washed cream was allowed to stand overnight at 5° C.

Both the high and low temperature washed creams were treated identically. The cream was brought to 13 - 14° C. and batch-churned in large glass jars placed horizontally in a mechanical shaker and agitated to rupture the fat globule membrane. The combined butter and buttermilk was warmed to 38° C. and run through a DeLaval Model No. 9 Laboratory Separator. The membrane-containing serum was collected and the butteroil discarded. Some of the warm separated cream achieved a plastic condition after overnight storage, and it was necessary to dilute it with distilled water at 13° C. in order to obtain churning consistency.

The serum was adjusted to room temperature and a saturated ammonium sulfate solution was slowly added with

stirring until a final salt concentration of 2.2 M was reached. Salted-out membrane material was concentrated by centrifuging at 2,000 R.P.M. in a Universal No. 2 Centrifuge. The membrane substance was finally concentrated in a Model SS-1 Servall Centrifuge by running at 25,000 x G for 30 minutes.

A solution of 35 percent ethanol in ethyl ether at -5° C. was added to the membrane material in the proportion of 100 milliliters to 50 grams of concentrated membrane. All ether used in the preparation was tested for formation of peroxides according to Weissberger, Proskauer, Riddick, and Toops (1955). The mixture was held at 0° to -5° C. and agitated for 15 minutes. Subsequently it was observed that for large quantities of membrane substance, the ratio of ethanol-ether to membrane must be increased; otherwise, the final ethanol concentration is insufficient to break the lipid-protein bonds. Following the cold ethanol treatment, the mixture was adjusted to -20° C. and filtered. The alcohol-treated membrane material was washed five times with 200 - 600 milliliter portions of -20° C. ethyl ether. Finally, the residual membrane lipids were extracted with three 250 - 800 milliliter portions of ether at 30° C. Solvent separation was achieved by centrifugation. The proteins were held overnight under a vacuum of 28 inches to remove residual ether.

Separation of the soluble from insoluble protein fraction was accomplished by the addition of successive 150 - 450 milliliter portions of 0.02 M sodium chloride solution. Following agitation at 2° C. for approximately 2 hours the suspension was centrifuged at 25,000 x G for 45 minutes. The supernatant from each extraction was drawn off and pooled. Extractions were continued until the supernatant gave a negative biuret test. No more than four extractions were required. The supernatant contained the "soluble" fraction while the residue in the bottom of the tube was the "insoluble" fraction. All procedures were carried out in glass or stainless steel containers.

Analytical Methods

Fat and total solids. Fat and total solids were determined by the method of Mojonnier and Troy (1925). In cases where only a small amount of a given sample could be used for testing purposes, a modified extraction technique was employed for determining fat. The extraction was carried out in a test tube using 0.25 - 0.50 gram of sample, 1 - 2 milliliters water, 1 milliliter concentrated ammonium hydroxide, 2 milliliters ethanol (two extractions), 3 milliliters ethyl ether, and 3 milliliters petroleum ether.

Ash. Samples to be ashed were exhaustively dialyzed against tap water, distilled water, and redistilled water.

Concentrated materials first were suspended in distilled water. Total solids determinations were run on the dialyzed samples. Fifty gram samples were dried on a steam bath and then placed in a muffle furnace at 550 - 600° C. overnight.

Alkaline phosphatase. The activity of this enzyme was determined according to the procedure of Sanders and Sager (1947). In every case, the protein precipitant used was 3.0 grams of zinc sulfate and 0.6 gram of cupric sulfate made up to 100 milliliters with distilled water. Optical density was read at 610 millimicrons in a Bausch and Lomb "Spectronic 20" Colorimeter. Calculations were based on the method of Zittle, DellaMonica, Custer, and Rudd (1956b) so that units of activity for both enzymes studied would be of the same order. The activity found was converted from optical density to units according to a standard curve with the equation

$$x - 2y = 0.$$

Ten and five-tenths units of activity is equivalent to the release of 1.0×10^{-7} moles of phenol.

Xanthine oxidase. A slightly modified method of Zittle et al. (1956a) was used for determining xanthine oxidase activity. This method of assay is based on the fact that tetrazolium salts are good oxidation-reduction indicators. They are colorless, soluble compounds in the oxidized form and colored, water insoluble, oxygen stable formazans in

the reduced state. In this test triphenyltetrazolium chloride is reduced by the action of xanthine oxidase. The principal reagents are a 0.5 M phosphate buffer to keep the reaction close to pH 7.5, 0.005 M xanthine to act as substrate for the enzyme, and 0.05 M triphenyltetrazolium chloride. Nitrogen gas was bubbled through the tube containing the reactants in order to prevent oxygen from inhibiting the reduction of the tetrazolium salt. To insure that the nitrogen was pure, three columns of Benedict's (1912) oxygen absorbent were placed in the nitrogen line. The assay was conducted at 30° C. and the triphenyltetrazolium allowed to be in contact with the enzyme for exactly 10 minutes. Mustakallio, Ahos, and Autio (1955) reported that triphenyltetrazolium chloride in solution is photosensitive and becomes red. They suggested using this salt only under condition of total darkness. But because of the nature of this particular test, Zittle (1956b) suggested using a room with a constant light source. The reduced triphenyltetrazolium was taken up in toluene and optical density was read at 485 millimicrons in the "Spectronic 20." The equation for the standard curve run for the xanthine oxidase had the equation

$$6x - 5y - \tfrac{1}{2} = 0.$$

One unit of activity is equivalent to the formation of 0.3×10^{-7} moles of the reduced triphenyltetrazolium.

Nitrogen. The samples tested were fractionated according to the method of Rowland (1938). Nitrogen was determined by means of a modification of the technique employed by Menefree and Overman (1940). Size of the Kjeldahl sample and amount of concentrated sulfuric acid used for digestion were determined by the nature of the sample and the specific protein for which one was analyzing. In general, 10 milliliters of nitrogen-free concentrated sulfuric acid and 5 grams of sodium sulfate-mercuric oxide (14:1) mixture were added to the sample. Digestion was allowed to proceed for 15 minutes after the solution became clear and colorless. The contents of the Kjeldahl flask were allowed to cool, the sides rinsed with distilled water, and the solution redigested for an additional one-half hour. One hundred fifty milliliters of distilled water and 40 milliliters of 50 percent sodium hydroxide plus sodium thiosulfate were added to the cooled contents. Approximately 100 milliliters were distilled into a flask containing 50 milliliters of use solution boric acid and a few drops of methyl red-methylene blue indicator. The receiving solution was made up as follows: 1 pound of reagent grade boric acid was dissolved in 10 liters of distilled water; the use solution contains 2 parts of the above diluted in 3 parts of water. The distillate was titrated with 0.05 N hydrochloric acid. Nitrogen was calculated as follows:

$$\frac{\text{ml. HCl} \times \underline{\text{N}}\ \text{HCl} \times .014}{\text{gm. eq.}} \times 1000 = \text{mg. N/gm. sample}$$

$$\text{mg. N/gm. sample} \times 100 = \text{mg./\% N}$$

$$\frac{\text{mg./\% N}}{\text{\% fat}} \times 100 = \text{mg. N/100 gm. fat}$$

where ml. HCl = milliliters of hydrochloric acid used in titration

$\underline{\text{N}}$ HCl = normality of hydrochloric acid

.014 = milliequivalent weight of Nitrogen

gm. eq. = grams of sample to which the aliquot taken is equivalent.

RESULTS

Yields

The final preparation was carried out with the DeLaval Model No. 9 Laboratory Separator, since its smaller size would permit a closer accounting of the materials and also allow for smaller losses due to milk, cream, etc. being held in the bowl.

Twenty-five pounds of 25 percent cream were obtained from 169 pounds of milk. After the first washing, 17 pounds of 36 percent cream remained. Succeeding washings changed the yield and percent fat only slightly, though it is obvious when looking at the final washed cream--15.5 pounds of 31 percent fat--that purely mechanical loss plays a part in the total picture which is obtained of the membrane. The yields from this preparation are tabulated in Table 1.

From this isolation 1.11 - 1.44 grams of lipoprotein material per 100 grams of fat were obtained. The final yield of membrane protein was 0.59 gram per 100 grams of fat. The soluble fraction appeared to make up 46 percent of the total proteins and the insoluble portion 54 percent.

Nitrogen

The results of a nitrogen distribution study on one series of washed creams are shown in Tables 2 and 3.

Determinations made for total nitrogen, casein nitrogen, non-casein nitrogen, proteose-peptone nitrogen, and non-protein nitrogen all displayed a sharp decrease after the first washing. From this point the various fractions lost nitrogen quite gradually. In fact, casein nitrogen leveled off so that the values for the final three washed creams were constant.

The amount of protein in each washed cream was calculated. These values followed the nitrogen curve (see Figure I) and are similar to those reported by Rimpila and Palmer (1935).

Fat and Total Solids

Tables 4 to 10 inclusive show, as one would expect, that the total solids and fat percentages in cream range higher from the warm than from the cold separations and washings. Conversely, the total solids content of the skim milk of the cold separations were slightly higher than from warm skimming. The first cold washing showed a marked decrease in solids content, while successive washing of the cold separation showed an increase. On the other hand, the total solids and fat content of the warm preparations increased markedly as a result of the first dilution and separation, reaching a maximum at this point in the washing procedure. Looking at the compositional data from another

viewpoint, the solids-not-fat of cold washed creams dropped after the first washing, whereas it increased after the first warm washing. Thus we have two distinct situations. After the first 4° C. washing the cream generally decreased in total solids and fat content, while the solids-not-fat increased proportionally. But the first warm washed cream usually showed an increase in the concentration of all three components.

In one trial a 12.7 percent sucrose solution was used for cold-washing the cream in order to increase the specific gravity of the mixture for separating. Data in Table 8 show that in this case a cream of considerably higher total solids content than the other cold separations was obtained, but with succeeding washing the cream's solids percentage never came within more than 15 percent of that from the 38° C. separation. To be certain that the sugar solution had no adverse effect on the plasma during washing, skim milk diluted to four times its volume with 12.7 percent sucrose was agitated and centrifuged. The casein was unaffected. Naturally, the increase in total solids was due to sugar from the wash solution entering the plasma. But the fat content also increased, indicating that sugar has some effect in preventing the loss of fat during the washing operation.

Membrane-containing serum had a total solids range of 0.8 - 2.4 percent. The sugar-washed preparation did not show a solids content out of proportion to the other trials. Approximately one-half of the total solids was fat. Concentrated membrane ranged from 36.4 - 42.5 percent total solids. Solids-not-fat at this phase was in the range from 27.2 - 33.3 percent. The membrane proteins were approximately 5 percent fat. In two out of three cases the soluble protein fraction from the cold separation was lower in total solids and solids-not-fat than the corresponding warm preparation. The cold insoluble fraction was higher in total solids and solids-not-fat content than the warm preparation.

Ash

For most trials the ash content was highest in the membrane-containing serum, the controlled trial yielding 5.43 percent total ash on the dry weight basis. The soluble protein fraction had a slightly higher ash content than the insoluble portion, 1.80 percent as opposed to 1.51 percent.

Enzyme Activity

Enzyme activities were not followed solely for the sake of studying the effects of various physical and chemical actions upon alkaline phosphatase and xanthine oxidase. The activities were used also as a means of observing

destruction or loss of membrane. Tables 4 through 10 show the gradual loss of these enzymes in terms of sample size and grams of fat. On the average, cream beginning with 146 units of phosphatase activity per gram of sample dropped to 76 units; xanthine oxidase activity fell from 51 to 7 units per gram.

Also one can see the relative effects of cold and warm separation and washing. Although the data were somewhat erratic, the general picture shows that with both enzymes, the cold washed membrane holds the phosphatase and xanthine oxidase more tightly than the warm washing during the earlier part of the procedure. But by the fourth dilution and separation there seems to be greater activity in the warm creams.

Most of the activity assays performed on the latter phases of the preparation proved to be unsatisfactory. However the author found that both phosphatase and xanthine oxidase activity were lost to a considerable extent as a result of the salting-out step and ethanol-ether treatment. Also observed was the fact reported by Zittle (1956a) that a majority of the alkaline phosphatase was concentrated in the soluble protein fraction.

TABLE 1

YIELD OF MATERIAL AT VARIOUS PHASES OF THE PREPARATION OF MILK FAT GLOBULE MEMBRANE PROTEINS

Phase	Yield	Composition		
		Total Solids	Fat	Solids-not-Fat
	(pounds)	(%)	(%)	(%)
Whole Milk	169	12.7	3.9	8.8
Cream	25	32.4	24.9	7.5
Washed Cream 1	17	37.9	36.4	1.5
Washed Cream 2	16	39.3	36.9	2.4
Washed Cream 3	16.5	33.9	33.8	0.1[c]
Washed Cream 4	15.5	35.0	32.8	2.2
Washed Cream 5	15.5	34.1	31.3	2.8
Membrane-containing Serum	9.5	0.8	0.4	0.4
	(grams)			
Concentrated Membrane[a]	164.6	40.2	13.0	27.2
Membrane Proteins	51.4	59.3	1.8	57.5
Soluble Protein Fraction	400[b]	3.5	0.1	3.4
Insoluble Protein Fraction	45.5	16.7	0.9	15.8

[a]Contains $(NH_4)_2SO_4$ from salting-out step.

[b]400 milliliters of NaCl solution containing this protein fraction.

[c]Value obviously low; probably due to error in analysis.

TABLE 2

NITROGEN CONTENT OF MILK, SKIM MILK, CREAM, AND WASHED CREAM

Phase	Nitrogen Distribution[a]				
	TN[b]	CN[c]	NCN[d]	PPN[e]	NPN[f]
	(mg. N/100 g. sample)				
Whole Milk	538	405	133	22	35
Skim Milk	542	404	138	27	33
Cream	378	226	152	41	28
Washed Cream 1	90.4	36	53.8	17	9.3
Washed Cream 2	38.3	28	10.2	3	5.2
Washed Cream 3	26.6	21	6.0	4	4.3
Washed Cream 4	22.6	21	1.0	-	3.7
Washed Cream 5	21.8	21	1.5	3	2.9

[a]According to Rowland (1938).
[b]Total nitrogen.
[c]Casein nitrogen.
[d]Non-casein (whey) nitrogen.
[e]Proteose-peptone nitrogen.
[f]Non-protein nitrogen.

TABLE 3

THEORETICAL AND ACTUAL TOTAL NITROGEN AND PROTEIN CONTENT OF WASHED CREAM

Phase	Nitrogen		Protein	
	Theoretical	Actual	[a]	[b]
	(mg. N/100 g. fat)		(g. protein/100 g. fat)	
Original Cream	1520	1520	9.70	11.4
Washed Cream 1	380	249	1.59	1.66
Washed Cream 2	80	104	0.66	0.73
Washed Cream 3	18	79	0.50	0.61
Washed Cream 4	4	69	0.44	0.59
Washed Cream 5	1	70	0.45	---

[a] Calculated to 15.68 g. Nitrogen.

[b] From Rimpila and Palmer (1935).

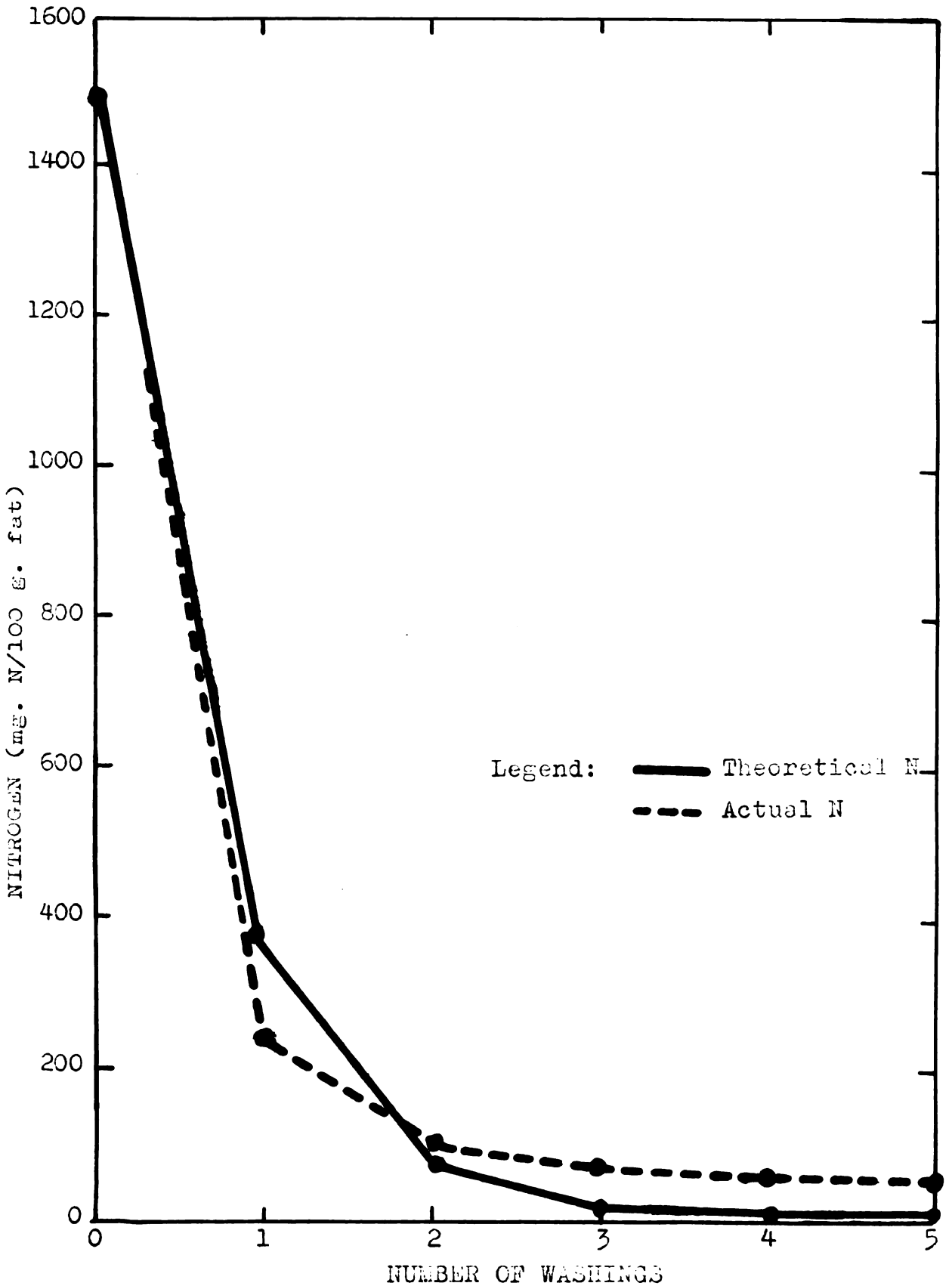

Figure I. Effect of successive washings on the total nitrogen content of cream.

TABLE 4

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 4° C. (Trial 1A)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids (%)	Fat (%)	Solids-not-Fat (%)	(Anhydrous) (%)	Alkaline Phosphatase (units/ml.)	Alkaline Phosphatase (units/g. fat)	Xanthine Oxidase (units/ml.)	Xanthine Oxidase (units/g. fat)
Whole Milk	12.4	3.3	9.1	---	---	---	10	318
Skim Milk	11.0	0.3	10.7	---	---	---	8.6	2870
Cream	40.9	35.0	5.9	---	201	574	59	168
Washed Cream 1	26.1	24.8	1.3	---	131	528	35	143
Washed Cream 2	35.9	29.8	6.1	---	86	289	14	48
Washed Cream 3	36.9	26.7	10.2	---	65	243	3.6	14
Washed Cream 4	---	---	---	---	28	---	6.6	---
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	0.8	0.4	0.4	12.7	65	19500	8.2	2450
Concentrated Membrane	38.9	11.2	27.7	---	111	800	0.7	5
Ethanol-treated Membrane	74.5	12.4	62.1	1.59	---	---	3.8	12
Membrane Proteins	79.3	7.7	71.6	4.12[a]	---	---	---	---
Soluble Protein Fraction	2.9	0.1	2.8	2.49	33	6960	2.2	459
Insoluble Protein Fraction	15.2	1.2	15.0	5.51	185	1980	6.2	67

[a]Calculated on basis of 46% soluble and 54% insoluble.

TABLE 5

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 38° C. (Trial 1B)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat	(Anhydrous)	Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.4	3.3	9.1	---	---	---	10	318
Skim Milk	10.4	0.5	9.9	---	---	---	5.2	1050
Cream	54.5	46.8	7.7	---	201	429	71	151
Washed Cream 1	55.1	46.2	8.9	---	118	255	62	134
Washed Cream 2	54.9	43.6	11.3	---	147	337	17	39
Washed Cream 3	53.0	45.2	7.8	---	121	268	20	43
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	2.4	1.7	0.7	2.82	70	1200	4.9	833
Concentrated Membrane	42.5	14.6	27.9	---	52	372	7.0	47
Ethanol-treated Membrane	70.2	21.1	49.1	2.04	270	860	2.4	14
Membrane Proteins	68.3	3.1	65.2	1.05[a]	---	---	8.4	26
Soluble Protein Fraction	3.9	0.2	3.7	2.03	83	13200	9.0	1215
Insoluble Protein Fraction	8.2	1.1	7.1	1.17	108	2280	1.3	30

[a]Calculated on basis of 46% soluble and 54% insoluble.

TABLE 6

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 4° C. (Trial 2A)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat	(Anhydrous)	Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.3	3.3	9.0	---	111	3360	7.4	224
Skim Milk	9.3	0.5	8.8	---	81	16200	6.3	1260
Cream	41.8	36.6	5.2	---	133	363	37	102
Washed Cream 1	36.9	32.3	4.6	---	74	229	20	61
Washed Cream 2	38.2	35.5	2.7	---	---	---	15	42
Washed Cream 3	41.0	36.7	4.3	---	---	---	---	---
Washed Cream 4	41.6	38.5	3.1	---	---	---	4.5	12
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	2.3	1.7	0.6	---	90	18000	12	2300
Concentrated Membrane	41.2	13.2	28.0	---	a	a	28	198
Ethanol-treated Membrane	61.3	5.0	56.3	---	a	a	a	a
Membrane Proteins	---	4.4	---	---	a	a	a	a
Soluble Protein Fraction	1.2	0.1	1.1	---	a	a	a	a
Insoluble Protein Fraction	24.2	3.6	20.6	1.13	a	a	a	a

[a]Enzyme activity lost during preparation.

TABLE 7

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 38° C. (Trial 2B)

Phase	Composition			Ash (Anhydrous)	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat		Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.3	3.3	9.0	---	111	3360	7.4	224
Skim Milk	8.4	0.3	8.1	---	109	36300	6.8	2250
Cream	59.2	55.9	3.3	---	172	308	51	91
Washed Cream 1	75.1	70.6	4.5	---	---	---	38	54
Washed Cream 2	74.3	71.6	2.7	---	185	258	16	22
Washed Cream 3	74.9	73.1	1.8	---	145	226	20	27
Washed Cream 4	71.1	70.9	0.2	---	111	157	8.1	11
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	1.9	1.2	0.7	3.70	29	4970	1.9	324
Concentrated Membrane	39.0	9.6	28.4	---	a	a	a	a
Ethanol-treated Membrane	68.1	10.8	57.3	---	a	a	a	a
Membrane Proteins	59.5	3.1	56.4	1.11[b]	a	a	a	a
Soluble Protein Fraction	2.9	0.2	2.7	1.32	a	a	a	a
Insoluble Protein Fraction	20.6	1.1	19.5	0.93	a	a	a	a

[a]Enzyme activity lost during preparation.

[b]Calculated on basis of 46% soluble and 54% insoluble.

TABLE 8

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 4° C. AND WASHED WITH A 12.7% SUCROSE SOLUTION (Trial 3a)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat	(Anhydrous)	Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.8	3.6	9.2	---	145	4030	14	403
Skim Milk	9.4	0.4	9.0	---	160	40000	9.0	2250
Cream	49.2	41.5	7.7	---	135	325	45	108
Washed Cream 1	53.6	48.4	4.2	---	---	---	29	60
Washed Cream 2	54.7	46.2	8.5	---	---	---	a	a
Washed Cream 3	55.7	47.5	8.2	---	80	178	14	30
Washed Cream 4	52.6	47.4	5.2	---	30	63	10	22
						(units/g. SNF)		(units/g. SNF)
Membrane Containing Serum	1.3	0.6	0.7	1.61	99	17300	7.7	1320
Concentrated Membrane	41.7	8.5	33.3	---	a	a	a	a
Ethanol-treated Membrane	49.9	5.6	44.3	1.68	109	492	a	a
Membrane Proteins	58.8	2.4	56.4	1.08[b]	132	468	a	a
Soluble Protein Fraction	4.0	0.2	3.8	0.26	122	18900	a	a
Insoluble Protein Fraction	12.4	1.8	10.6	1.78	120	1700	a	a

[a]Enzyme activity lost during preparation.

[b]Calculated on basis of 46% soluble and 54% insoluble.

TABLE 9

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 38° C. (Trial 3B)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat	(Anhydrous)	Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.8	3.6	9.2	---	145	4030	14	403
Skim Milk	9.5	0.2	9.3	---	130	65000	2.2	1080
Cream	59.3	54.4	4.9	---	124	228	53	97
Washed Cream 1	75.1	66.2	8.9	---	102	154	35	53
Washed Cream 2	72.3	70.1	2.2	---	---	---	---	---
Washed Cream 3	70.5	69.5	1.0	---	124	178	---	---
Washed Cream 4	71.2	65.3	5.9	---	134	205	5.4	8.3
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	1.2	0.6	0.6	4.65	30	6000	12	2460
Concentrated Membrane	36.4	7.0	29.4	---	---	---	18	120
Ethanol-treated Membrane	41.1	5.0	36.0	0.64[b]	206	1140	a	a
Membrane Proteins	58.8	2.6	56.2	0.78[b]	78	278	41	142
Soluble Protein Fraction	3.4	0.2	3.2	0.17	113	20800	15	2750
Insoluble Protein Fraction	11.9	1.0	10.9	1.31	176	2440	a	a

[a]Enzyme activity lost during preparation.

[b]Calculated on basis of 46% soluble and 54% insoluble.

TABLE 10

COMPOSITION, ASH, AND ENZYME ACTIVITY OF THE VARIOUS PHASES OF THE QUANTITATIVELY CONTROLLED FAT GLOBULE MEMBRANE PROTEINS PREPARATION USING MILK SEPARATED AT 38° C. (Trial 4)

Phase	Composition			Ash	Enzyme Activity			
	Total Solids	Fat	Solids-not-Fat	(Anhydrous)	Alkaline Phosphatase		Xanthine Oxidase	
	(%)	(%)	(%)	(%)	(units/ml.)	(units/g. fat)	(units/ml.)	(units/g. fat)
Whole Milk	12.7	3.9	8.8	---	140	3590	7.9	203
Skim Milk	9.3	0.2	9.1	---	124	82700	a	a
Cream	32.4	24.9	7.5	---	128	514	40	163
Washed Cream 1	37.9	36.4	1.5	---	89	245	a	a
Washed Cream 2	39.3	36.9	2.4	---	84	228	a	a
Washed Cream 3	33.9	33.8	0.1	---	94	278	a	a
Washed Cream 4	35.0	32.8	2.2	---	101	308	a	a
Washed Cream 5	34.1	31.3	2.8	---	71	227	a	a
						(units/g. SNF)		(units/g. SNF)
Membrane-containing Serum	0.8	0.4	0.4	5.43	122	36600	a	a
Concentrated Membrane	40.2	13.0	27.2	---	---	---	a	a
Ethanol-treated Membrane	57.3	9.3	48.0	---	208	904	a	a
Membrane Proteins	59.3	1.8	57.5	1.84[b]	749	2610	a	a
Soluble Protein Fraction	3.5	0.1	3.4	1.80	23	3990	a	a
Insoluble Protein Fraction	16.7	0.9	15.8	1.51	177	1590	a	a

[a]Enzyme activity lost during preparation.

[b]Calculated on basis of 46% soluble and 54% insoluble.

DISCUSSION

Yields

The results from this study indicated a concentration of 1.11 - 1.44 grams of membrane material per 100 grams of fat. These values compare favorably with data reported by others. Palmer and Wiese (1933) isolated 0.66 - 0.89 gram of membrane substance per 100 grams of fat; Jenness and Palmer (1945) reported 0.71 - 1.20 grams per 100 grams of fat; and Herald (1956) found 1.27 grams per 100 grams of fat. Storch (1897) and Schwarz and Fischer (1937) isolated 38 grams and 0.12 gram of lipoprotein material per 100 grams of fat, respectively--values which are not coincident with other data. Also it should be noted that the values reported by Jenness and Palmer do not include the high-melting triglyceride fraction of the membrane found by Palmer and Wiese. Nevertheless the data of the latter indicates values somewhat lower than those of Jenness and Palmer.

A yield of 0.59 gram of membrane protein per 100 grams of fat was isolated in the present work. This is close to the 0.51 gram per 100 grams of milk fat reported by Herald (1956). Rimpila and Palmer (1935) and Jenness and Palmer (1945) found 0.46 - 0.71 gram and 0.38 - 0.86 gram of membrane protein per 100 grams of fat, respectively. Roland (1956) calculated the mean of a number of determinations to

be 1.15 grams of protein per 100 grams of fat. He based this figure on his formula

$$x = \frac{(n-e)\ (100-f)}{f}$$

where x = percent nitrogen in protein

n = percent nitrogen in fat-free liquid

e = percent nitrogen in skim milk

f = percent fat in cream and

7.4x = percent protein in membrane. Even with the exaggerated figure of 0.12 gram of membrane protein per 100 grams of fat reported by Schwarz and Fischer (1937), the amounts of lipoprotein material and membrane protein would indicate that workers in this field are fairly well agreed on the amount of these substances which is present in normal cow's milk.

Since Herald (1956) was the first to fractionate the membrane proteins according to their solubility in dilute salt solution or water, an extensive comparison with results found in this present study cannot be made. Forty-six percent of the membrane protein was found in the supernatant solution while 54 percent remained as the insoluble fraction. Herald's results were almost identical. His soluble fraction accounted for 44 percent and the insoluble portion 56 percent of the total membrane protein.

Brunner, Duncan, Trout, and Mackenzie (1953b) provisionally classified the entire fat globule membrane protein

as one of a globulin-like nature. Interestingly, Herald (1956), on the basis of sedimentation velocity and other properties, tentatively classified his soluble proteins as globulin in nature. On the basis of their solubility in 0.02 M sodium chloride, one might go so far as to call them euglobulins. Herald also concluded that the insoluble fraction should be provisionally classified as a "pseudokeratin."

Nitrogen

Table 3 and Figure I show the theoretical and actual effects of the washing procedure on the total nitrogen content of the cream. Theoretically the plasma (and its nitrogen) of the cream should be reduced by three-fourths after each washing. The curve in Figure I showing the expected distribution of nitrogen over five cream washings was calculated on the basis of the results obtained in this study. As can be seen in Table 10, the plasma content of the various washed creams was not constant, so the theoretical curve was based on the changing values rather than on the original 24.9 percent cream. Since fat is hydrophobic, it cannot be diluted by the water, and one assumes that the plasma in the first washed cream has been reduced to 25 percent of its value in the original cream. Therefore the total nitrogen content of the plasma portion of the first washed cream should be only one-fourth that of the original.

The aqueous phase of the second washed cream then would have 25 percent of the nitrogen which was in the previous washing, and so forth.

Brunner et al. (1953a) showed a nitrogen curve for nonhomogenized milk similar to the "Actual N" in Figure I. After the second washing the nitrogen content is slightly higher than the expected level. Then the actual value levels off so that the curve is practically flat. The theoretical curve also levels off but can never become horizontal to the abscissa since the nitrogen would continue to be lost until only an infinite amount remained. Therefore the actual total nitrogen content of the washed creams has increased in comparison to the expected values. This is due to the fact that not only plasma nitrogen but also membrane nitrogen has been measured.

Palmer (1944) pointed out that washing cream probably removes from the membrane all but the most tenaciously held materials. The curves in Figure I bear out this hypothesis. It is interesting to note that Brunner et al. (1953a) found that the protein of homogenized milk membrane differed markedly from that of nonhomogenized milk. Trout (1950) recognized the probability of such a change when he mentioned that due to the effects of homogenization, one may presume ". . . that a film of protein material is adsorbed to the surfaces of the . . . fat globules." Trout (1957) also

mentioned the possibility that some of the original membrane might be enveloped by the fat globule during homogenization. This could account for the change in amino acid composition reported by Brunner et al. All of the above mentioned is yet another indication that the membrane proteins obtained by means of the rather harsh treatments used in this preparation procedure are changed materially from the natural membrane proteins.

A final note on the amount of protein found in the fat globule membrane might be made. A comparison between the results of Rimpila and Palmer (1935) and those from the present study showed the latter to be slightly lower in absolute value, but the trend was similar. For the sake of uniformity, protein values were calculated on the basis that an average nitrogen content is 15.68 percent per protein. The author realizes that such a figure must be used critically, but as already stated, this provides a common ground for discussion. Also the present calculation assumes (since it was not specifically stated) that Rimpila and Palmer computed their protein results to 15.68 grams of nitrogen.

Fat and Total Solids

When Storch (1897) first devised and used the method of repeated dilutions and separations, he found that the cream from each washing had a considerably smaller amount of fat

than the cream from the previous separation. If he meant that the percentage of fat in the cream lessened, such results are not corroborated by this present study, for the fat dropped after the first washing only; succeeding washings showed a fairly constant level of fat. Although his work was performed at room temperature while the studies presented here were carried out at 4° and 38° C., the milk fat in Storch's experiments was largely in a solid state and should have behaved somewhat similarly to the cold separation of this report.

Therefore one may assume Storch (1897) meant that the total amount of fat in the cream decreased after each dilution and separation. Using the figures from Trial 4 (Table 10), we find the following:

Washed cream 1	2.81 kilograms of fat
Washed cream 2	2.68 kilograms of fat
Washed cream 3	2.53 kilograms of fat
Washed cream 4	2.31 kilograms of fat
Washed cream 5	2.20 kilograms of fat.

These values do indicate that fat was lost with each succeeding washing. Data from the second through fifth washed creams show gram of membrane protein per gram of fat ratio values of 0.0057, 0.015, 0.018, and 0.020. The ratio of protein to fat over the same period was 0.025, 0.020,

0.019, and 0.020. Jack and Dahle (1937) and Brunner et al. (1953a) also found that the nitrogen/fat ratio was fairly constant after the third washing, demonstrating that almost all of the plasma protein had been removed. Therefore one may presume that membrane material was lost to only a slight extent. Furthermore we may conclude that the lipoprotein complex still adsorbed on the fat globule after the third washing will not be changed to any great extent until the emulsion is broken by ". . . twelve or more . . ." (Rimpila and Palmer, 1935) successive washings.

Again referring to Storch (1897), one finds that he, too, used a strong sugar solution in some of his washings. He observed that using this technique left most of the membrane around the fat globule, whereas washing the cream with plain water resulted in the loss of a considerable part of the lipoprotein complex. The sucrose solution was used in the present study in order to increase the difference of specific gravity between the cream and wash solution to permit the physical separation of fat from wash water during cold washings. When water alone was used, we found it difficult to obtain good yields of cream from operations at 4° C. A comparison of Tables 8 and 9 points out the advantage of a sugar wash. One must agree with Storch that much more fat and accompanying membrane material is lost with plain water washings. Skim milk washed with the same concentration

sucrose solution was not affected, indicating that this procedure did not change the results by preventing adsorbed casein from being released by the fat globule surface even though the specific gravities of the two are close. A sucrose solution was not used for any of the warm washings, since it was felt that the mechanical separation was efficient; therefore, no comparison can be made between the amount of membrane left under the different conditions discussed. In all probability an even greater yield of lipoprotein would have been obtained from warm sugar-washed cream.

The membrane proteins after the ethanol-ether treatment contained approximately 5 percent fat. This was probably phospholipid from the lipoprotein complex which would have been removed during the early stages of the preparation had a more drastic method than churning been used for freeing the membrane from the milk fat. However it was thought desirable to employ this technique so as to obtain proteins as close to those of the original membrane as possible.

The results in Tables 4 through 9 demonstrate that perhaps cold separation and washing of cream removes more adsorbed material from the fat globule surface. This would agree with the findings of Jenness and Palmer (1945) wherein they stated that both chilling and aging of cream tend to

increase the dissociation of the membrane material from the cream. The reason for this is not clearly understood, though it is probably merely a physical phenomenon whereby the colder temperature causes the fat globule surface to become more "brittle" and loose membrane into the plasma. Using this same line of reasoning, warm fat surfaces are quite pliable, since the fat is completely melted, and lipoprotein material is less likely to be washed off by the mechanical action of the wash water or separator.

Ash

Titus et al. (1928) reported 3.11 percent ash in the membrane proteins, and Hare et al. (1952) found 3.22 percent. Herald (1956) noted a considerably higher percentage of ash, 4.27 percent. The present study found an average of 1.63 percent ash, quite a bit lower than other reported values. In the light of the lower enzyme activities reported for the latter phases of the preparation, the ash values are not as far out of line as they might appear at first glance. Herald, Brunner, and Bass (1957) made the comment that both protein fractions contained mineral elements in concentrations higher than reported for other milk proteins, suggesting that the fat globule membrane may be the spot at which the trace elements in milk are located.

The ash values shown in Tables 4 through 10 indicate no definite pattern with regard to differences occurring in cold and warm washed creams.

Enzyme Activity

Enzyme activity results as complete as might be desired were not obtained. This was due partially to mechanical problems encountered with the assay methods themselves and partly to loss of activity during the preparation.

Zittle et al. (1956b) have shown the enzyme activity distribution through successive cream washings. The present study agrees with their findings. On the average, 42 percent of the original cream's phosphatase activity remains after four washings, while only 18 percent of the xanthine oxidase activity is present. Sharp (1940) commented that over 50 percent of the xanthine oxidase originally present in the membrane is removed by washing. This indicates that alkaline phosphatase is more tenaciously held by the lipoprotein complex. Zittle et al. note that the loss is a mechanical one into the wash water rather than destruction of the enzymes. The large drop in xanthine oxidase activity may be because this enzyme is associated with the plasma as well as the fat globule surface. On the other hand Zittle et al. (1956a) found only a low level of activity in skim milk.

Rimpila and Palmer (1935) reported that 72 percent of the original alkaline phosphatase activity remained after four washings, a figure somewhat higher than reported here and by Zittle et al. (1956b). The latter postulate the cause to be due to chilling of the original cream before washing. The present study tends to support such a theory, for when cream was separated and washed in the cold, 49 and 13 percent of the phosphatase and xanthine oxidase activity, respectively, remained, whereas 56 and 25 percent, respectively, were retained by the warm cream. Jenness and Palmer (1945) said that ". . . mere aging of milk at low temperature followed by rewarming before separation greatly decreases the retention of protein and phospholipide during washing." This would explain to some extent the reason for this author's washed creams loosing so much more enzyme than that of Rimpila and Palmer, for all milk used had been in the University Dairy's holding tanks for 16 - 48 hours at 3.3° C. before separation. But the warm washed cream retained more enzyme than the cold. Perhaps the rewarming allows a portion of the enzymes to be readsorbed onto the fat globule surface, while separating and washing directly at the colder temperature permits a maximum amount to remain in the milk plasma.

Loss of enzyme activity after the churning process may be due to chemical as well as physical changes. Whereas

during separation, washing, and churning, the cream came in contact with nothing that could destroy the enzyme or inhibit activity, subsequent treatments conceivably were detrimental to the enzyme. In one preparation the butter and buttermilk, instead of being held at room temperature until all was ready to be warmed and separated, was brought to approximately 38° C. and held there for from 20 to 90 minutes before the butteroil was removed from the serum. Neither xanthine oxidase nor alkaline phosphatase activity could be detected in subsequent stages of the preparation with the exception of a considerably reduced amount of activity for phosphatase in the membrane-containing serum. An explanation of this phenomenon is difficult. We know that phosphatase is only 96 percent inactivated after being held at 61.5° C. for 30 minutes (Kay and Graham, 1934), and Zittle et al. (1956a) have shown that xanthine oxidase is even more resistant to heat. In addition, the work of Zittle et al. was performed on skim milk, a low fat product, while the butter and buttermilk in the preparation under discussion had a combined fat content of 71 percent, thus providing good insulation against the relatively high temperature.

As can be seen in Tables 4 through 10, the recovery of enzyme (activity) after treatment with cold ethanol-ether is quite low. Evidently, organic solvents take their toll

of phosphatase and xanthine oxidase activity. This indicates that perhaps other proteins of the lipoprotein complex also may be harmed. If further trials were to show the same results, this would demonstrate that the final products of the preparation are quite drastically changed from those of the original membrane. Zittle et al. (1956b) obtained membrane from washed cream buttermilk by means of ultracentrifugation and recovered 89 and 82 percent, respectively, of the xanthine oxidase and alkaline phosphatase in the original buttermilk fraction. The present study recovered an average of less than 1 percent of each enzyme in the original serum.

Another point of interest was observed during this series of preparations. Sharp (1940) noted that the reddish-brown color of buttermilk is due to the presence of xanthine oxidase. Herald (1956) found that when this color disappears, xanthine oxidase activity is gone, also. The present study proves, however, that the reverse is not true; namely, the presence of this characteristic color does not guarantee that any activity will be found.

In the final membrane protein fractions, Zittle (1956a) found most of the phosphatase activity concentrated in the soluble portion, 84 and 90 percent of that in both fractions for the two samples reported. Present results show 78, 85, 92, and 90 percent of the phosphatase in the soluble

fraction. The absolute amount of activity is considerably smaller in the latter series of determinations, but this might be explained by normal variations in the milk samples.

SUMMARY AND CONCLUSION

The primary objective of this study was to observe some of the changes which occur in the fat globule membrane proteins during the isolation procedure. Factors studied included nitrogen distribution in the washed creams, variations in composition due to different temperatures of separation and washing and type of wash solution, and alkaline phosphatase and xanthine oxidase activity of the various phases throughout the preparation. Where the data obtained made it possible, further comments were given concerning the results of other workers in this area. Such items as yield and general composition were observed.

Yields of 1.11 - 1.44 grams of membrane per 100 grams of fat and 0.59 gram of membrane protein per 100 grams of fat were obtained. These values agree with the results of other researchers. Likewise the soluble and insoluble protein fractions were in the same proportions as found by Herold (1956).

Determinations of the nitrogen distribution by means of Rowland's (1938) fractionation showed a loss in every fraction studied, but this loss leveled off quite rapidly after the first washing. More nitrogen was lost from the cream after the first washing than was expected from calculations. Washed creams had more than the theoretical amount

of nitrogen due to the fact that membrane as well as plasma nitrogen was measured.

Washing cold cream with a sucrose solution instead of plain water resulted in an increase in the separation efficiency and consequently an increase in the yield of membrane material. Separation and washing at 38° C. resulted in a higher recovery of lipoprotein complex than did working at 4° C.

The results of ash analyses were somewhat lower than those obtained by other researchers but may have been due to normal variations in milk.

Enzyme activities were reduced quite drastically during washing, 58 percent of the alkaline phosphatase and 82 percent of the xanthine oxidase having been removed. This indicates that some of the phosphatase in milk is more closely associated with the fat globule surface than is xanthine oxidase. One cannot say "all phosphatase" because it is found in skim milk as well as in cream. Again the warm separated and washed cream retained more enzyme activity than the cold products, 56 percent compared to 49 percent of the phosphatase and 25 percent as opposed to 13 percent of the xanthine oxidase. Loss of enzyme activity after salting-out with ammonium sulfate was quite pronounced. Use of organic solvents also appeared to inhibit activity or destroy these enzymes.

Should further enzyme studies of the preparation yield results similar to those obtained in the present work, one may possibly conclude that the membrane material of normal cow's milk is altered as a result of the techniques employed in the isolation procedure.

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