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Preparation of "natural" cow-milk fat globules: preliminary investigation of materials adsorbed at their surfaces

Eugene Harry Sander
Iowa State University

PREPARATION OF "NATURAL" COW-MILK FAT GLOBULES; PRELIMINARY INVESTIGATION OF MATERIALS ADSORBED AT THEIR SURFACES

by

Eugene Harry Sander

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In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
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INTRODUCTION

The chemical and physical properties of the materials found at the milk fat globule interphase in milk have been of interest to investigators seeking answers to some of the practical and theoretical problems encountered in the commercial processing of dairy products. Emulsion stability of fat globules following homogenization, reconstitution of dried whole milk powder, development of oxidative and other undesirable flavors in fluid milk and butter, and the churning of cream are typical problems in which the interphase materials (IM) play an important role.

It has been suggested that the materials present at the fat globule interphase can be divided into two classes, labile and non-labile. The former are easily removed from the interphase by physical means, whereas the latter resist removal to a greater degree. The labile materials have been considered to be the constituents of skimmilk, whereas partially classified lipoprotein and glycoprotein complexes represent, in part, the non-labile class of IM. The combined hydrophobic and hydrophilic properties of both classes of materials play a major role in the "emulsion" stability of fat globules in milk serum.

To obtain the small amount of IM in whole milk a number of investigators have used the cream separator to prepare "washed" fat globules, from which non-interphase materials were considered to have been removed, by repeated dilution of cream with water and reseparation. When the washed creams were churned, sizable quantities of IM were obtained but uncertainty prevailed as to how nearly these preparations represented the natural IM. At an earlier date, some investigators "washed" the fat glob-

ules by allowing them to rise through long columns of water. They believed that fat globules washed by this more "gentle" method retained more of the complete IM at their surfaces. Casein was identified as one of the major interphase components. Investigators, who favored the use of the cream separator, claimed that IM obtained by the gravity washing technique were contaminated with milk serum proteins. However, they were unable to identify the protein components in the IM obtained by the washing technique. Based on chemical and physical analyses, they concluded that interphase proteins were unlike the known milk proteins, the presence of lipoprotein complexes made characterization difficult, and the natural state of the protein fraction remained in doubt when methods used to cleave the lipoprotein complex were applied.

Studies relating the churning losses and the pH of the cream churned suggested that casein not only influenced their isoelectric point but contributed considerable stability to the fat globules during the churning process.

The information, discussed above, suggested this problem. The objectives of the investigation were: 1) to evaluate gravity and separator methods of preparing washed fat globules, 2) to develop a method of washing cream whereby the bulk of the IM were recovered, 3) to demonstrate the effect of repeated washing of cream on the lability of IM by following: a) variation in the amounts of acid- and centrifugally-precipitable materials, b) changes in the lipid and nitrogen contents, and c) changes in trace metal contents of centrifugally-precipitable IM preparations.

REVIEW OF LITERATURE

Comprehensive reviews of the literature concerning the fat globule-milk serum interphase, and of the practical and theoretical explanations regarding the chemical and physical behavior of the IM have been presented by Bird, Breazeale and Bartle (10), and King (49). The review of literature presented here will summarize the more recent work in the field and include earlier work pertinent to this problem.

Classical Methods of Preparing Washed Fat Globules

Non cream separator-washing methods

Völtz (108) and Abderhalden and Völtz (1) were the first to allow the fat globules in milk to rise through columns (50 cm long) of distilled water or a salt and sugar solution in the preparation of washed creams. A 10-cm layer of milk was placed on the bottom of the column. Washed cream was periodically, up to three days, siphoned from the top of the column. A variety of preservatives were added to both the milk and washing solutions. Following hot ether extraction of the filtered washed cream, the ether-insoluble material remaining was reported to be casein.

Titus, Sommer and Hart (105) washed an 8% cream through a column of water 120 cm high. Washed cream was removed from the top after 24- and 48-hr intervals. Following removal of lipids from filtered, washed cream, by ethanol-ether extraction and carbohydrates by repeated washing with distilled water, the chemical composition of the dried residue was determined. Based on the sulfur, nitrogen, phosphorus and tryptophane content and its antigenic properties, they concluded that protein portion of IM was casein.

Mulder (71) recommended starting with uncooled fresh milk and allowing it to separate by gravity at less than 10 C. The fat globules concentrated in the cream retained all of the natural IM present. Based on the distribution of any component among the milk, cream and skimmilk, its concentration at the fat globule surface could be calculated.

Hattori (35) treated milk with nine volumes of chloroform-saturated water. After two to three days the chloroform-saturated fat globules settled out. This procedure was repeated four times after which the fat was extracted from the isolated fat globules with ether. Later Hattori and Ogimura (36) precipitated the casein from cream with acid and rennet, washed the fat globules from the precipitate with ether-saturated water, and studied the ether-insoluble IM obtained from the isolated fat globules. In both cases IM was reported as mostly protein which differed chemically from all known milk proteins.

Cream separator-washing procedure

In 1897 Storch (95) washed fat globules four to five times using a farm cream separator. The cream from 10 liters of milk was repeatedly mixed with 8 liters of distilled water at 35 C and separated. After four washes the presence of "albuminoids" in the cream suggested that IM did exist at the fat globule surface.

Wiese and Palmer (110) washed fat globules as many as eight times by repeated dilution of the "cream" in four volumes of distilled water at 38-43 C and separation of the washed cream in a Sharples centrifuge at 10,000 rev/min. They observed churned particles of fat in the cream following the fourth pass through the centrifuge.

More recently Tarassuk, Koops and Pette (98) washed cream up to ten or 15 times with four volumes of distilled water at 37 C by repeated dilution and separation of the cream in a cream separator. They reported that emulsion stability was retained throughout the washing procedure by maintaining a constant fat content, 25%, and starting with fresh, uncooled raw milk.

The cream separator was used more frequently to prepare washed fat globules because it was faster than other methods and higher yields of washed cream were obtained. Distilled water generally was used to wash cream. The effects of using saline and sugar solutions on the final IM obtained were also studied (49).

Recently Sasaki and Koyama (87) washed cream up to four times with nine volumes of a 0.95% sodium chloride solution at 40 C. Jackson and Brunner (40) employed a 3% sucrose solution through the first three washes and removed all traces of sugar from the washed cream with three additional washes with distilled water. Cole, Kluepfel and Lusena (24) washed a freshly prepared 45 - 55% cream twice at 35 C with ten volumes of a 0.01% ash-free gelatin solution. A cream separator was employed in all these investigations.

Effect of repeated washing of fat globules on the chemical and physical properties of the interphase materials

The use of the gravity washing technique (1, 105, 108) was criticized from the viewpoint that the final preparation of IM was contaminated with milk serum constituents. In the absence of natural milk salts the ascending fat globules clumped readily, thereby trapping milk serum constituents with-

in the interstices of the cluster (49). Mulder (71) commented that centrifugal washing techniques employing water or salt solutions disturb an adsorption equilibrium at the milk serum-fat globule interphase resulting in the loss of important constituents. He favored the use of the gravity washing technique.

Rimpila and Palmer (82) were able to wash cream up to 24 times using a Sharples centrifuge. Beyond the 12th wash destabilization of the washed cream increased and the total protein to fat ratio began to decrease, after remaining constant from the fourth wash. Negative Biuret and Fehling's tests on the wash water together with constant protein to fat ratios in the washed cream were the criteria used to estimate the number of washes required. Using rennet whey in place of several of the distilled water washes during the washing sequence resulted in a reduction in the lipid-phosphorus content of the washed cream. Cream washed six times with distilled water retained only 50% of its original phosphatase activity. Later Palmer (75) reported that 50% of the xanthine oxidase activity was also lost by washing cream six times with distilled water. Both enzymes are known to be components of IM (49).

Recently Zittle et al. (112) used the washing procedure of Rimpila and Palmer (82). Based on the amount in the original cream, 14% and 15% of the xanthine oxidase and alkaline phosphatase activities, respectively, were recovered in buttermilk obtained from cream washed four times. In each wash the enzyme activity of the cream and wash water approximated the total assay of the previous cream. As washing progressed, the xanthine oxidase activity decreased at a faster rate than the alkaline phosphatase activity. Reduction in the activity of these enzymes during repeated

washing of the cream was confirmed by others (9, 24, 89).

The susceptability of washed cream to the development of fishy flavor was investigated by Tarassuk, Koops and Pette (98). Copper-catalyzed oxidation of the phosphatides contained in IM was believed to be responsible for this defect. As the milk serum was removed by repeated cream separator-washing of cream, the development of fishy flavor reached a maximum after four washes and thereafter decreased. This decrease was attributed to the removal of a copper-bearing protein from the IM.

Isolation of Interphase Materials from Washed Fat Globules and their Characterization

Isolation of the interphase materials

Early investigators were content to fractionate the lipid and non-lipid components from filtered, washed cream by solvent extraction with hot ether (1, 108) or ethanol treatment followed by solvent extraction with ether (105).

Palmer and his co-workers (43, 44, 75, 82, 110, 111) churned the washed cream and recovered IM from the resulting buttermilk and butterserum. This procedure was widely used by later investigators (49). Palmer (75) admitted that protein denaturation could have occurred during churning. Recently (24) washed cream was frozen and thawed in order to dislodge IM from the fat globule surface.

Concentration of IM from buttermilk, butterserum or their combination has been accomplished in many ways: acidification to pH 4.0 (37, 40, 76), salting out the crude IM with ammonium sulfate (20, 37), pervaporation followed by freeze drying (104) or direct ultracentrifugation (67, 68, 89).

Subsequent fractionation of the lipid and non-lipid components in the concentrated IM has been achieved by extraction with ethanol and ether (19, 37, 38, 44, 49, 76, 87, 102, 103, 104), in either 1:1 or 1:2 ratios. Morton (69) used butanol to dissociate the lipoprotein particles he isolated from milk. He was able to recover all of the original enzyme activity in true solution and free of lipids. Quantitative recovery of enzyme activity suggested that lipoprotein complexes had been cleaved without denaturation of the associated enzyme systems.

Identification of the components of the interphase materials

Lipids Rimpila and Palmer (82) reported that the IM contained 13 - 26% phospholipid, 38.52 - 52.01% ether-soluble non-phospholipid and 21.98 - 43.96% protein. Earlier Palmer and Wiese (76) identified lecithin, cephalin and sphingomyelin by ether and acetone fractionation of an ethanol extract of crude IM. Their determined phosphorus to nitrogen ratios of the isolated phospholipids agreed well with other reported values, for similar phospholipid fractions. Jenness and Palmer (44) crystallized a "high-melting glyceride fraction" (HMGF) from the ethanol-ether extracts of butterfat, buttermilk and butterserum. Yields of 3.5 - 5.1%, 4.1% and 37.4%, respectively, were obtained. The melting point, 52 - 53 C, iodine value, 5.0 - 7.1, and saponification value, 198 - 204 were similar for the HMGF from the three sources. The high yield of HMGF from butterserum suggest that it was closely associated with the phospholipid-protein complex in IM.

Recently Patton and Keeney (77) demonstrated that crystallization at 22 C of the ethanol-ether extract of IM gave a HMGF identical in yield and

composition when either acetone or ethanol was used as the solvent. Thompson, Brunner and Stine (102) presented a more detailed characterization of a HMGF isolated from crude IM. The iodine value, 5.0, melting point range, 50 - 51.8 C, and saponification value, 201 - 202 were in good agreement with the values reported by Jenness and Palmer (44). Analysis of the fatty acids obtained from the HMGF fraction indicated the following mole percentages of acids: 11.0 C_{14}, 59.6 C_{16} and 16.5 C_{18} acids; C_{18} unsaturated acids represented only 6.1 mole % of the total. These values were in good agreement with those reported by Patton and Keeney (77).

Thompson, Brunner and Stine (103) reported that the total lipid extract, obtained by ethanol-ether treatment and subsequently fractionated using acetone and absolute ethanol, contained 43.5% phospholipids and 44.6% HMGF. Thompson, et al. (104) fractionated the ethanol-ether extract of IM from combined buttermilk and butterserum on silicic acid columns. Carotenoids, squalene, cholesterol esters, triglycerides, free fatty acids, cholesterol, di- and monoglycerides and phospholipids were evaluated quantitatively. Phospholipids and triglycerides represented 13.76 and 36.12%, respectively, of the total IM; 67.51% of the total IM was lipid and 32.49%, protein. When the buttermilk-butterserum combination was extracted with ether prior to freeze drying and subsequent ethanol-ether extraction was applied, only the last five of the above mentioned components were recovered in smaller amounts; the total lipid represented only 43.76% and the protein, 56.24% of the IM.

Mulder and Zuidhof (74) estimated from the fat and cholesterol contents of milk, cream and skimmilk, that 36 $\pm$ 16 mg of cholesterol/100 g

of fat were present in IM. Using the same techniques and calculations, Mulder, Menger and Koops (73) showed that 60% of the total phospholipids in milk are concentrated on the fat globule surface. The phospholipid content of IM was 20 - 30%. Koops and Tarassuk (52) studied the effects of various processing treatments on the desorption of phospholipids from the fat globule surface. Eighty per cent of the total phospholipids present were firmly bound and they were not removed by normal plant processing other than homogenization. Homogenization did not reduce the total phospholipids retained but did reduce the concentration per unit of fat globule surface two to three times. This latter observation may be an important factor in the inhibition of oxidized flavor in milk as a result of homogenization (97).

Proteins The amount of protein reported in IM obtained from cream washed repeatedly with water varied from 0.46 - 0.71 g (82) and 0.38 - 0.86 g/100 g of fat (43). These values are in good agreement with the values, 0.2 - 0.8 g/100 g of fat, reported by Mulder and Menger (72). They calculated their values from the protein differences, on a fat free basis, between the cream and skimmilk obtained by gravity separation of milk. Roland (84) developed an equation, based on this same difference, for estimating the protein content of IM. He reported an average protein content of 1.15 g/100 g of fat, with a range of 0.44 - 2.22 g/100 g of fat, in milk separated in a cream separator.

Studies on the whole protein fraction Based on the low nitrogen, 11 - 14%, high sulfur, 1.2 - 2.6%, low phosphorus, 0.33 - 0.64%, and differences in amino acid composition, it was concluded that the protein

in IM was different from known milk proteins. Furthermore, it was shown to be heterogeneous, partially composed of a variety of enzyme systems. The method of preparing the IM varied among investigators which in turn may have influenced variations in the results obtained (45, 49).

Mulder and Menger (72) concluded from simple serological experiments that casein, albumin, and globulin were components of IM. They were unable to solublize their preparation with sodium hydroxide (pH 9.0) following hydrochloric acid treatment (pH 4.0).

Sasaki and Koyama (87) compared interphase protein (IP) preparations from cream washed two and four times with a 0.95% sodium chloride solution, with proteins isolated from unwashed cream, isoelectric casein and whey proteins from skimmilk. The paper electrophoretic patterns of IP were different from those of casein and whey proteins. From the results of a later study (88) they concluded that casein, representing the outermost protein in the interphase, was completely removed after four washes. Whey proteins closely resembled the remaining IP of which β-lactoglobulin was definitely not a component.

Lipoproteins Morton (67) ultracentrifuged buttermilk from churned, washed cream at 14,000g for 45 min. The sedimented reddish-brown pellet contained 22% lipid and traces of nucleic acid and succinic dehydrogenase activity. Based on the high amounts of enzyme activities he concluded that small lipoprotein particles were attached to the protein on the fat globule surface. Since the enzyme activities were similar to those reported for microsomal particles from animal tissue, he assumed the lipoprotein or "microsomal particles" originated in the secretory cells of the

udder and were released to the milk as a result of physiological changes within the cell. More recently Bailie and Morton (7, 8) compared the chemical composition of microsomal particles in milk with those isolated from the mammary gland. Enzyme activities of the two were different; milk microsomal particles contained less lipid and nucleic acid. Incubation of gland microsomes in milk caused a drop in lipid and nucleic acid and changes in the enzyme activities so that they closely resembled milk microsomes. They concluded that following secretion the gland microsomes were changed.

Sasaki and Koyama (89) isolated lipoprotein material from combined buttermilk and butterserum from washed and unwashed cream by ultracentrifugation and ammonium sulfate fractionation followed by ultracentrifugation, respectively. Paper electrophoresis of the resulting fractions followed by differential staining for lipids and proteins revealed the presence of two or three lipoproteins in the unwashed cream including interphase lipoprotein material. They considered that lipoproteins, other than the interphase lipoprotein were loosely adsorbed on the surface of fat globules.

Fractionation of interphase protein and characterization of individual fractions Herald and Brunner (37, 38) resolved "the most tenacious membrane proteins" into soluble and insoluble fractions by salting out the IM from combined buttermilk and butterserum with ammonium sulfate, followed by ultracentrifugation, extraction of the lipids and ultracentrifugation of the "lipid-free" IP dispersed in a dilute sodium chloride solution. An insoluble fraction sedimented as a reddish-brown pellet whereas a soluble fraction remained in the supernatant. The soluble fraction produced two peaks of similar mobilities when studied electrophoretically.

The insoluble fraction was solublized using sodium sulfide and Duponal PC revealing one and two peaks, respectively.

In subsequent studies, Brunner and Herald (17, 18), detergents were employed to solublize the insoluble fraction. The electrophoretic properties of both fractions were then compared in a variety of buffers. The isoelectric point of the soluble fraction was pH 5.0. The unfractionated IP preparations contained 12.2 to 12.6% nitrogen whereas the subsequent soluble and insoluble fractions contained 9.5 - 11.5% and 12.9 - 13.9% nitrogen, respectively; both fractions had similar amino acid compositions. The soluble fraction gave a positive Molisch and a negative nitroprusside test, whereas the insoluble fraction gave a negative Molisch and a positive nitroprusside test (37). Brunner and Thompson (19) reported that the reddish-brown pellet contained 8% lipid with a 1:1 ratio of triglycerides to phospholipids and the ultracentrifuge supernatant contained lipoproteins containing 45% lipid with a 2:1 ratio of triglycerides to phospholipids.

Ramachandran and Whitney (80) isolated a soluble IP in a manner resembling that of Herald and Brunner (37). It represented 17% of the total IP and was electrophoretically homogeneous in buffers above pH 7.0. Detailed elemental analysis of this fraction was also reported.

Brunner and Thompson (20) suggested that the soluble IP fraction was closely related in chemical composition and electrophoretic behavior to some of the minor protein fractions obtained by different treatments of skimmilk. The soluble IP contained 2.69 mg hexose, 1.08 mg hexosamine, 0.71 mg fucose, and 2.00 mg of sialic acid/100 g of protein (101). Recently Jackson, Coulson and Clark (41) found that the soluble IP contained a variety of carbohydrates including hexosamine and sialic acid, and con-

tained 11.28% nitrogen, 0.68% phosphorus, 6.12% ash and 2.1% lipid. They classified it as a muco-protein. In a second paper Coulson and Jackson (26) demonstrated that the isolated mucoprotein differed immunologically from other known milk proteins.

Jackson and Brunner (40), starting with homogenized milk prepared lipid-free IP according to the method of Herald and Brunner (37). The soluble IP fraction was found to contain an acid-precipitable caseinate complex and heat-denaturable whey proteins. They concluded that caseinates as well as whey proteins were adsorbed onto the fat globules during homogenization. Sasaki, Tsugo and Miyazawa (91) reported that only the whey proteins were adsorbed.

Loewenstein (62) found that heat treatments (momentarily to 40 C, 62 C for 30 min and 82 C for 15 min) of milk caused changes among the materials adsorbed on the milk fat globule surface in approximate proportion to the severity of the heat. As the heat intensity increased, the amount of protein, phospholipid, and phosphorus-free lipids retained decreased. He concluded that casein was replaced by other proteins and protein-fat complexes were formed as a result of heating milk. Earlier Dunkley and Sommer (31) discussed the role of euglobulins in the "agglutination" of individual fat globules into clusters. They suggested that euglobulins may be an important IM component but their presence or absence on the fat globule surface depends on the physical state of the fat. Solid or partially solidified fat (warmed to 5 and 25 C, respectively) retains the euglobulin, whereas warming milk to 50 C results in the loss of euglobulin from the fat globule surface.

Enzymes associated with the interphase materials Aldolase, alkaline phosphatase and xanthine oxidase (49, 67), as well as diaphorase and diphosphopyridine nucleotide-cytochrome C-reductase activities (67) have been reported for the isolated IM. Active lipase, which is present in the milk serum of fresh, uncooled milk, is associated with the IM when milk is cooled (98).

Zittle, et al. (112) demonstrated that 60% of the alkaline phosphatase and xanthine oxidase activities in milk were in skimmilk. Although they showed that alkaline phosphatase was associated to a greater degree with the casein particles in milk than was xanthine oxidase, they believed that cream washing procedures and subsequent fractionation techniques dislodged considerable amounts of lipoproteins containing both enzymes from IM. Robert and Polonovski (83) studied the effects of various treatments on the activity of xanthine oxidase in milk. They postulated from their results that the cooling of milk dislodged "liposomes" from the fat globule surface with subsequent dissociation of a portion of the xanthine oxidase from the "liposomes" producing a soluble enzyme in the milk serum.

Kiermeier and Vogt (48) showed that when milk was separated most of the xanthine oxidase activity was concentrated in the cream. Rennet coagulation of milk yielded whey and curd containing equal amounts of the enzyme. Later Kiermeier and Meinl (47) indicated the presence of alkaline phosphatase in the IM and the presence of an acid phosphatase in the milk serum. Stage of lactation influenced the concentrations of both enzymes in milk. Bredholt, Hansson and Sjöström (15) reported on the chemical and physical properties of acid phosphatase. They also demonstrated that creaming, and acid or rennet coagulation of the milk concentrated acid phosphatase activ-

ity in the cream, whey and precipitate, respectively. Acid phosphatase was reported to be associated with the IM as well as with the skimmilk by Bingham, Jasewicz and Zittle (9). Cream washed only twice with water or sodium chloride and sucrose solutions retained only 50% of the initial enzyme activity. They concluded that the enzyme was easily dissociated from the IM.

Herald and Brunner (37) showed that the alkaline phosphatase and xanthine oxidase activities were concentrated in the soluble and insoluble IP fractions, respectively. Sasaki and Koyama (89) sedimented the interphase lipoproteins from buttermilk by ultracentrifugation. The alkaline phosphatase activity was 30 times greater in the resulting pellet than in the supernatant. Ethanol-ether extraction of the lipoprotein pellet resulted in a 20% loss in enzyme activity.

Lipoproteins separated from cream contained two or three fractions, each showing alkaline phosphatase and xanthine oxidase activity (90). Alexander and Lusena (2) fractionated IM into five sedimentable fractions representing 75% of the total and 25% of one soluble fraction. The sedimented fractions varied in their xanthine oxidase and alkaline phosphatase activities.

A series of investigations was conducted by Koyama in which he reported on the effect of divalent ions and heat (61) and the effect of pH (60) on the alkaline phosphatase activity of soluble and sedimented insoluble lipoprotein fractions from IM. He also demonstrated that the soluble fraction was more soluble than the insoluble lipoprotein fraction in 0.08 M NaCl (56). Only 13% of the original alkaline phosphatase activity remained in cream washed four times (54).

Aurand and Woods (6) concluded that development of spontaneous oxidized flavor in milk depended on the presence of a high level of xanthine oxidase alone; milk lactoperoxidase or catalase alone or combined with xanthine oxidase has no effect. Smith and Dunkley (92) observed no correlation between xanthine oxidase activity and the development of spontaneous oxidized flavor in milk.

Trace metals and metal containing proteins The presence of natural, and the fate of added copper and iron in milk were determined by King, et al. (51) by injecting isotopes of the two metals intraveneously, prior to milking, and adding the isotopes directly to the milk. Following isolation of IM by the method of Herald and Brunner (37) and separation of the casein by acid precipitation or ultracentrifugation from skimmilk, it was found that less than 3% of the added copper and 35% of the natural copper was associated with the IM. Natural copper in the skimmilk was divided between the whey and casein fractions of skimmilk in proportion to their total nitrogen contents; 75% of the added copper was associated with IM and that present in the skimmilk preferentially associated with the whey proteins; all of the added iron was present in the skimmilk. Additions of EDTA (50) chelated 37% and 50% of the natural copper and 50% and 100% of the added copper in skimmilk and IM, respectively.

Menger and Mulder (66) had also shown that the natural copper in milk was associated with IM. The copper content in milk was highest (> 200 mg/liter) early in lactation and diminished to normal levels (20 - 40 mg/liter) during the first two months.

Herald, Brunner and Bass (39) analyzed the soluble and insoluble IP

fractions spectrographically; these were prepared by the method of Herald and Brunner (37). Calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, and zinc were found in both fractions. The soluble fraction contained more calcium, copper, magnesium and phosphorus whereas the insoluble fraction contained more iron and molybdenum. Total ash contents of the soluble and insoluble fractions were 7.06 and 2.08%, respectively.

Ramachandran and Whitney (80) analyzed the soluble IP fraction spectrographically for trace metals. They reported traces of cobalt and silicon, significant amounts of sodium, in addition to the trace metals reported by Herald, Brunner and Bass (39). Ramachandran and Whitney (80) did not detect molybdenum and reported only trace amounts of metals except for copper which was 10 times higher than the value reported by Herald, Brunner and Bass (39).

Xanthine oxidase from milk was found to contain iron and molybdenum in an atomic ratio of 8:1 (16). Other metal-containing enzymes, iron in catalase and lactoperoxidase, zinc in carbonic anhydrase and magnesium in inorganic pyrophosphatases, have been isolated from milk. Their association with IM was not confirmed (45). Trubowitz, et al. (106) isolated an alkaline phosphatase from human leucocytes and found that the zinc content increased as the enzyme was purified. Metal cofactors of milk acid or alkaline phosphatases have not been reported (45).

The presence of copper in IM has been reported to catalyze the oxidation of interphase phospholipids under certain environmental conditions (98).

Although enzymes have been the only metal-containing proteins shown to be present in IM, it was interesting to note the isolation of an iron-containing glycoprotein by treating casein with acid (34). Johanson (46) also

reported on the properties of an iron-containing protein "salted out" of milk serum with ammonium sulfate. Derechin and Johnson (27) isolated two red protein fractions during the chromatographic separation of a whey albumin fraction on DEAE cellulose columns. The two fractions were closely related to the red protein of Groves (34).

Blanc and Isliker (13) isolated an iron-containing glycoprotein which they called "lactotransferrin" from fat-free milk serum by ultracentrifugation and ammonium sulfate fractionation of the resulting supernatant. They found it was associated with isolated β- and γ-globulin fractions. They present an excellent summary of the chemical and physical properties of iron-containing proteins isolated from milk by several investigators.

Dills and Nelson (30) reported on the characteristics of a copper-bearing protein isolated from whole milk by ammonium sulfate fractionation. The protein contained 0.19% of nondializable copper and 15% nitrogen. After purification it produced a deep red color in solution.

Preparation of Model Emulsions in an Attempt to Simulate the Fat Globule Milk Serum Interphase

The behavior of fat globules under acid conditions is largely influenced by the types of IM present. Bird, Breazeale and Bartle (10) reviewed the work of early investigators who measured the isoelectric pH of natural fat globules in milk or cream as well as the isoelectric point of butterfat in artificial emulsions. The isoelectric point of natural fat globules in milk ranged from pH 4.1 to 4.3. Reported isoelectric points of artificial emulsions were pH 3.2 in butteroil-water emulsions; pH 4.7 in butteroil-3% casein sol emulsions; pH 4.6 in butteroil-casein sol emulsions

to which phospholipids, lactose and milk salts were added; pH 4.3 in butteroil-phospholipid sol emulsions to which casein, lactose and milk salts were added and pH 2.0 for butteroil-phospholipid sols.

Palmer and Weise (76) "supercentrifuged" the proteinaceous material from buttermilk-butterserum from cream washed six to eight times. In addition to containing lipid phosphorus, the material began to precipitate at pH 4.6 and a clear serum separated at pH 3.9 - 4.0. They concluded that the final isoelectric point, pH 3.9 - 4.0, of the material was due to the influence of the components in a phospholipid-protein complex. Since this value was lower than the reported isoelectric point, pH 4.1 - 4.5, of natural fat globules, they concluded that materials commonly found in the milk serum influence the final isoelectric point of natural fat globules. This conclusion would be in agreement with the isoelectric pH values obtained on the artificial emulsions of butteroil in milk serum constituents.

More recently King (49) pointed out in his review that the isoelectric point of natural fat globules in unwashed cream was pH 4.5 which was close to the isoelectric point of casein, pH 4.6 - 4.7. Following repeated washing the isoelectric point dropped to 3.7 - 3.8 suggesting that materials influencing the isoelectric point of the natural fat globules had been removed.

Payens (78) studied the interaction of mixed monolayers of β-lactoglobulin with milk phosphatides at different pH values (1.0, 3.9 and 5.9) by determining the surface area pressure curves. The surface pressure: area ratios were calculated for ideal mixtures in which the two components do not interact. At pH 1.0 and 5.7 the measured and calculated curves coincided, indicating no interaction, but at 3.9 the measured curve lay

below the calculated one, indicating strong interaction between the components. β-lactoglobulin is isoelectric at pH 5.1, so at 3.9 it is positive. At this pH lecithin and sphingomyelin are ionized and carry no net charge, while cephalin maintains a negative charge to pH 2.0; so interaction between cephalin and β-lactoglobulin could occur between pH 2.0 and 5.1. He considers that the isoelectric point of IM results from the presence of phosphatides and proteins. If protein-phospholipid interaction is strongest at pH 3.8, it may explain the high susceptibility of phosphatides to oxidize at this pH. The strong interaction could explain the long churning times at pH 3.8.

More recently Jackson and Pallansch (42) compared the effects of α-, β- and γ-casein, from isoelectric casein, with those of a "monodispersed" casein preparation, as regards their reduction of the interfacial tension of a butteroil protein-free "milk plasma" and a butteroil-water interphase. The α-, β- and γ-caseins behaved alike but differently from the "monodispersed" preparation in the milk-plasma system and all behaved differently in the water system. When skimmilk was centrifuged to remove calcium-caseinates, their progressive removal had little effect on the interfacial tension. Preparations of serum proteins showed more variations. Globulins were more effective in the milk-plasma-butteroil interface, whereas the albumins were most effective in the water-butteroil interface. The ability of all these milk proteins was negligible in comparison with that of a soluble protein fraction (37) from IM.

Sasaki and Koyama (89) prepared emulsions of butteroil in prepared Ca^{45} caseinate solutions in the absence and presence of added lecithin and calcium phosphate. Similar emulsions were prepared using acid whey from

skimmilk. Measurement of radioactivity in the prepared washed creams indicated casein was removed from the interphase after four washes. Paper electrophoresis of the whey proteins isolated from the washed creams indicated that β-lactoglobulin was removed by the end of two washes. Other whey proteins were more representative of the real IP; casein constituted the outermost protein layer at the interphase.

Recently Koyama (55) demonstrated that the emulsifying powers of sodium caseinate and lactalbumin were the same in the presence and absence of lecithin and highest in the absence of lecithin, respectively. Lactoglobulin was effective only when the lecithin was dissolved in the butterfat prior to emulsification. He concluded that lecithin functioned only to aid the adsorbtion of lactoglobulin on the surface of the fat globule.

Further studies by Koyama (57, 58, 59) on an artificial lipoprotein prepared by emulsifying butterfat in whey in the presence of lecithin showed it had a nitrogen content similar to isolated interphase lipoprotein but less than that of lactalbumin and lactoglobulin. This artificial lipoprotein was sedimented at 19,000g. Removal of the lipid yielded a protein fraction which was hardly soluble in dilute sodium chloride. Phosphatase remained in the insoluble protein fraction, whereas whey phosphatase was soluble in dilute sodium chloride. He concluded that lactoglobulin served as the major protein fraction in interfacial lipoprotein complexes.

EXPERIMENTAL

Materials

Milk

Raw milk was obtained from the University dairy farm. Samples were taken from the bulk tank after 80 to 100 cows, including Holstein, Guernsey, Jersey and Brown Swiss breeds, had been milked. The milk was never more than 4 hr old when washing procedures were started.

Water

Distilled water Tap water softened by ion exchange treatment was distilled in a Barnstead, hard-water model, laboratory still and stored in Pyrex, 18-liter carboys. For use in the cream separator-washing procedure distilled water was stored in stainless steel 5 and 10-gal cans until a sufficient quantity had been accumulated. It was brought to a boil in the cans and boiled for 1 min by means of a stainless steel coil, through which steam was passed, for the standardized runs. Distilled water was boiled for 5 min in 4-liter Erlynmeyer flasks, cooled to 4.4 C and stored in 18-liter carboys in the walk-in cooler; after about three days the chilled water was used in the washing procedures in which the Size 2 International centrifuges were used.

Redistilled water Redistilled water was prepared according to the procedure described by Bird, et al. (12).

Reagents

All reagents used were reagent grade. Aqueous solutions were prepared

using redistilled water.

Technical grade sodium hydroxide was used in the nitrogen determination and in the 50% solutions employed in the traps in the wet ashing procedure.

The 0.01% dithiozone in carbon tetrachloride used in the determination of zinc had a molar absorbancy index of 1.57 which conformed to the A.C.S. specifications (3).

Solvents

Acetone Acetone was purified by refluxing over ferric chloride (0.5 g/liter) for 2 - 3 hr, distilling, refluxing over calcium chloride (60 g/liter) for 3 hr, and distilling; the first and last 200-ml portions of a 5-liter batch were discarded.

Carbon tetrachloride Reagent grade carbon tetrachloride was used. It was evaluated for spectral purity in accordance with A.C.S. specifications (3) and was found to be satisfactory.

95% Ethanol Commercial ethyl alcohol was refluxed over potassium hydroxide (10 g/liter) and aluminum (10 g/liter) for 2 hr and distilled.

Petroleum ether Commercial Skelly B was refluxed over potassium carbonate (25 g/liter) for 2 hr and distilled; the first and last 200-ml portions from a 5-liter batch were discarded.

Redistilled nitric acid and ammonium hydroxide

Nitric acid Two liters of reagent grade nitric acid were distilled from an all-glass system. A constant boiling fraction (119 ± 0.5 C) was collected; the first and last 100-ml portions were discarded.

Ammonium hydroxide Fifteen hundred ml of reagent grade ammonium hydroxide was distilled from all-glass distillation apparatus. The gas evolved was collected in an initial charge of 500 ml of redistilled water chilled in an ice plus water bath. Distillation was continued until the final volume in the receiver was 1 liter. The final concentration was 22.78% ammonium hydroxide. For use in trace metal procedures, redistilled ammonium hydroxide was diluted 1:1 (v:v) with redistilled water.

Buffers for electrophoresis and solubility studies

Sodium hydroxide-glycine-sodium chloride (pH 9.2 and 12.2), disodium phosphate-citric acid (pH 2.6) and potassium chloride hydrochloric acid (pH 1.8) buffers were prepared according to Clark (21).

The veronal (pH 8.6) and phosphate (pH 7.5) buffers were described elsewhere (78).

Apparatus

Gravity washing of cream

Design of water columns A piece of Pyrex glass tubing, 4.5 (O.D.) x 124 cm, was sealed off at one end and fitted with a short perpendicular side arm sealed with a self-sealing rubber stopper. Eight cm from the open end of the tube a right-angle side arm was attached which paralleled the glass tube. This side arm extended into a 50-ml graduate cylinder which

served as a reservoir to collect washed cream. A rubber stopper was used to attach the graduated cylinder to the side arm. A cotton plug was inserted in the open end of the glass column.

Design of Erlynmeyer flasks Three-liter Erlynmeyer flasks were fitted with 1.5 cm (O.D.) pieces of glass tubing inserted through a stopper. The lower end of the glass tubing was 0.5 cm from the bottom of the flask. The diameter of the top of the glass tubing was increased to 3 cm (O.D.) to allow for easier entrance of the sample.

Cream separator

A De Laval farm cream separator, Model 100 AE, was purchased (Montgomery Ward, Chicago, Ill.) for the study of the separator washing technique. The entire assembly contacting the milk was made of tinned metal except for the bowl discs which were stainless steel.

No cream screw settings were available with this machine. As a result a "zero point" was established. The cream screw was lined up flush with the inside of the cream divider disc; adjustments in or out were made from this starting point.

Centrifugation

International Size 2 centrifuges Room temperature (Size 2) and refrigerated (model PR-2) International centrifuges (International Equipment Company, Boston, Mass.) were used to establish a method of centrifugally washing cream. The latter machine was equipped with an automatic timer and refrigeration system. Selected temperatures were controlled to $\pm$ 1 C automatically. The head and cups which were purchased could be oper-

ated at a maximum of 2300 rev/min, and were large enough for 600 or 1000-ml bottles. One-liter polyethylene reagent bottles fitted the cups satisfactorily; the threaded neck was cut off so that the bottles cleared the yoke of the head during centrifugation. Rheostat speed control on the refrigerated model was calibrated using the large head, cups and water-filled 1-liter bottles.

Ultracentrifugation A Spinco model L ultracentrifuge (Spinco Division, Beekman Instruments, Inc., Belmont, Calif.) equipped with a No. 21 head was used to concentrate IM for buttermilk and/or butterserum. Plastic tubes fitted with aluminum caps and holding approximately 91 g of liquid were used.

Churning

Washed and unwashed creams were churned in quart glass Mason jars, with glass lids and rubber gaskets, placed horizontally on a home-made, motor-driven, reciprocal action shaker with 75 - 80 oscillations per min.

Pervaporation

Pervaporation was conducted at room temperature by filling cellulose casing, size 36/32, (The Visking Corp., Chicago, Ill.) to a depth of about 3 ft with the buttermilk-butterserum sample. The casings were suspended from a framework of 0.5-in steel rods in front of a 16-in fan. The lower ends of the filled casings were anchored to a steel rod to prevent movement by the strong air currents.

Freeze drying

Freeze drying apparatus was constructed from glass; it had two reservoirs, each fitted with four 24/40 inner standard taper joints. The reservoirs were set into two 4-liter Dewer flasks containing a dry ice-acetone mixture. Both reservoirs were connected to a Welch Duo Seal vacuum pump (Welch Manufacturing Co., Chicago, Ill.) via a condensate trap, through glass tubing fitted with two stopcocks which permitted individual or simultaneous operation of the two units. Round bottom 24/40 ST flasks (the size depended upon the amount of sample) containing the samples were attached to the reservoir inlets after coating the joints with Apiezon N grease. A residual pressure of 0.07 mm was achieved under actual operating conditions. Approximately 500 ml of water could be sublimed per reservoir.

Electrophoresis

The free electrophoresis apparatus employed was model 38A Tiselius unit (Perkin Elmer Corp., Norwalk, Conn.) equipped with a Polaroid Land camera for photographing patterns. Two-ml closed Tiselius cells were used for all the runs. Dialysis was carried out in 1-liter dialysis units (American Instrument Co., Silver Springs, Md.) fitted with a stirring motor and stirrer and an attachment for holding the cellulose casing. The sample in the casing was dialyzed against three changes of buffer during a 24-hr period. The sample and buffer outside the casing were stirred (magnetically) throughout dialysis.

pH measurements

pH measurements were made with a portable Beckman glass electrode pH meter, model G (Beckman Instruments, Inc., Pasadena, Calif.).

Spectrophotometry

Absorption spectra and optical density readings were made in Pyrex cells using a Beckman spectrophotometer, model DU (Beckman Instruments, Inc., Pasadena, Calif.). Most wave lengths used in trace metal procedures were in the visible region (436 - 820 mμ), and required no filters. A red filter, Corning 9863, was used in the near ultra-violet region, 320 - 400 mμ, when the absorption spectra of metal chelates were determined. Reflectance measurements were made on a similar Beckman spectrophotometer, model DU, equipped with a reflectance attachment containing solid calcium oxide as a reference standard.

Wet ashing

Two 6-in hot plates were placed under the hood in such a manner that their heights could be adjusted. The hot plates were connected to variacs which were calibrated using thermometers set in mercury wells on top of the hot plates; with the hot plates set at "medium," 64 - 68 volts and 85 - 89 volts produced temperatures of 175 ± 5 C and 235 ± 5 C, respectively.

Fume heads placed above the hot plates were connected to 500-ml suction flasks containing 150 - 200 ml of a 50% sodium hydroxide solution. The flasks were connected to an aspirator with Tygon tubing. The aspirator was tapped with a second nipple thus permitting direct connection of both suction flasks. Bubbling acid fumes through the caustic solution prevented corrosion of the aspirator. Glassware and thermometers were supported on a 0.5-in aluminum rod. Diehl and Smith (29) present a picture of the apparatus on page 369.

Excess perchloric acid was rapidly evaporated, whenever desired, by

fitting a separate pair of fume heads with a piece of glass tubing (0.5 cm O.D.) extending through the fume head into the Vycor flask. The space between the fume head and the neck of the flask was packed with Pyrex wool filtering fiber. The current of air drawn through the glass tube quickly evaporated the excess acid. For the sample sizes used in this study 125-ml Vycor Erlynmeyer flasks were satisfactory.

Separatory funnels

One hundred and twenty-five-ml Squibb, centrifuge separatory funnels equipped with Teflon stopcocks were used in trace metal analyses. The conventional top opening was replaced with a fitted g.s., No. 13 stopper to avoid the use of rubber stoppers during organic solvent extraction of metal chelates.

Methods

Preparation of washed creams

Gravity washing of cream using water columns Boiling distilled water was poured into the column supported on a ring stand. The water level was adjusted to accommodate the cream sample without causing an overflow of water into the graduated cylinder attached to the upper side arm. After inserting the cotton plug on top, the filled column was stored in the 4.4 C walk-in cooler for 24 hr prior to use.

Raw milk was placed in 3-liter Erlynmeyer flasks previously sterilized with boiled distilled water. After 18 hr the resulting skimmilk was siphoned off and the cream was transferred to a sterilized 400-ml beaker.

Fifty or 100 ml of cream were layered onto the bottom of the water

column using a 50-ml syringe equipped with a large diameter, pointed needle. The self-sealing rubber stoppers survived many runs. Washed cream samples were removed from the top of the water column after 24 hr; a sufficient amount of 4.4 C distilled water was injected through the self-sealing stopper causing the cream layer to overflow through the side arm into the graduated cylinder.

Gravity washing of cream using Erlynmeyer flasks A series of four 3-liter Erlynmeyer flasks were each filled with 2 liters of distilled water. The water was brought to a boil, an inverted beaker was placed on top and the Erlynmeyers were stored for 48 hr at 4.4 C. Approximately 1100 ml of gravity-separated, raw cream were prepared as described under the previous section. One liter of the cream was carefully layered under the 2 liters of distilled water in an Erlynmeyer flask. After 24 hr at 4.4 C the washed "cream" was recovered, after removing the "skimmilk" through a glass siphon. The washed cream then was layered under the water a second time, and the washing procedure repeated until the cream had been washed the desired number of times.

Centrifugal washing of creams using the International centrifuges One thousand grams of raw milk were weighed into each of four polyethylene bottles. The bottles were centrifuged at 450 or 2250 rev/min for 90 min in the room temperature or refrigerated centrifuge, respectively. The resulting skimmilk was removed through a glass siphon introduced through a hole punctured in the cream layer. The glass tubing was bent allowing it to touch the perimeter of the bottle bottom. The glass tubing was held stationary by a rubber stopper containing a vent hole. The bottle was

placed in front of a light source during the siphoning procedure to help determine when the last traces of skimmilk were removed. A sample of well-mixed skimmilk was retained for later analysis.

To the cream remaining in the bottle, 10 ml of 4.4 C distilled water were added and the bottle was gently rotated. Lumps of cream which remained were dispersed gently with the aid of a glass rod. Three additional 10-ml aliquots of water were added with rotation of the bottle between additions. Lumps of cream clinging to the walls of the bottle were dispersed with the aid of a glass rod. The dispersed cream was made to the original milk volume with 4.4 C water. The bottles were placed in an ice plus water bath (about 1.1 C) in the walk-in cooler for 30 - 40 min prior to centrifugation. For each wash this procedure was repeated. When the final wash was completed, the creams (about 60 g/bottle) were redispersed using three 10-ml aliquots of distilled water so that each bottle contained about 90 g of cream. The washed creams were combined into one bottle; each bottle was rinsed with 10 ml of distilled water which was added to the washed cream. The combined washed cream sample was weighed. This procedure produced approximately 360 g of cream containing from 34 - 40% fat.

In order to prepare a sufficient quantity of cream washed as many as six times, 4000 g of raw milk were used for each wash. Washes were paired to make effecient use of time. The paired washes were rotated so that one series of washed cream samples was being prepared for centrifugation while the other series was in the centrifuge. Forty-eight hr were required to prepare samples of cream washed zero to six times.

Separator washing of creams The raw milk was preheated to 37.7 C in 5 or 10-gal cans with hot tap water in the sink. The cream screw on the separator was given one full turn in. After allowing one min for the bowl to reach maximum speed, 3 gal of distilled water at 43.0 C were used to preheat the bowl. In subsequent washes a 5.6 C differential between the cream separation and the distilled water preheat temperatures was used in each wash, thus permitting separation of the cream at the desired temperature. Cream was collected in 3-liter beakers and weighed. Skimmilk was collected in stainless steel cans and discarded after it was mixed and a sample was taken. The cream was redispersed in distilled water which was two degrees higher than the desired separation temperature. Agitation and foaming were minimized by allowing the cream to run down the walls of the milk can. Gentle hand stirring followed to completely redisperse the cream in the water; the volume was made to the original volume of the milk. Prior to each redilution procedure, the separator bowl had been dismantled and the sludge removed from the bowl. Following assembly of the bowl after the first wash, the cream screw was set at two full turns in. Prewarming the bowl with distilled water at 43.0 C was followed by reseparation of the redispersed cream. The redilution and reseparation procedure was repeated up to six times. If more than three washes were used the temperatures of separation and preheating were lowered to 34.9 and 40.5 C, respectively, for subsequent washes in order to prevent serious oiling off; other conditions remained unchanged.

Although no sludge accumulated in the bowl beyond the first wash, the bowl was dismantled and rinsed after each separation because clumps of fat occasionally plugged the cream screw and cream cover spout. Distilled water

used for redispersing washed cream was heated to the desired temperature in 5 or 10-gal cans using stainless steel coils connected to the steam line in the laboratory.

Separation of interphase materials from washed cream

Washed and unwashed cream samples were tempered for 12 hr at 4.4 C. From 350 - 450 g of cream were placed in glass quart containers and churned. Churning times were noted. Buttermilk was poured from the churns through cheese cloth to remove butter granules. The butter was retained in the glass container which was placed in a 40 C water bath for 30 - 45 min. Most of the resulting butteroil was poured off. The remaining butterserum-butteroil mixture was centrifuged at 700 rev/min for 10 min in 125-ml separatory funnels. The butterserum layer was removed and combined with the buttermilk when desired.

Concentration of interphase materials

Acid precipitation The pH of the buttermilk-butterserum combination was adjusted to 4.6 - 4.7 with 0.01 N hydrochloric acid during constant agitation. The acidified samples were allowed to stand overnight at 4.4 C to allow the precipitate to settle completely. A sample of the whey was removed and the remainder was siphoned off and discarded. The precipitate slurry was transferred to a round bottom flask and freeze dried.

Pervaporation Cellulose casing was filled with the combined buttermilk-butterserum to a height of about 3 ft. The casings were suspended in front of a fan at room temperature. The final volume was determined by the number of centrifuge tubes available for handling the concentrated

solution. The casings were inverted several times when evaporation was completed to mix the sample which was then collected in a tared beaker and weighed.

Ultracentrifugation followed by freeze drying Either the pervaporated concentrates of combined buttermilk-butterserum or the buttermilk directly from the churn were placed in centrifuge tubes using a 50-ml syringe. Each tube held 91 g of sample. The filled tubes along with the No. 21 head were chilled for 60 - 90 min in an ice plus water bath at approximately 1.1 C. The samples were ultracentrifuged at 20,000 rev/min for 20 hr. When the centrifuge had stopped, the tubes were removed immediately and the caps taken off. The whey and fat plug were poured off and traces of fat were wiped from the walls with facial tissue.

Redistilled water was added in small portions to the pellet in the centrifuge tube. Between additions the pellet was rubbed gently with a blunt stirring rod to facilitate redispersion of the pellet. The redispersed pellet was then freeze dried in round bottom flasks. Redispersed samples were continuously rotated in a dry ice-acetone bath (-70 C) until the sample was frozen. It was necessary to have only a thin film on the bottom of the flask to prevent the water vapor from the bottom from carrying loose, dried materials that settled, into the head of the water collector. The samples were considered freeze dried when the flasks were at room temperature.

Separation of lipid and non-lipid fractions

Soxhlet extraction Freeze dried buttermilk-butterserum samples were weighed directly into tared extraction thimbles. The thimbles were

fitted with a cotton plug and placed in the extraction chamber of an all-glass Soxhlet apparatus. The extraction chamber was attached to a water-cooled condenser, fitted with a drying tube containing indicating drierite, and to an amber, 350-ml flask containing 250 ml of ethyl ether. All the standard taper joints were lubricated with glycerine to prevent loss of ether. Heat was supplied by a heating mantle and controlled by a variac. Extraction was complete after 24 hr at which time the thimble was removed, placed in a beaker and dried under vacuum for 3 hr at room temperature. The Soxhlet residue was transferred to a sample bottle which was stored in a vacuum over phosphorus pentoxide.

The Soxhlet extract remaining in the amber flask was evaporated to a volume of about 30 - 50 ml on the Mojonnier hot plate (100 C) and was transferred to a tared Mojonnier fat dish. The flask was rinsed three times with 30 to 50-ml portions of ethyl ether which were transferred to the fat dish and evaporated. Residual solvent was removed in the Mojonnier oven (70 C, 26-in vacuum, 15 min) after which the fat dishes were cooled for 4 hr in a desiccator and weighed. The weight of the Soxhlet residue was calculated by difference from the weight of the initial sample and the Soxhlet residue.

Modified Bloor extraction of Soxhlet residue The extraction of lipids from blood serum by the method of Bloor (14) involves dropwise addition of 50 ml of blood plasma to 750 ml of an alcohol-ether mixture (3:1 v:v). Proteins not precipitated by the ethanol are precipitated by boiling the plasma-solvent mixture. Since our Soxhlet residues were dry and it was desired to save the non-lipid portion, the following modified

procedure was followed. The Soxhlet residue (0.8507 g) was suspended in 50 ml of redistilled water in a 250-ml centrifuge bottle using a stirring bar and magnetic stirrer. After 30 min, 200 ml of a 3:1 ethanol-ethyl ether mixture (added at the rate of 1000 ml per g of sample) was added and stirring continued for an additional 30 min. The samples were centrifuged at 2000 rev/min for 10 min and the resulting solvent supernatant was decanted into an amber 350-ml flask and evaporated under vacuum and a stream of nitrogen at 60 C. The aqueous residue remaining in the centrifuge bottle was treated with four additional solvent extractions, each of which was added to the amber flask and evaporated to dryness. The lipid residue was retained for subsequent fractionation by Bloor's method (14).

The aqueous supernatant was separated from the non-lipid residue in the centrifuge bottle by decantation. The residue was dried under vacuum over phosphorus pentoxide for 48 hr.

Modified Mojonnier extraction of the Soxhlet residue The Mojonnier procedure (5) for the extraction of total lipid from milk and related products calls for the addition of ammonium hydroxide (1.5 ml concentrated ammonium hydroxide/10 ml final aqueous sample) to facilitate peptization of proteinaceous material and subsequent release of lipids. The procedure was modified by substituting desired buffers for ammonium hydroxide to effect peptization of the sample. From 0.12 - 0.15 g of Soxhlet residue was peptized in 7 ml of sodium hydroxide glycine buffer (pH 12.23) in a 15-ml glass mortar with the help of a blunt stirring rod and magnetic stirring.

After stirring for 60 min the peptized sample was transferred to a

Mojonnier-type extraction flask. Three ml of buffer were used to rinse the mortar. A Mojonnier fat determination was run on the combined peptized sample and buffer rinses, which were made to 10 ml. The residual lipid in the Soxhlet residue was calculated as a percentage of the Soxhlet residue.

After stirring for 60 min the peptized sample was transferred to a Mojonnier-type extraction flask. Three ml of buffer were used to rinse the mortar. The combined peptized sample and buffer rinses totaling 10 ml were then treated according to the Mojonnier extraction procedure. The residual lipid in the Soxhlet residue was calculated from the total lipid extracted.

Characterization of the interphase lipids

Separation of ether-soluble and-insoluble fractions from the total Soxhlet extract The Soxhlet extract (in the Mojonnier fat dish) was dissolved in ethyl ether on the Mojonnier hot plate (100 C), transferred to a tared 15-ml centrifuge tube and centrifuged at 2000 rev/min for 10 min. The clear supernatant was poured into a tared Mojonnier fat dish. The insoluble material in the centrifuge tube was washed twice with 15-ml portions of ethyl ether. The resulting supernatants were added to the initial ethyl ether supernatant in the fat dish. Following solvent evaporation on the hot plate and in the vacuum oven as with the Soxhlet extract, the residual, solvent-soluble, lipid fraction was cooled in a metal desiccator and weighed.

The centrifuge tube containing the solvent-insoluble fraction was dried under vacuum for 3 hr and weighed.

Fractionation of the total Bloor extract from the Soxhlet residue

The method of Bloor (14) was used to fractionate the free fatty acids, neutral lipids and phospholipids contained in the total Bloor lipid extract from the Soxhlet residue. Mojonnier-type extraction flasks were employed.

Fractionation of the Soxhlet extract The ether-free Soxhlet extract (in a Mojonnier fat dish) was dissolved in petroleum ether on the Mojonnier hot plate (100 C). The petroleum ether soluble and-insoluble fractions were separated as outlined for the separation of ether soluble and-insoluble fractions from the Soxhlet extract. Petroleum ether was used in place of ethyl ether in an attempt to improve the solubility of the whole Soxhlet extract.

The petroleum ether-soluble, lipid fraction was redissolved in 15 ml of acetone and allowed to stand at room temperature for 60 min. The acetone-insoluble material was separated by centrifugation in a 15-ml centrifuge tube at 2000 rev/min for 25 min. The acetone supernatant was removed and the insoluble material washed twice with 15-ml portions of acetone. The acetone-insoluble fraction was dried under vacuum for 3 hr and weighed. The combined supernatants containing the acetone-soluble, neutral lipids and phospholipids were evaporated to a 10-ml volume and transferred to a 15-ml tared centrifuge tube. After standing for 30 min at 4.4 C, 3 ml of cold, saturated magnesium chloride in ethanol was added. The well-mixed sample was centrifuged at 2000 rev/min for 25 min. The precipitate was washed three times with acetone. The supernatants were combined, evaporated to dryness and the acetone-soluble, neutral lipid fraction was weighed. The precipitated phospholipids were dissolved in 15 ml of ethyl

ether; excess magnesium chloride was separated from the solvent by centrifugation at 2000 rev/min for 25 min. The precipitate was washed twice more with 15-ml portions of ethyl ether which were combined with the initial extract, evaporated to dryness and weighed in a Mojonnier fat dish. The combined weights of the initial acetone-insoluble fraction and the ether-soluble fraction removed from the magnesium chloride precipitate were considered to represent the total phospholipids in the Soxhlet extract.

Characterization of the ether-insoluble fraction from the Soxhlet extract The dried ether-insoluble residue was peptized in 7 ml of sodium hydroxide-glycine buffer (pH 12.23) in a 15-ml mortar using a blunt stirring rod and mechanical stirring on a magnetic stirrer. After 60 min the sample was transferred to a Mojonnier-type extraction flask and the mortar was rinsed with 3 ml of buffer which was added to the flask. Lipid was removed by a Mojonnier extraction. The residual aqueous layer was returned to the mortar and residual solvent was removed by mechanical stirring in the mortar on a magnetic stirrer for 3 hr. The sample volume was measured in a graduated cylinder and a 1-ml aliquot removed for the total nitrogen determination. Another 1-ml aliquot was removed for the Molisch test.

Characterization of the non-lipid interphase materials

Solubility of the non-lipid fraction from interphase materials Soxhlet residues were peptized in a variety of buffers and residual lipids were extracted by the Mojonnier procedure. The method of peptization was described in an earlier section. In addition to using buffers alone, a variety of chemical agents added to the buffers were used in attempts to

solubilize the non-lipid IM. A summary is presented in Table 8.

After vigorous stirring at room temperature to remove traces of Mojonnier solvents, the peptized samples were transferred to test tubes and held at 4.4 C overnight. The clarity of the sample was observed. In some cases centrifugation at 2000 rev/min for 15 min in a 15-ml centrifuge tube was applied and the amount of insoluble material present was compared among buffers.

Electrophoresis of non-lipid interphase materials A sample of Soxhlet residue, which would yield an approximately 1% concentration of non-lipid material in buffer was peptized, the lipid extracted by the Mojonnier method and the residual solvent was evaporated. This material was transferred to a 10-ml, graduated cylinder and the volume was measured. A 1-ml sample was removed for total nitrogen determination. The remaining sample was dialyzed against three changes of buffer for 24 hr at 4.4 C. The final volume of the dialysate was measured and insoluble material was removed by centrifugation at 2000 rev/min for 15 min. A 2-ml aliquot of the supernatant was used for electrophoresis. Subsequent electrophoretic patterns were obtained according to the method described in the Perkin Elmer manual (79).

Preparation of bulk amounts of interphase materials from different washes

Five to 10-gal lots of raw milk were washed from zero to six times using the farm cream separator. The washed creams were churned and the buttermilk weighed and pervaporated. The concentrated buttermilk was weighed and ultracentrifuged at 20,000 rev/min for 20 hr. The resulting

pellets were freeze dried and the yields of dried material determined.

The IM from each wash was used for solubility studies in various buffers. Reflectance measurements before and after Soxhlet extraction were determined. The distribution of ether insoluble and-soluble fractions among the Soxhlet extracts and the Mojonnier-extractable lipid retained by the Soxhlet residues were measured. The nitrogen contents of the Soxhlet residues before and after Soxhlet extraction were determined. The non-lipid residues then were subjected to free electrophoresis and a comparison of the effect of repeated washing was made. Finally the bulk IM was used to evaluate the reliability of trace metal methods in recovery runs and was subsequently analyzed for calcium, copper, iron, magnesium, phosphorus and zinc contents.

Comparative runs: separator vs. centrifugal washing techniques

Washed creams (zero to six washes), prepared with the cream separator and the refrigerated centrifuge, were compared. In each case, the two series were prepared from the same large lot of raw milk. The washed creams were churned and aliquots of the buttermilk were ultracentrifuged at 20,000 rev/min for 20 hr. Color photographs of the resulting precipitates were taken. The total nitrogen, "non-casein" nitrogen and total lipid distribution among the washed and unwashed creams, skimmilks and buttermilks was determined.

The comparison was repeated. In the second comparison the washed and unwashed creams were standardized to 30% "fat," using redistilled water, prior to churning. Churning times were recorded for each sample. The total nitrogen distribution among the washed and unwashed buttermilks and the

supernatants resulting from ultracentrifugation of these buttermilks was determined. The total nitrogen contained in the pellet was calculated by difference. The freeze dried pellets were wet ashed and the calcium, copper, iron, magnesium, phosphorus and zinc contents were determined.

Qualitative tests

The Molisch, Buiret and Fehlings tests were used according to the procedures described by Anderson (4).

Reflectance measurements

The sediment pad on a milk sediment assay card was replaced with a piece of cellophane similar to the cover. A sufficient amount of dried sample was placed on the cellophane and the cover was folded down and glued. The sample between the two cellophane surfaces was spread out, completely covering the cellophane surface. The assay card was trimmed to a size which would fit the reflectance attachment of the Beckman Model DU spectrophotometer. Reflectance readings (% transmittancy read on the machine) were taken from 310 - 1000 mμ.

Photography

Colored pictures of the tubes containing the washed and unwashed buttermilk precipitates obtained by ultracentrifugation were taken with a 35 mm camera, by the author, for the first comparative run and by the experiment station photographer for the second one. The tubes were removed from the centrifuge head immediately after it had stopped. They were placed between aluminum bars supported by wooden blocks on a shelf in the 4.4 C walk-in cooler. Moisture on the outside of the tubes was wiped off and

pictures were taken of front and side views of the precipitates in the tubes.

Standardization of cuvettes

The method of Van Devender, Lyon and Bird (107) was used to standardize the Pyrex cuvettes. A 0.95% solution of chromic chloride in ethanol and an aqueous solution of potassium permanganate (0.025 g/liter) were used to standardize the cuvettes at 436 mμ, and at 535 and 538 mμ, respectively.

Cleaning of glassware

All glassware was cleaned by soaking in a hot sulfuric-nitric acid bath followed by rinsing with a sufficient amount of distilled water to remove traces of acid. Glassware, used in trace metal analyses, was given an additional soaking in dilute hydrochloric acid (1:1 v:v with redistilled water) for 4 hr to remove traces of metals, then rinsed with a sufficient amount of redistilled water to remove traces of acid.

Cuvettes were rinsed thoroughly with the solvent in use followed by ethanol and then soaked in a mild detergent solution overnight. After rinsing with distilled water the cuvettes were allowed to dry before using again.

Wet ashing procedure

Dry samples of the IM were weighed into 125-ml Vycor flasks. Three ml of redistilled water were added followed by 3 ml of redistilled nitric and 4 ml of 70% perchloric acid. The acids were added in this ratio to samples ranging in size from 0.05 - 0.5 g. The flasks were placed on the hot plates under the fume heads and heated to 175 $\pm$ 5 C. After 1 hr the

variacs were adjusted to produce a temperature of 235 ± 5 C. Digestion was continued for an additional 1.5 hr. At this point heavy white fumes were evolved and the digest was clear. Sometimes the digest had a greenish color which disappeared when the flasks were cooled. When the flasks had cooled, 20 ml of redistilled water were added and the flasks were returned to the hot plate for an additional 25 min. The dissolved ash was transferred to a 50-ml volumetric flask while still hot. The Vycor flask was rinsed three times with 10-ml portions of redistilled water. These rinses were added to the volumetric flask. After rinsing the funnel and walls of the volumetric flask, the wet ash digest was cooled overnight and made to volume. The samples were mixed well before aliquots were removed for analysis.

A 10-ml aliquot was pipetted into a 125-ml Vycor Erlenmeyer flask. The flask was placed under the fume head fitted with a piece of glass tubing extending into the flask. The open space between the head and the neck of the flask was packed with Pyrex wool filtering fiber. The variacs were set to produce a temperature of 235 ± 5 C. After 2 hr the ash was completely dry. Twenty ml of 10% (27 ml cond. HCl/100 ml redistilled water) hydrochloric acid was added to the cooled flask. The glass tube was allowed to remain in the fume head, and the flask was returned to the hot plate. After 25 min the dissolved ash was transferred to a 125-ml separatory funnel. The flask was rinsed three times with enough redistilled water to increase the volume of the ash sample to 20 ml. The final ash solution in the funnel was used in the copper and zinc determinations. Phosphorus, iron, calcium and magnesium analyses were made using 1, 10, 10 and 10-ml aliquots, respectively, of the original wet ash digest, *per se*.

Analytical methods

Total lipid The official Mojonnier procedure (5) was followed for the determination of the total lipid contents of milk, washed and unwashed cream, skimmilk and buttermilk samples. One-g samples were used for unwashed and one to three times, washed creams, and 2-g samples for creams washed four to six times. Ten-g samples of unwashed and washed skimmilks and buttermilks were used.

The Mojonnier extraction procedure, exclusive of the ammonium hydroxide addition, was used to extract the lipids from freeze dried, Soxhlet-extracted IM, peptized in buffers and determine them quantitatively.

The official Babcock test (5) was used to determine the lipid content of one series of washed and unwashed creams before and after standardization to a specific lipid content.

Total and "non-casein" nitrogen The separation procedures for the non-casein nitrogen were carried out according to the method of Rowland (85). The distillation procedures and reagents employed were those suggested by Menefee and Overman (65).

The total nitrogen contents of milk and of washed and unwashed cream, skimmilk and buttermilk samples were determined using 300-ml Kjeldahl flasks. Fluid samples were weighed out in Mojonnier 1, 2, 5 and 10-g pipettes after thorough mixing. Washed skimmilk and buttermilk samples beyond the fourth wash contained small amounts of protein; 50 - 200-g samples were weighed out directly into 800-ml Kjeldahl flasks using a large Torsion balance. A summary of sample sizes used for all samples is presented in Table 1.

Table 1. Sample sizes used in total nitrogen (TN) and "non-casein" nitrogen (NCN) determinations on unwashed and washed cream, skimmilk and buttermilk samples

Wash[a]	Cream		Skimmilk		Buttermilk	
	TN (g)	NCN (g)	TN (g)	NCN (g)	TN (g)	NCN (g)
WO	1	10	1[b]	10[b]	1	10
1W	1	20	5	20	2	10
2W	1	20	100	90	5	20
3W	1	20	200	90	10	20
4W	2	30	200	90	10	50
5W	2	30	200	90	10	50
6W	2	30	200	90	10	50

[a]WO was the abbreviation used for the original or unwashed sample; 1W was the first wash, 2W, the second, etc.

[b]Sample sizes used for whole milk.

Samples for the "non-casein" nitrogen determination were weighed into 100-ml volumetric flasks. Filtrate aliquots, digested in duplicate, were 25 ml for milk and unwashed cream, skimmilk and buttermilk samples, and 40 ml for all the washed samples.

Total nitrogen contents of the isolated IM and subsequent non-lipid fractions were determined on weighed, dry material samples or on a 1-ml aliquot of peptized samples; 300-ml Kjeldahl flasks were employed.

Trace metals The method for the analysis of the metals in IM is a combination of the following procedures, modified in some instances; Iron, Collins and Diehl (25); phosphorus, Fontaine (32); copper, Lusas,

Bird and Rosenberger (63); zinc, Sandell (86); and calcium and magnesium, Bird, et al. (12). The composite method employed was as follows:

Samples of 100 mg to 520 mg were wet ashed, as previously described, and made to 50 ml with redistilled water.

Iron Place a 10-ml aliquot of the wet ash digest into a 125-ml Squibb, centrifuge, separatory funnel. Add 8 ml of 10% (w/v) hydroxylamine hydrochloride (HAHC) and allow 60 min for reduction of the iron. Add a piece, 2mm x 2mm, of congo red paper and enough ammonium hydroxide (NH_4OH) (1:2 v:v) until the paper is just pink. Add 3 ml of 0.002 M bathophenanthroline (BPA) and 5 ml of acetate buffer (113.4 ml glacial acetic acid and 164 g anhydrous sodium acetate/liter), pH 4.80, and allow the samples to stand for 30 min to chelate the iron. Add 4 ml of nitrobenzene and shake vigorously for 60 sec. Dislodge droplets of nitrobenzene from the walls of the funnel by swirling the funnel contents and tapping the funnel gently against the funnel rack. Run the solvent into a 10-ml clear, g.s., graduated cylinder. Extract remaining ferrous-bathophenanthroline-perchlorate salts from the aqueous layer remaining in the funnel with subsequent 2.0 and 1.9-ml portions of nitrobenzene. Add the second and third extracts to the cylinder containing the first extract. Transfer droplets of extract, that may be in the funnel tip after each extraction, to the cylinder by gently tapping the funnel tip against the cylinder wall. Add enough nitrobenzene to make the volume in the cylinder to 8 ml. Rinse the lip and inner wall of the cylinder with 2 ml of ethanol while making to volume. Mix well, rinse the cuvette with 3 ml of the sample and read the optical density in 1-cm cuvettes at 538 mμ vs. nitrobenzene-ethanol (8:2

v:v). Carry a 10-ml aliquot of the blank wet ash digest through the above procedure and use it as a reagent blank for correction of the optical densities of the samples.

Iron-free HAHC and acetate buffer were prepared according to the methods described by Collins and Diehl (25). Add 20 ml of 0.002 M BPA to 500 ml of 10% HAHC and extract with 50-ml aliquots of nitrobenzene until the solvent layer is yellow. Add 5 ml of 0.002 M BPA to the aqueous layer and extract any traces of red color with additional 20-ml aliquots of nitrobenzene until the solvent layer is yellow. Centrifuge the remaining aqueous layer in 250-ml centrifuge bottles for 15 min at 2000 rev/min. Decant the clear supernatant into a glass-stoppered bottle. Add 10 ml of 10% HAHC and 20 ml of 0.002 M BPA to each 500 ml of acetate buffer. Extract the iron with nitrobenzene and centrifuge as described above. The final solution will have yellow color.

A standard iron solution containing 0.99995 μg iron/ml was prepared by the method of Collins and Diehl (25). Analyses were made on aliquots containing from 1 - 25 μg of the standard. Ten ml of 10% (w/v) ammonium perchlorate were added to each aliquot prior to following the procedure as described above.

Phosphorus Place a 1-ml aliquot of the wet ash digest in a 25-ml, g.s., volumetric flask. Add 5 ml of 10 N sulfuric acid (300 ml concentrated sulfuric acid plus 671 ml of redistilled water). Add 2.5 ml of 7.5% (w/v) sodium molybdate followed by enough redistilled water to made the volume to 20 - 22 ml. Mix the sample well after each addition of reagents and water. Add 2.5 ml of a fresh stannous chloride solution (1 ml

of a concentrated stannous chloride solution, 10 g/25 ml of concentrated hydrochloric acid, diluted to 200 ml; use a concentrated stannous chloride solution which is not more than four weeks old) and mix well. Place the sample in a boiling water bath for 20 min to develop the blue color. Cool in a tap water bath for 30 min and make the samples to volume after holding an additional 60 min at room temperature. Mix well and use 6 ml of sample to rinse the cuvettes. Read the optical density in 1-cm cuvettes at 820 mμ vs. a reagent blank consisting of a 1-ml aliquot of the wet digest of the redistilled water sample carried through the above procedure.

A standard phosphorus solution containing 0.01 mg of phosphorus/ml was prepared according to the method of Fontaine (32). Analyses were made as described above of dilute aliquots containing 0.001 mg of the standard. Good agreement between the transmittancies obtained and the calculated regression equation of Fortney (33) eliminated the need for preparing another standard curve. The regression equation reported by Fortney (33) was used.

Copper Evaporate a 10-ml aliquot of the wet ash digest in a 125-ml Vycor Erlenmeyer flask under the modified fume head. Dissolve the residue in 20 ml of 10% (27 ml conc HCl/100 ml redistilled water) hydrochloric acid and transfer quantitatively to a 125-ml Squibb, centrifuge, separatory funnel. Add 8 ml of 10% (w/v) solution of HAHC, mix and allow to stand 60 min to reduce the copper. Add a 2mm x 2mm piece of Congo red paper and enough NH_4OH (1:2 v/v) until the paper is just pink. Add 3 ml of acetate buffer (36.2 ml glacial acetic acid and 30.35 g anhydrous sodium acetate/liter), pH 4.42, and 8 ml of 1% aqueous 2, 2′-bipyridyl solution (1 g in 100 ml) to chelate the iron. After 30 min add 5 ml of 0.1% aqueous

sodium diethyldithiocarbamate (DEDTC). Allow 5 min for chelation of the copper. Add 5 ml of carbon tetrachloride (CCl_4) and shake vigorously for 30 sec. Dislodge droplets of CCl_4 from the walls of the funnel and the surface of the aqueous layer by swirling the funnel contents and tapping the funnel gently against the funnel rack. Run the solvent layer into a 10-ml, actinic red, g.s., graduated cylinder and add 3 ml additional DEDTC. After 5 min, extract additional copper-DEDTC with 2.5 and 2.0 ml CCl_4. Run the second and third extracts into the cylinder containing the first extract. Save the aqueous solution in the separatory funnel for the zinc analysis. Transfer droplets of extract, that may be in the funnel tip after each extraction, to the cylinder by gently tapping the cylinder wall against the funnel tip. Rinse the lip and inner wall of the cylinder with CCl_4 (about 0.5 ml) while making to volume. Mix well, rinse the cuvette with 3 ml of the sample and read the optical density in 1-cm cuvettes at 436 mμ vs. CCl_4. Carry two 10-ml samples of the wet ash digest through the above procedure and use one as a reagent blank for correction of the optical densities of the samples.

A standard copper solution containing 1.00098 μg copper/ml was prepared by the method of Lusas, Bird and Rosenberger (63). Analyses were made, as described above, of dilutions containing from 1 - 25 ml of the standard. A regression was calculated from the data and used in determining the amounts of copper in the samples analyzed.

Zinc To the aqueous solution in the separatory funnels (samples and blanks), add 10 ml of a 10% sodium citrate buffer [make 10% sodium citrate solution alkaline with NH_4OH and extract with 0.01% dithi-

zone until the solvent layer remains green. Filter through Whatman No. 42 filter paper treated previously with 250 ml of 6N hydrochloric acid and enough redistilled water to remove traces of the acid; Sandell (86 p. 637)]. To one of the blanks add 4 drops 0.1% ethanolic bromthymol blue, and then 1:2 (v:v) NH_4OH until a greenish color develops. Continue addition of NH_4OH until the pH of the blank is 7.85 $\pm$ 0.05, as determined with a pH meter. Record the total ml of NH_4OH employed and add this amount to the second blank and the samples.

Add 5 ml of 0.01% dithizone in CCl_4 and shake vigorously for 30 sec. Run the dithizone extract into a 25-ml, actinic red, g.s., graduated cylinder. Extract twice more with 1-ml portions of dithizone solution and add the extracts to the graduated cylinder. Again be certain that no drops of extract remain in the funnel tip. Make the extracts, containing zinc dithizonate, to volume in the graduated cylinder and mix well. Rinse the cuvettes with 6 ml of this solution and measure the optical density, in 1-cm cuvettes at 535 mμ against the second blank which is carried through the same procedure as the sample.

A standard zinc solution was prepared as follows: Dissolve 0.1257 g zinc oxide A.C.S. specifications (3) in some redistilled water, to which 10 ml concentrated HCl has been added and make to volume. This solution contains 1.0 μg zinc/ml. Aliquots of the standard (0 - 22 ml) were added to 125-ml separatory funnels and were treated as though copper and zinc were to be determined. A regression for optical density on zinc concentration was determined and used in calculating the zinc in the samples analyzed.

Calcium and magnesium Pipette two 10-ml aliquots (for calcium, and calcium plus magnesium) into 250-ml centrifuge bottles. To each aliquot add a magnetic stirring bar and 2 ml of nitric acid (1:2 v:v). Add 20 ml of potassium metastannate solution with constant magnetic stirring. Continue stirring for an additional 15 min followed by centrifugation at 2000 rev/min for 20 min. Pour the resulting supernatants into a 125-ml Erlynmeyer flask and rinse the lips of the centrifuge bottles into the flasks as well as back into the bottles. Redisperse the precipitates in redistilled water with the help of a glass stirring rod and magnetic stirring for 20 - 30 min. Make the final slurries to the original sample plus reagents volume (32 ml) and centrifuge as above. Remove the supernatants and wash the precipitates four times to remove all calcium, and calcium plus magnesium.

For calcium add a 2mm x 2mm piece of litmus to the supernatant in the 125-ml flask and neutralize with 1.5 N sodium hydroxide (NaOH). Add an additional 2 ml of NaOH and 0.02 g Erio SE mixed indicator (0.1 g Eriochrome Blue S.E.[1], 0.17 g napthol green B and 20.0 g sodium chloride ground to a fine powder) to produce a definite red color. Titrate with EDTA solution (2 g of disodium dihydrogen ethylenediaminetetraacetate/liter) until the red color is just changed to a clear blue.

Use the second series of supernatants to determine calcium plus magnesium. Add 1 drop of methyl red indicator (0.1 g methyl red in 500 ml of ethanol) and 1.5 N NaOH until the color changes to yellow. Add one drop of

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[1]Geigy Dyestuff Division, Geigy Chemical Corp., Saw Mill River Road, Ardsley, N. Y.

nitric acid (1:2 v:v) to change the color back to red. Add 0.05 N NaOH until the color changes to yellow. Add 1 ml of buffer (mix 4.0 g of sodium tetraborate in 80 ml of water and 1.0 g of NaOH plus 0.5 g sodium sulfide in 10 ml of water and make to 100 ml) to maintain the pH at 8 - 10 and 2 drops of Eriochrome Black T indicator (1 g in 30 ml of water containing 1 ml of 1 N sodium carbonate; make to 100 ml with 2-propanol). Titrate immediately from red to light blue color.

Carry two 10-ml blanks through the precipitate washing procedure and use the supernatant from one for a calcium blank and the other for a calcium plus magnesium blank.

Calcium and magnesium standards as well as the potassium metastannate reagent were prepared by the method of Bird et al. (12). The concentration of magnesium in the standard was determined by precipitating the magnesium as pyrophosphate by the method of Diehl and Smith (29).

The mg of calcium equivalent to each ml of EDTA solution was determined by titrating a series of three aliquots of the calcium standard solution. The mg of magnesium equivalent to each ml of EDTA was determined by multiplying the calcium equivalent by the factor, 0.60679 (atomic weight magnesium/atomic weight of calcium). The titration of the supernatant and washings from each sample corrected for the blank titration was employed in calculating the calcium and magnesium contents of the samples:

$$\text{mg Ca/g of sample} = \frac{(\text{ml EDTA}_{Ca})\ (\text{mg Ca} \eqsim 1.0\ \text{ml EDTA})}{\text{g sample} \eqsim \text{aliquot size used}}$$

$$\text{mg Mg/g of sample} = \frac{(\text{ml EDTA}_{Ca\ +\ mg} - \text{ml EDTA}_{Ca})\ (\text{mg Mg} \eqsim 1\text{ml EDTA})}{\text{g sample} \eqsim \text{aliquot size used}}$$

RESULTS AND DISCUSSION

Evaluation of the Accuracy of Trace Metal Analytical Methods

Calcium and magnesium

In the method of Bird, et al. (12) for the determination of calcium and magnesium, phosphate is removed by addition of potassium metastannate, centrifuging and determining calcium and magnesium of the supernatant. Because the amounts of calcium and magnesium involved were small it was considered necessary to determine the number of times the metastannate precipitate must be washed to remove all the calcium and magnesium; four washings were found to be sufficient. In checking the determination, 40-ml aliquots containing 0.12708 mg of calcium and 0.03324 mg of magnesium were employed. The means of the recoveries of four trials were: Calcium, 100.68 (SEM[2], 0.65)% and magnesium, 102,48 (SEM, 0.61)%. Although the magnesium recoveries were slightly high, it was considered that the accuracy of the method was satisfactory for the purpose at hand.

Iron

A regression equation and a correlation coefficient, r, were calculated from optical density values (corrected for cuvette variation) obtained with solutions containing 0 - 22 μg of iron/22 ml of solution.

$$\text{mg Iron/10 ml of solution} = \frac{\text{optical density} + 0.0016}{0.0420}$$

$r = 0.9997$

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[2]Standard error of the mean

According to Diehl and Smith (28) the cations and anions commonly found in milk would not interfere in the use of bathophenanthroline to determine iron. Cobalt forms a yellow chelate which is not extracted under acid conditions; copper forms a colorless chelate which is extracted by nitrobenzene but does not interfere. To insure the presence of an excess of bathophenanthroline in samples containing high amounts of copper and possibly cobalt, a 0.002 M solution was used.

In a preliminary recovery, using only a standard solution containing calcium, magnesium, phosphorus and iron, only 93% of the iron was recovered from the nitric-perchloric acid digest. Increasing the amount of reducing agent, hydroxylamine hydrochloride (HAHC), from 4 to 8 ml increased the recovery to 97% in the same sample. Sandell (86, p. 369) indicated that the effectiveness of the reducing agent may be impaired by the presence of high amounts of phosphorus in the wet ash digest. Ferric pyrophosphates may be formed; these are not easily dissociated unless the pH is close to 1. The pH was measured and found to be 0.5. Increasing the amount of HAHC added to 8 ml improved the iron recovery and was used in subsequent iron determinations.

Phosphorus

The influence of the perchlorate ion on the final color intensity of the phosphorus sample was studied. Perchloric acid in concentrations approximating those expected in the final 50 ml of wet ash digest (from 0 to 4 ml/50 ml) was added to a series of phosphorus determinations using aliquots of the phosphorus standard. Phosphorus recoveries decreased from 100 - 96% as the perchloric acid concentration increased. The presence of per-

chloric acid did not influence the color development in the phosphorus determination.

Subsequently, in phosphorus recovery determinations, a 1-ml aliquot of standard phosphorus solution (0.01 mg/ml) was added to 1 ml of the blank wet ash digest in a 25-ml volumetric flask. The phosphorus determination was carried out and the recovery of the standard measured. A mean recovery of 100.39 (SEM, 0.98)% was obtained from 16 standard phosphorus samples using the regression equation reported by Fortney (33). Since the average recovery and SEM were within the experimental error of the method, the regression equation calculated by Fortney (33) was used in all the phosphorus determinations:

$$\text{mg Phosphorus/25 ml of solution} = \frac{1.99817 - \log \% \text{ transmittancy}}{30.5874}$$

Recoveries of calcium, magnesium, iron and phosphorus in the presence of interphase materials

Fifty ml of a standard solution containing 0.050 mg of iron, 0.500 mg phosphorus, 0.7545 mg calcium and 0.096 mg of magnesium were wet ashed in the presence of 0.0831 to 0.1202 g of freeze dried, ultracentrifuged, unwashed buttermilk samples (WOB).[3] In each case WOB samples without added standards were wet ashed. Fifty ml of redistilled water was used as a blank and accompanied the samples through the wet ashing and subsequent analyses.

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[3]The following abbreviations were employed: The number of washes from zero to six by WO, 1W....6W; creams washed zero to six times, WOC, 1WC....6WC; buttermilks from those creams, WOB, 1WB....6WB, and skimmilks obtained in separating the creams, WOS, 1WS....6WS.

The mean recoveries and (SEM) in three separate runs were: calcium 99.39 (2.64), magnesium 100.41 (4.94), iron 98.73 (1.13) and phosphorus 99.32 (0.86)%. The mean composition and (SEM) of the three WOB samples alone were calcium 18.26 (0.07) mg/g of sample, magnesium 3.167 (0.108) mg/g, iron 0.0256 (0.0010) mg/g and phosphorus 17.12 (0.30) mg/g.

The recoveries of added calcium, phosphorus and iron were acceptable. In view of the small amounts of magnesium added to the samples the variation, 100.41 (SEM, 4.94)%, in quantitative recoveries could be expected. A large variation, 3.167 (SEM, 0.108) mg/g, was also obtained among the compositional analyses of WOB for magnesium. However, the recovery runs and compositional analyses of WOB suggested that the methods employed to determine calcium, magnesium, phosphorus and iron were satisfactory. Furthermore, each of the analyses could be made directly on the nitric-perchloric acid digests of the samples.

The levels of added iron, 0.050 mg/0.0831 g of sample and 0.050 mg/0.1202 g of sample, were equivalent to 0.6017 and 0.4160 mg/g of sample, respectively. In each 10-ml aliquot of wet ash digest used for the iron determination, the concentration of added iron was 0.050 mg/50 ml of wet ash digest (0.0001 mg/ml). The amounts of added iron, 0.4160 and 0.6017 mg/g, exceeded the average amounts of iron found, 0.0256 (SEM, 0.0010) mg/g, among three WOB samples which suggested that the amounts of added iron should be reduced to a level closer to the actual amount present in WOB. However, the other alternative was to increase the sample size of WOB used, thereby increasing the concentration of iron in the final aliquot of wet ash digest used in the actual determination of iron. In later recovery runs both changes were made.

Copper

Using the optical density values obtained (corrected for cuvette variation) with aliquots containing 0 - 23 μg of copper/23 ml of solution carried through the copper determination, the regression equation and correlation coefficient, r, were calculated.

$$\text{mg Copper/10 ml of solution} = \frac{\text{optical density} - 0.0001}{0.02003};$$

$r = 0.9967$

The absorption spectra of copper diethyldithiocarbamate in carbon tetrachloride showed a single peak with a maximum at 436 mμ which agreed with Snell and Snell (94, p. 62). Cobalt has been reported (79) to be present in trace amounts in IM. Clinton (22,23) extracted the diethyldithiocarbamates of copper, cobalt and nickel at pH 8.5 and 4.0 and determined them quantitatively by measuring the optical densities of the mixed chelates at 436, 367 and 328 mμ, respectively. During the actual determination of copper in IM from different washes, the absorption spectra of the solvent extracts were determined. The absence of a peak at 367 mμ suggested that cobalt was not present or was present in very small amounts undetected by the procedure employed.

Zinc

The optical density values obtained with solutions containing up to 12 μg of zinc were used to calculate the regression equation and correlation coefficient, r.

$$\text{mg Zinc/25 ml of solution} = \frac{\text{optical density} + 0.00499;}{0.05701}$$

$r = 0.9984$

Above 13 μg of zinc/25 ml of carbon tetrachloride solution (0.52 ppm) deviation from Beer's Law was observed. Sandell (86, p. 622) reported that above a value of 0.4 ppm deviation from Beer's Law occurred.

Dithizone is a very sensitive reagent and will form metal dithizonates with a variety of divalent metals; copper, cobalt, iron, manganese and zinc were of interest in this study. The zinc dithizonate yields an intense reddish-violet color which is not very stable. Snell and Snell (93, p. 412) reported that as little as 0.005 ppm of zinc could be detected using dithizone and that from 1 - 100 μg of zinc could be quantitatively measured with an accuracy of 5 - 10%.

The determination of zinc following copper on the same aliquot of wet ash digest seemed logical in view of the fact that two interfering metals, copper and iron, had already been eliminated. Moss and Mellon (70) reported that the bipyridyl chelate of iron was stable over a pH range of 3.0 to 9.0. An aliquot of iron standard solution was carried through the copper and zinc determinations. The red-colored bipyridyl chelate remained in the aqueous phase and the absorption spectrum of the dithizone extracts was characteristic of pure dithizone. Manganese dithizonate is unstable above pH 7.0 and the presence of an excess of pyrophosphates eliminates their interference (86). If it can be assumed that pyrophosphates are formed during the wet ashing procedure, their presence would eliminate possible manganese interference. Possible cobalt interference was eliminated by the presence of an excess of sodium citrate buffer. Sandell (86, p. 282) points out that the extraction of cobalt becomes more difficult as the molarity of added sodium citrate is increased beyond 0.02. Absence of cobalt interference was confirmed by treating an

aliquot of cobalt standard solution, 5 - 10 μg, according to the copper and zinc procedures but eliminating the addition of diethyldithiocarbamate in the copper procedure. The lack of red color and a characteristic absorption spectrum in the dithizone extract suggested that the concentration of sodium citrate (0.074 M in the final aqueous solution) was high enough to prevent the interference of cobalt.

The absorption spectrum of dithizone extracts from wet ash digests of IM from different washes showed a single peak with a maximum at 535 mμ which is the maximum reported by Welcher (109, p. 479) for zinc dithizonate.

The method appeared to be quantitative for zinc. Because sodium diethyldithiocarbamate chelates zinc (86, 93, 94, 109) the loss of zinc during the copper determination was checked. Possible loss of zinc in the described copper plus zinc determination was evaluated by analyzing two identical aliquots of zinc standard (10 μg/10 ml), one by the copper plus zinc procedure and one by the zinc method only. The former recovered 97.31% of the zinc found by the latter. This loss was considered to be within an expected experimental error of the method. Zinc dithizonates were observed to be unstable unless an excess of dithizone was present. When the extracted zinc concentration exceeded 5 μg of zinc/25 ml of carbon tetrachloride solution read (characterized by a deep reddish-violet color), optical density values decreased by more than 2% during the time required to take three readings. Increasing the amount of 0.01% dithizone added to 7 ml during the extraction of zinc, plus the amount contributed by the previously dithizone-treated sodium citrate buffer (see zinc method for preparation of buffer) afforded an adequate excess of dithizone in the

final extract (characterized by a blue-green to purple color). Under these conditions the optical densities showed greater consistency during three readings.

Recoveries of combined trace metal standards in the presence of interphase materials

After establishing satisfactory methods for the determination of copper and zinc, samples of the bulk ultracentrifuge buttermilk precipitates (UPS) from unwashed and washed separator-prepared creams were analyzed for iron, copper, zinc, calcium, magnesium and phosphorus. Since the approximate levels of each metal present were known, recovery runs were set up in which the concentrations of the added metals represented the highest and lowest amounts (except for phosphorus) actually present in the samples. Aliquots of phosphorus (5 ml), calcium (1 and 5 ml), magnesium (1 and 3 ml), iron, copper and zinc (5 and 10 ml) standards were added to 125-ml Vycor flasks; three of the flasks contained the smaller aliquots, and the other three contained the larger aliquots of added standards. The combined aliquots were evaporated nearly to dryness. Varying amounts, 0.0632 - 0.1222 g of 3UPS, were weighed into the flasks. The final concentrations of added metals ranged from 0.075 - 0.140 mg of iron, copper, and zinc, 4.09 - 7.90 mg of phosphorus, 6.57 - 15.71 mg of calcium and 0.88 - 2.20 mg of magnesium/g of 3UPS. The amount of element present (except for copper and zinc) did not influence the recovery data. Consequently the recoveries obtained on the six samples were averaged together. The mean recoveries and (SEM) were 99.64 (0.24) for calcium, 100.62 (5.26) for magnesium, 99.58 (2.58) for iron, 91.63 (2.18) for copper, 93.30 (3.09) for zinc and 99.06

(1.79)% for phosphorus. The average calcium, magnesium, iron and phosphorus recoveries agreed well with the values obtained in the absence of added copper and zinc. The presence of added copper and zinc was, therefore, considered to have no effect on the analysis of the other elements.

A high mean recovery, 109.05 (3.44)% of copper, as well as a wide range of values (SEM = 3.44%) were obtained when aliquots of the wet ash digest were analyzed directly. Lusas, Bird and Rosenberger (63) found that the presence of the perchlorate ion in the copper determination appeared to influence the recovery of added copper; elimination of the perchlorate ion by taking the wet ash digest to dryness improved the consistency of the copper recoveries. This was tried in this study as described in the wet ashing procedure.

Recoveries of zinc in the absence of the perchlorate ion were not improved, 93.21 (2.84)%. However, copper recoveries were reduced to an average of 91.19 (1.99)% and were more consistent (SEM = 1.99%).

Aliquots (10-ml) of the wet ash digests from six 3UPS samples plus added standards were taken to dryness and redissolved in 10% (w/v) hydrochloric acid; the ash solutions were transferred to 125-ml separatory funnels and the copper and zinc determined. The average zinc recovery, 92.37 (3.44)%, was lower than the value, 93.30 (3.09)%, obtained when the wet ash digest was analyzed directly. The average recovery of copper was considerably lower, 91.19 (1.99)% vs. 109.05 (3.44)%, but more consistent as indicated by the lower SEM value of 1.99%.

The copper and zinc recoveries were generally lower when the levels of added zinc ranged from 0.075 - 0.090 mg/g of 3UPS. In order to determine whether or not the recoveries were affected by the amounts of added

copper or zinc present, five additional recovery runs were set up in which the added copper and zinc varied from 0.007 mg - 0.086 mg/g of 3UPS. The recoveries obtained were plotted against the corresponding amounts of copper and zinc contained in the 10-ml aliquot of ash solution analyzed. Above 0.002 mg of copper and 0.003 mg of zinc per 10-ml aliquot analyzed, the plotted curves asymptotically approached recoveries of 95.2 and 96.0%, respectively. When the concentration of copper or zinc in the final 10-ml aliquot of wet ash digest was known, recoveries were obtained from the plotted curves.

Evaluation of the Methods of Washing Cream

Gravity washing

The use of water columns Gravity separated cream (18 hr, 4.4 C) was used in all gravity washing experiments. The initial cream, layered on the bottom of the water column at 4.4 C, formed a distinct cream-water interface. Gradually the cream dispersed into the water layer, and after 24 hr only a thin cream layer was visible at the top. After 48 hr the increase in the washed cream layer depth was negligible. Increasing the creaming time to 72 or 96 hr, doubling the amount of cream, varying the lipid percentage of the cream from 8 - 40%, or adjusting the cream to higher temperatures (9.9, 15.6, 21.0, 26.8 and 32.1 C) before washing failed to increase the amounts of washed cream obtained. The formation of a strong cream-water interface was believed to be the limiting factor. Attempts to hasten the dispersion of the cream layer by magnetically stirring the cream or bubbling air through the cream layer had little effect. Addition of detergents to the water apparently reduced the interfacial tension at the

interface; however, the cream rose to the top in large clumps. This type of gravity separation was considered unsatisfactory. A final attempt was made to wash cream through water columns at room temperature. A sizable washed cream layer was obtained but the cream was sour. The use of water columns was abandoned.

The use of Erlynmeyer flasks An attempt was made to increase the surface area of the cream-water interface by using Erlynmeyer flasks to gravity wash cream. When 300 ml of 14% cream and 2700 ml of distilled water were employed about 50 ml of washed cream were obtained within 24 hr at 4.4 C. The increased surface area appeared to facilitate gravity washing of cream. Attempts to wash the 1W gravity cream a second time were unsuccessful. By reducing the cream to water volume ratio from 1:9 to 1:2, cream could be washed as many as two times at 4.4 C. However, on the third pass the cream soured. By this time the sample was 96 hr old. Attempts to wash cream by gravity techniques were abandoned because of the bacterial decomposition encountered.

Centrifugal washing of cream

An effort was made to simulate a gravity washing technique by using the room temperature Size 2 International Centrifuge. Centrifugation of raw milk at 450 rev/min for 90 min provided a sizable cream sample possessing the desired physical characteristics of plasticity and emulsion stability. The original cream could be redispersed in 4.4 C redistilled water and washed as many as three times without appreciable loss of desirable physical characteristics; some oiling off was observed on top of the washed cream layers. From the 4W through 6W, successive redispersions of

the washed cream in 4.4 C water resulted in partial churning of the fat and increased oiling off during subsequent centrifugation. It was considered that temperature changes during redispersion and centrifugation resulted in considerable destabilization of the cream as the number of washes increased beyond four.

Model PR-2, International Centrifuge The idea of using centrifugal force to accelerate the gravity washing of fat globules appeared sound. The partial success achieved using the room temperature centrifuge suggested the use of a refrigerated centrifuge to overcome the effect of temperature fluctuations on the emulsion stability and physical appearance of the washed creams.

Two lots of raw milk were centrifuged at 450 rev/min for 90 min using the room temperature and refrigerated centrifuges. The refrigerated centrifuge produced a plastic cream layer closely resembling that obtained by gravity separation in Erlynmeyer flasks. The room temperature centrifuge produced a cream layer composed of tightly packed fat globules. The yellow color in the latter cream suggested that either IM was removed more rapidly than with the refrigerated centrifuge or that incipient oiling off occurred during the initial preparation of the cream sample; this defect was overcome by using the refrigerated centrifuge. Although these results were encouraging, subsequent analyses of the skimmilks revealed that 8.00 and 47.55% of the total lipid in the initial milk sample was lost to the skimmilk using the room temperature and refrigerated centrifuges, respectively.

A series of raw milk samples was centrifuged at speeds of 450, 900, 1350, 1800 and 2250 rev/min for 45 and 90 min, respectively, in the refrig-

erated centrifuge. The macroscopic and microscopic appearances of the resulting creams were observed. The cream yields and lipid contents of the cream and skimmilk fractions were determined. The amount of total milk lipid lost to the skimmilk was calculated. These data are summarized in Table 2.

As the centrifuge speed and time increased the cream layers became more compact and yellow; the number of small fat globules increased; the number of fat globule clusters increased; and the loss of total milk lipids to the skimmilk decreased. Regardless of the speed or time used the cream layers were plastic and redispersed readily into 4.4 C redistilled water. No

Table 2. Centrifuge (Model PR-2) speed and time, and the amount of total milk lipid lost in the skimmilk

Rev/min	Cent. time	Total lipid		yield[a] (g)	Original milk lipid lost to skimmilk (%)
		cream (g/100 g)	skimmilk (g/100 g)		
450	90[b]	33.66	1.89	48	47.55
900	90[b]	41.65	0.50	68	12.29
1350	90[b]	40.52	0.16	77	3.89
1800	90[c]	48.52	0.12	75	3.06
2250	90[c]	48.68	0.05	74	1.27
900	45[b]	39.22	1.00	60	24.78
1350	45[c]	40.48	0.66	75	16.83
1800	45[c]	36.08	0.23	95	5.73
2250	45[c]	49.68	0.10	71	2.55

[a]From 1000 g of milk

[b]Original milk tested 3.51%

[c]Original milk tested 3.61%

visual oiling off was evident in the cream layer following centrifugation at 2250 rev/min for 90 min.

The effect of repeated washing on the macroscopic and microscopic appearance of the washed creams and the total fat lost to the resulting skimmilks was considered. Creams were washed from zero to six times at speeds of 900, 1350, 1800 and 2250 rev/min for 90 min. At each wash (to six) the fat globules yielded a more compact cream layer the higher the centrifuge speed. Microscopic examination of the dispersed creams revealed the presence of individual spheres and very few clusters (two to four spheres/cluster) regardless of the speed used. However, as the speed increased, a greater proportion of small fat globules were recovered in the washed creams. The presence of a low proportion of clusters in the redispersed creams indicated each globule was being washed and the chances of milk serum components being trapped in cluster intersticies and carried through the washing procedure was not likely. Despite the formation of compact cream layers at higher speeds (1800-2250 rev/min), the creams retained their plastic appearance and were readily dispersed into 4.4 C distilled water. Oiling off was absent and partial churning during redispersion was not encountered.

The amount of original milk lipid lost to the skimmilk during each wash of the sequence was determined at each of the four speeds. The highest loss occurred during the first wash, and decreased as the speed increased.

The percentage of the total lipid in the original milk lost to the skimmilk during the entire washing sequence (0W to 6W) decreased from 56.3% at 900 to 11.5% at 2250 rev/min. The latter value compared favorably with the loss (10.4%), obtained using the cream separator. Based on these deter-

creased as washing progressed showing only a small increase in the fourth wash. Although the degree of oiling off was the same in both runs, at 32.1 C, most of the lipid gathered in increasing amounts in the foam. As a result, it was not mixed homogeneously with the liquid passing through the separator. The lipid contents of the skimmilks from both runs are presented in Figure 1.

An attempt to wash cream using a separation temperature of 21.0 C was tried. By the end of the fourth wash very little oiling off was evident but the cream was churned badly; butter granules were visible in the cream. A compromise temperature gradient was tried. In the original separation and first three washes 37.7 C was used, and during the final three washes 34.9 C was employed. Oiling off was not avoided but destabilization was not observed during four washes. This greater stability is indicated by the data presented in Figure 1. The lipid lost to the skimmilk gradually decreased and showed no marked increase during the fourth wash. The total amount of original milk lipid lost in this series of skimmilks was 10.35% of the original fat content of the milk in contrast with 11.81% at 37.7 C and 5.217% at 32.1 C. The low value of 5.217% of the total fat at 32.1 C agreed with the observation that most of the oiled off lipid remained in the churned foam and was not incorporated in the liquid passing through the separator.

Tarassuk, Koops and Pette (98) maintained the lipid content of successively washed creams close to 25% which helped prevent destabilization during washing. Using a 22.9% cream, the proposed temperature gradient and a cream screw setting of 1TI, the lipid content of the cream gradually decreased to 4.3% by the end of six washes. A gradual increase in oiling

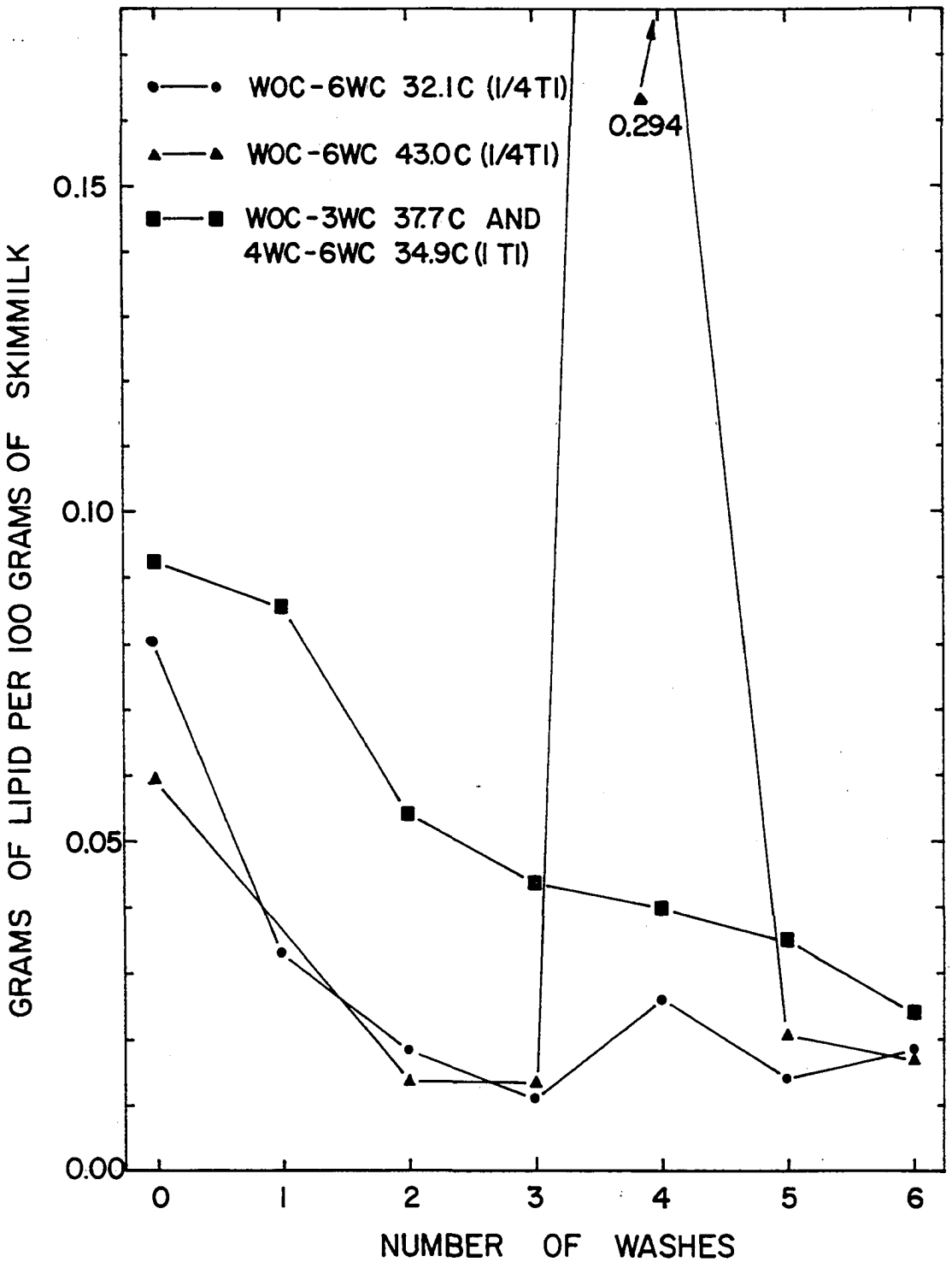

Figure 1. Effect of separating temperature on the lipid losses to the skimmilk during repeated washing of cream

off was observed. By adjusting the cream screw setting to 2TI following the initial separation of milk, the subsequent washed creams contained from 33.5 - 43.0% lipid. Visual oiling off was reduced and the resulting washed fat globules were concentrated into a smaller volume of washed cream. Lipid losses to the skimmilk were not determined.

As a result of the foregoing observations, physical destabilization was accepted as a characteristic of cream washed with the cream separator. Previous investigators failed to report similar observations; however, recent workers suggest that if uncooled raw milk is used this problem is eliminated entirely. This latter idea was never tried because of the inability to obtain a sample of uncooled raw milk at the dairy farm. Milk supplied to the processor is cooled. Consequently, it would seem that a study of the IM and their relationship to processing problems should begin with a cooled raw milk sample.

Finally, for all comparative runs involving the use of the refrigerated centrifuge and the cream separator, 37.7 C was the separation temperature used in the initial separation and first three washes, and 34.9 C was used during the final three washes.

Comparison of the Methods of Washing Cream: Use of Separator vs. the Refrigerated Centrifuge

Two methods of washing cream were compared by the use of a sequence of studies each of which was suggested, in part, by its predecessor. The physical appearance of the resulting washed creams suggested that by using the refrigerated centrifuge, emulsion stability was retained throughout the washing sequence. The interphase materials nitrogen (IMN): lipid

ratios were calculated to determine the effect of repeated washing on the loss of labile IM. When a constant IMN: lipid ratio was achieved after repeated washing it was considered that only the non-labile IM remained. If casein was the principal labile component in the IM it should have been possible to precipitate it from buttermilks at pH 4.6 - 4.7 and leave "non-casein" proteins in the acid whey. The high acid precipitable (pH 4.6 - 4.7): total nitrogen ratios among washed buttermilks showed that a much larger portion of IM behaved like casein than had been expected. Direct acidification of a buttermilk-butterserum combination was used to concentrate crude IM. The casein-like behavior of a large portion of IM suggested that it might be present in large enough micelles to be concentrated by ultracentrifugation under conditions similar to those previously used in this laboratory to prepare natural caseinates. The color sequences that were obtained among the ultracentrifuge buttermilk precipitates, obtained from centrifuge and cream separator-prepared washed creams, were photographed so that a permanent record of the changes in relative amounts of precipitable IM constituents would be available. Attempts were made to confirm visual color differences by measuring the reflectance spectra of freeze dried, ultracentrifuge buttermilk precipitates (unextracted and Soxhlet-extracted) obtained from cream separator-prepared unwashed and washed creams. The appearance of colored materials among the ultracentrifuge buttermilk precipitates indicated the possible presence of metal-containing fractions.

Because the lipid percentages of the creams in the first color comparison varied greatly for the separator creams, an attempt was made to increase the fairness of the comparison of washing techniques by standard-

izing the lipid content of the unwashed and washed creams to 30%. Because all of the creams had the same lipid content, churning times were noted as a measure of the "normalcy" of the creams obtained; the total nitrogen contents of the subsequent buttermilks (TNB) were determined and the TNB:cream lipid ratios were calculated and compared. The ultracentrifuge buttermilk precipitates were photographed; the color differences among subsequent washes and between washing methods were compared as were the corresponding yields of freeze dried, ultracentrifuge buttermilk precipitates. Finally the calcium, magnesium, phosphorus, iron, copper and zinc contents of the precipitates were determined and compared.

Physical appearances of the washed creams

Washed creams, which could not be distinguished from the original unwashed sample, were obtained with the refrigerated centrifuge. After standing for 12 hr at 4.4 C the washed creams were plastic in appearance, and no free lipid had separated. Washed creams prepared with the cream separator contained an increasing amount of free lipid as the number of washes increased. These observations suggest that the former method is the less rigorous of the two procedures. A high degree of emulsion stability was retained throughout centrifuge washing, suggesting that IM retained by the fat globules might be more representative of the natural materials present. The presence of free lipid in the creams washed by the cream separator suggests that the labile materials may have been removed at a much faster rate and that non-labile IM may have been lost.

Quantitative distribution of total interphase materials nitrogen and the calculated interphase nitrogen to lipid ratios among unstandardized, unwashed and washed creams

An accepted criterion for determining the number of washes needed to completely remove traces of milk serum constituents from washed creams has been the total nitrogen (TN):lipid ratios among the unwashed and washed creams. Rimpila and Palmer (82) used this criterion and showed that beyond four washes the ratio became constant. The TN:lipid ratios among the washed creams obtained with the separator gave values which were in good agreement with those of Rimpila and Palmer (82) to the third wash. At this point the ratio was lower, 0.77 vs. 0.96 mg/100 g lipid; it remained constant, 0.74 $\pm$ 0.03 mg/100 g lipid, through the sixth wash. The TN:lipid ratios among the washed creams obtained using the refrigerated centrifuge were lower than those from the separator samples through the second wash. However, the third wash yielded a cream with a higher ratio, 1.19 vs. 0.77 mg/100 g lipid. Beyond the third wash the ratio was lower but remained constant, 0.33 $\pm$ 0.03 mg/100 g lipid, through the sixth wash.

Roland (84) developed an equation to calculate interphase materials nitrogen (IMN) from the difference between the total nitrogen contained in separated cream, calculated on a lipid-free basis, and the total nitrogen contained in the resulting skimmilk:

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

in which n = g of nitrogen in 100 g of lipid-free cream

e = g of nitrogen in 100 g of skimmilk

f = g of lipid in 100 g of cream

x = g of IMN per 100 g of lipid in the cream

The IMN:lipid ratios were calculated with the above equation. Using the relationship, (n - e)/n, the ratio of IMN:TN in the cream was calculated. Values obtained for the creams prepared using the separator and centrifuge are plotted in Figure 2. The IMN/100 g of lipid value (scale on left side of Figure 2) was higher in the WOC, 1WC, 2WC and 3WC samples obtained by centrifugal than by separator washing. In centrifuge-washed creams IMN/100 g of lipid decreased from 1WC - 4WC and then remained constant. Among creams prepared using the cream separator, IMN/100 g of lipid also showed a sharp increase in 1WC, the ratio remained constant through 2WC, decreased in 3WC and remained constant through 6WC. The equilibrium ratios, IMN/100 g of lipid, were higher for separator than for centrifuge-prepared creams. The higher IMN:TN ratios after the second wash, 0.982 vs. 0.935 (for separator-prepared creams), suggested that essentially all of the milk serum proteins had been removed by the centrifuge washing procedure after the second wash. Only after three washes had been completed did the IMN:TN ratio (0.970) of the separator-prepared washed cream became constant; they were lower than the ratio (0.996) obtained in the centrifuge prepared washed cream. The separator washing technique appears to be less efficient than the centrifuge procedure in removing milk serum constituents in the early washes; the curves suggest that some serum proteins may persist to a greater degree through the sixth wash in the separator samples as compared to the centrifuged samples. Perhaps the higher IMN: lipid ratio from the fourth through sixth washes resulted from the inclusion of traces of "soluble" milk serum proteins during the separator washing procedure. The presence of free lipid among these washed creams

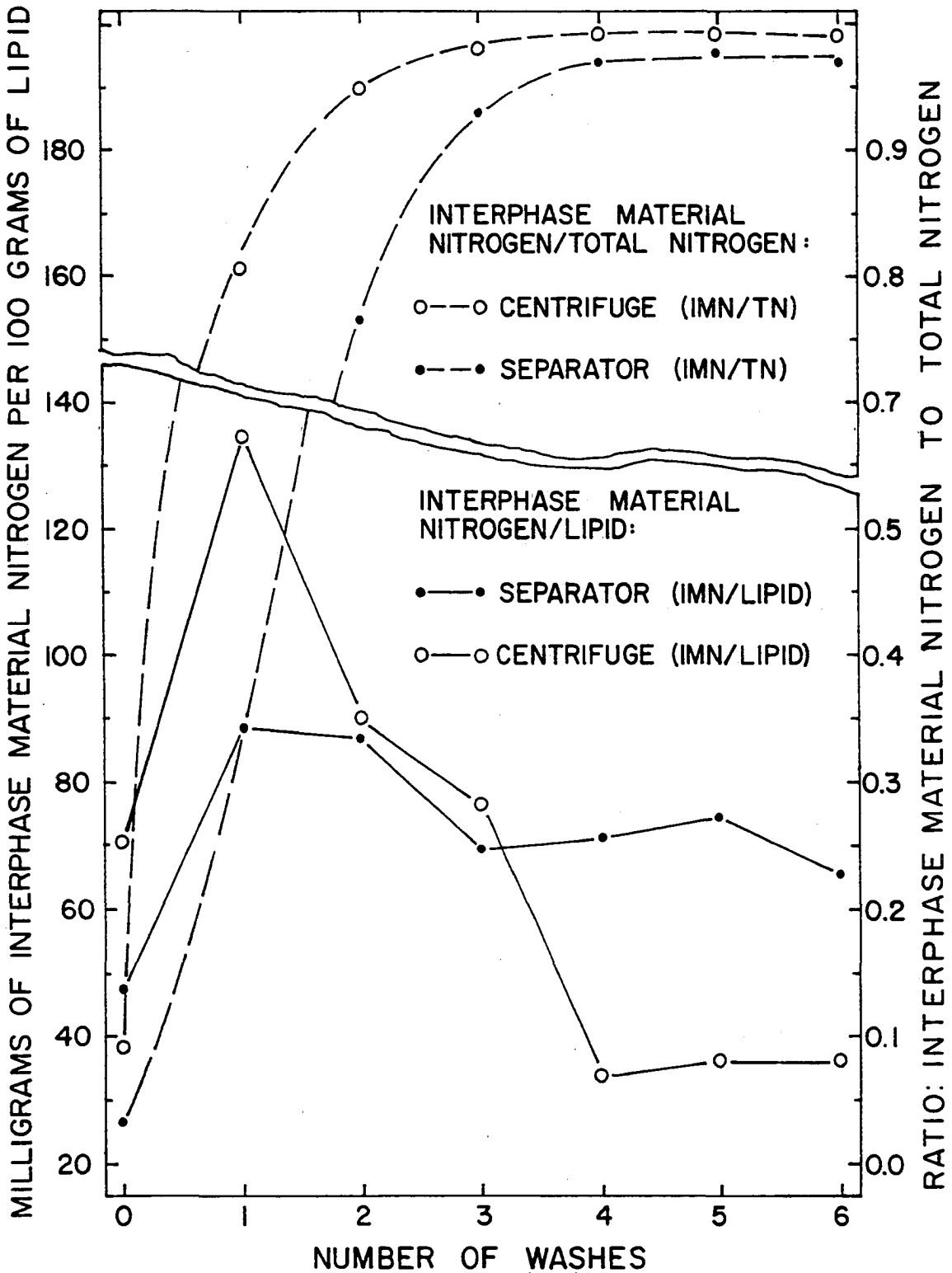

Figure 2. Interphase materials nitrogen (IMN):lipid and IMN:total nitrogen (TN) ratios among centrifuge- and separator-prepared unwashed and washed creams

could lead to partial emulsification of lipids with the proteins present. This occurrance is not difficult to visualize in view of some of the previous studies (55, 82, 88, 110) of artificial emulsions of butteroil with various milk proteins.

On the basis of the IMN:lipid ratios, a greater amount of IM was retained through three washes using the refrigerated centrifuge than was retained using the cream separator.

Concentration of interphase materials following churning of unwashed and washed cream

Amounts of acid-precipitable interphase materials contained in "buttermilks" from cream separator- and centrifuge-prepared unstandardized, unwashed and washed creams The total nitrogen determination involved the use of heat, concentrated sulfuric acid and a catalyst (mercuric oxide and sodium sulfate) to digest the organic matter in the sample and convert organic nitrogen to ammonium sulfate (29). The nitrogen is freed as ammonia by adding a mixture of sodium hydroxide and sodium thiosulfate, the ammonia was distilled into 2% boric acid solution and the distillate was titrated with dilute hydrochloric acid (65). Determination of the "non-casein" nitrogen in the sample involves precipitation of casein at pH 4.6 - 4.7 with an acetic acid-sodium acetate buffer. The precipitate was removed by filtration; the total nitrogen content of the filtrate was determined as described above. The total "casein" acid-precipitable nitrogen was then calculated by difference. The total and "non-casein" nitrogen contents of the buttermilks obtained from unwashed and washed creams were used to calculate the amount of pH 4.6 - 4.7, acid-precipitable nitrogen

(APN) present. The APN:total nitrogen (TNB) ratios were calculated for both the centrifuge- and cream separator-prepared buttermilks and are plotted in Figure 3. The APN:TNB ratios among the buttermilks from unstandardized centrifuge-prepared creams continued to increase beyond the second wash reaching values of 0.9642 - 0.9687 in the third and fourth washes, respectively; beyond the fourth wash the ratio decreased to an average value of 0.9028 $\pm$ 0.0186 in the fifth and sixth washes. These ratios follow the changes among the MMN:lipid ratios (Figure 2).

The higher APN:TNB ratios in the 2WB, 3WB and 4WB buttermilk samples from centrifuge-prepared than from the separator-washed creams indicate that more acid-precipitable MMN was retained using the centrifuge. Beyond the fourth wash the APN:TNB decreased to an average of 0.9152 through the sixth wash, suggesting that the relative lability of APN as compared to other constituents in MMN increased beyond four washes. A comparison of the APN:TNB ratios, from 2W - 6W, separator- and centrifuge-prepared creams indicates that possibly the most labile components are washed from the globules by the separator more quickly than by the centrifuge.

If the changes among the APN:TNB ratios were representative of the lability of a major portion of the MMN, three washes would be sufficient by either method; the use of the refrigerated centrifuge would be preferred since the APN:TNB is higher, 0.9642, than the corresponding value, 0.9152, obtained using the cream separator.

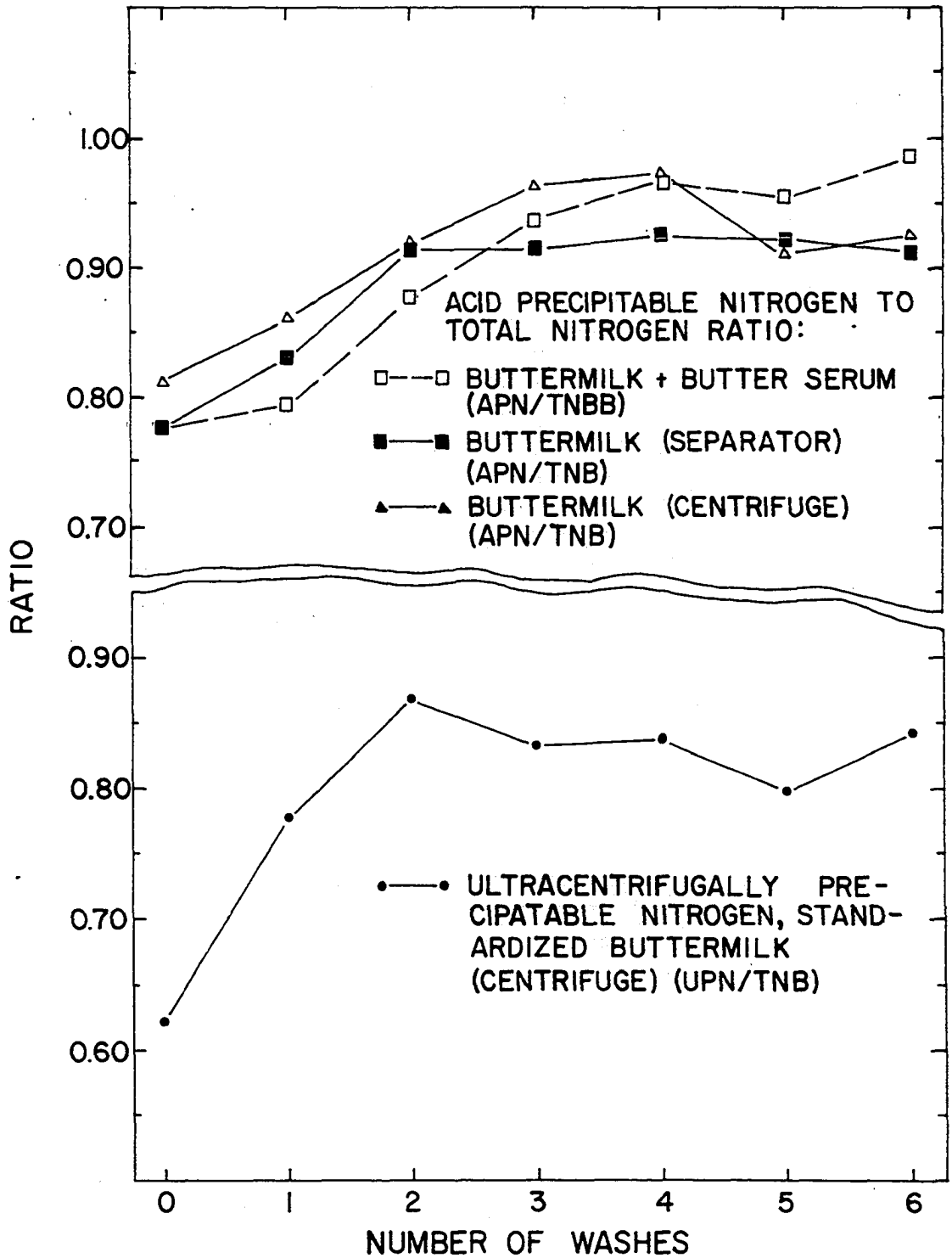

Figure 3. Concentration of interphase materials from buttermilk and buttermilk-butterserum mixtures by acid precipitation (pH 4.6 - 4.7) and ultracentrifugation

Concentration of interphase materials by the direct acidification of buttermilk and butterserum mixtures with hydrochloric acid Acidification (pH 4.6 - 4.7) of a series of combined buttermilk and butterserum (BMBS) preparations from separator-prepared washed creams resulted in the formation of a casein-like precipitate after 24 hr at 4.4 C. The amount of APN was determined from the differences between the nitrogen contents of the resulting clear supernatants and the total nitrogen in the BMBS (TNBB). The calculated APN:TNBB ratios are plotted in Figure 3. Beyond the third wash 94 - 98% of the TNBB was APN. The supernatants were Biuret negative beyond the second and Fehlings negative beyond the first wash suggesting that acid soluble (pH 4.6 - 4.7) milk proteins and lactose were removed after three and two washes, respectively. A strong positive Molisch test was obtained with the supernatants through the fourth wash; faint positive tests were recorded through the fifth and sixth washes. The presence of carbohydrates in the IM have been reported (41, 101).

The presence of materials yielding a positive Molisch test throughout the washing sequence and a negative Fehlings test beyond the second wash suggested that acid precipitation at pH 4.6 - 4.7 may have caused mild acid hydrolysis of IM glycoproteins, thereby releasing carbohydrates to the supernatant; the negative Fehlings tests suggested that free lactose was not present beyond the second wash and that such carbohydrates as were released were non-reducing. The existence of an acid-soluble glycoprotein in the supernatants was also considered since the Molisch test is sensitive to glycoproteins (4). The faint positive Molisch test obtained on the supernatants from the fifth and sixth washes suggested that possible glycoprotein concentration in the IM was eventually reduced by repeated washing

of the fat globules.

The behavior of the IM under acid conditions was remarkable in view of the fact that 70% of the crude IM in buttermilk was found by others to be lipid (82, 103, 104) (free lipid, unchurned fat globules and bound interphase lipid in the BMBS). The casein-like behavior of the small non-lipid portion suggested the use of ultracentrifugation as a means of concentrating the IM from buttermilk. By centrifuging buttermilk alone, the lipid content of the final ultracentrifuge precipitate would be reduced since a large portion of the free lipid contained in the buttermilk could rise to the top of the tube.

Ultracentrifugation of buttermilk A series of buttermilks from churned, standardized (lipid content), centrifuge-prepared, unwashed and washed creams were ultracentrifuged at 20,000 rev/min for 20 hr. The combined resulting supernatants and lipid layers and the whole "buttermilks" (TNB) were analyzed for total nitrogen; the total nitrogen in the ultracentrifuge precipitate (UPN) was calculated by difference. The UPN:TNB ratio is plotted in Figure 3. A maximum ratio of 0.8673 was obtained by the end of two washes and remained almost constant (average 0.8253 ± 0.0146) beyond the second wash. A comparison of the UPN:TNB and APN:TNB ratios indicates that ultracentrifugation does not concentrate all the APN into the precipitate. A portion of the nitrogen was either soluble in the supernatant, complexed as a low density lipoprotein or was present on unchurned fat globules concentrated in the lipid plug. It was interesting to find that in the second wash the UPN:TNB ratio, 0.8673, was closer to equilibrium APN:TNB ratios than were any other UPN:TNB values. This finding sug-

gests that certain UPN constituents are lost beyond the second wash, which apparently are acid-precipitable. The loss of UPN would seem to occur at a greater rate than that of APN constituents to the sixth wash with separator washing. Analysis of the ultracentrifuge precipitates for total lipids indicated that 47% of the final freeze dried solids were lipid. The lipid analyses will be discussed in a later section. The data suggest that the non-lipid portion of the IM influenced the density of the IM to a greater degree during ultracentrifugation than did the lipid portion.

Color changes among the ultracentrifuge precipitates from separator and centrifuge buttermilks obtained from unstandardized, unwashed and washed creams

The changes in colors obtained among the OW - 6W precipitates during the study of ultracentrifugation as a means of concentrating IM from buttermilks indicated considerable change among the precipitate constituents during washing. Colored photographs of the precipitates are presented in Figure 4, A and B. The presence of colored materials was detected among all the precipitates regardless of the washing method used. As most of the skimmilk caseinate appears to have been removed by the end of the first wash, a red-brown material represented a major component of the precipitate of 1WB. A comparison of the precipitates obtained from centrifuge-prepared WOB and WOS (Figure 4, B) showed that the red material was present in higher amounts in the buttermilk than in its corresponding skimmilk. This indicates that the red material was a component of the IM. A close look at the side and front views of the ultracentrifuge precipitates (OUPC and 1UPC) from centrifuge-prepared buttermilks (WOBC and 1WBC) revealed the

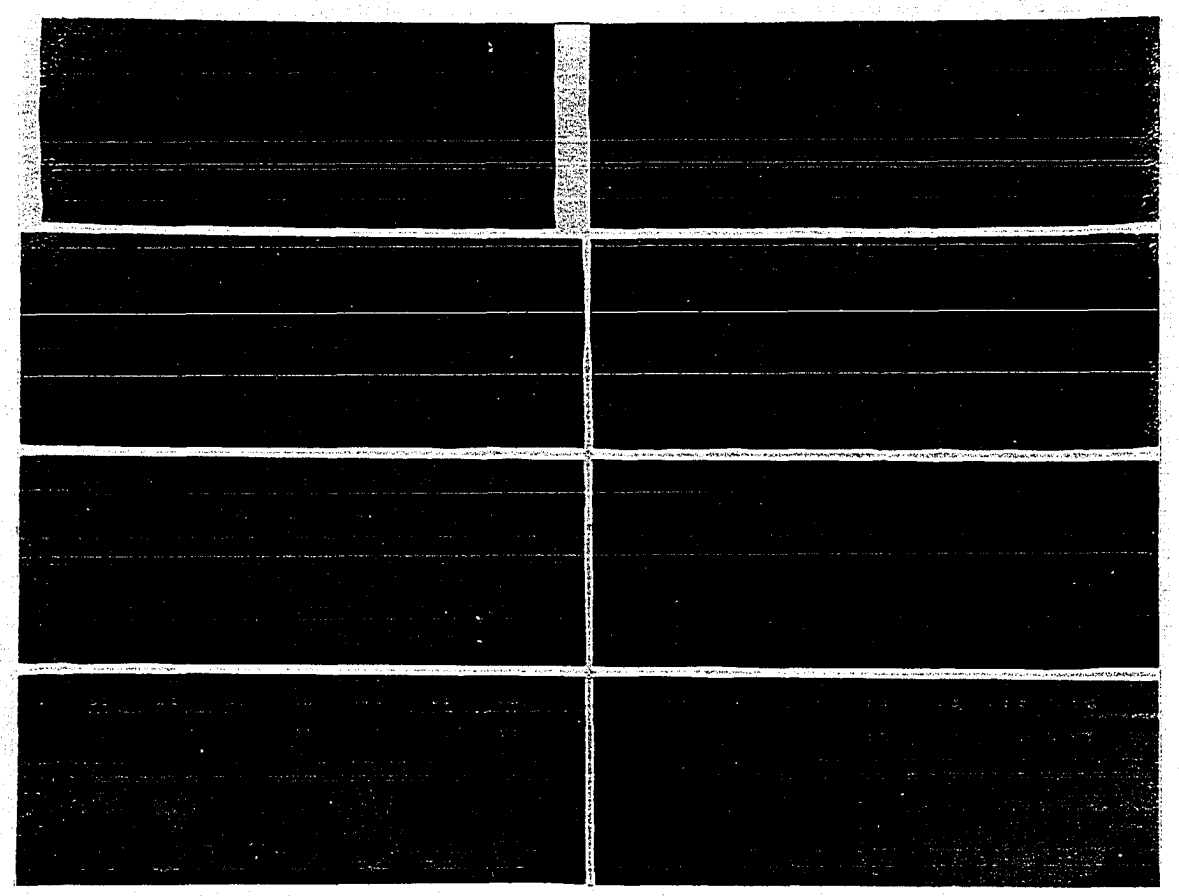

Figure 4. Photographs of colored ultracentrifuge precipitates of buttermilks

Separator, unstandardized creams: A front, a side view

Centrifuge, unstandardized creams: B front, b side view

Separator, standardized creams: C front, c side view

Centrifuge, standardized creams: D front, d side view

Except in B,b the creams from which the buttermilks were obtained were washed zero to six times, from left to right; in B,b, the extra tube on the extreme left represents skimmilk from the original milk

presence of several layers of sedimentable material. One thin red layer seems to lie between two white, non-caseinate layers and is lost to a considerable degree in the first wash. The larger one persists, in part, at least to 6UP. The original degree of layering was lost as washing progressed suggesting that uncolored and colored materials with lower densities either were lost during successive washes or became integrated with each other in the subsequent precipitates. The multilayer effect was not observed among the ultracentrifuge precipitates (0UPS and 1UPS) obtained from buttermilks (WOBS and 1WBS) prepared using the cream separator, regardless of whether or not the creams were standardized.

Visual differences between the two series of ultracentrifuge precipitates were the greater proportion of red material to caseinate and other white components in the precipitate (0UPC) from centrifugally-washed buttermilk (WOBC), the larger and more uniform sizes of the 2UPC through 6UPC precipitates, the progressive, more rapid reduction in the ratio of red to white materials in 4UPS through 6UPS than in 4UPC through 6UPC, and the intensity of the red color among all the precipitates.

The smaller amount of caseinate and the larger amount of red material in 0UPC than in 0UPS suggested that the initial centrifugation of milk removed more skimmilk from the unwashed cream than did the initial cream separator treatment. Furthermore, the higher IMN:lipid and IMN:TN ratios (see Figure 2) agreed with the presence of higher amounts of red material in 0UPC.

The progressive decrease in the visual ratio of red to white materials from 3UPS - 6UPS suggested that the red material was removed from the IM at a faster rate by using the separator rather than the centrifuge to wash

cream. The high ratio of red to white materials among 3UPC - 6UPC samples suggested that centrifugally-washed fat globules retain a greater proportion of labile, centrifugally-precipitable IM at their surfaces than do those obtained by the cream separator washing method. However, the fluctuations among the IMN:lipid ratios (Figure 2) of the corresponding washed creams do not support this interpretation. This may result from the fact that the separator-prepared creams could have been less representative of the fat globule system of milk than were the centrifuge-prepared creams as indicated below.

When the lipid percentages of the two series of creams (Figure 4, A and B) were calculated, it was found that the lipid content decreased from 22.93 - 4.31% for the separator and from 38.28 - 30.01% for the centrifuged samples. This indicated that the net result of the differences shown in Figure 4, A and B, was an unfair comparison of the effects of the washing techniques if color and size of the precipitate are used as criteria among the ultracentrifuge precipitates.

Standardization of the lipid contents of cream separator- and centrifuge-prepared, unwashed and washed creams

A second series of unwashed and washed creams was prepared from a single lot of raw milk using the cream separator and refrigerated centrifuge. Dilution of the separator-prepared creams during washing was prevented by readjusting the cream screw (2TI) following the separation of the raw milk. Resulting creams (from both series) were standardized to a 30% lipid content using redistilled water. The creams were churned and the buttermilks ultracentrifuged. Photographs of the resulting precipitates

were taken. Both front and side views are presented in Figure 4, C and D. The purpose of this comparative run was to further study the effect of washing by both methods, in a manner that should have given comparative results, on the appearances of the ultracentrifuge precipitates. The churning times of the standardized creams and the approximate yields of freeze dried ultracentrifuge precipitates from each wash were determined. In addition, TNB:cream lipid ratios were calculated. This ratio was calculated using the following equation:

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

x = mg total nitrogen per 100 g of lipid

n = mg nitrogen in 100 g of buttermilk

f = g of lipid in 100 g of cream

100 - f = g of lipid-free serum in 100 g of cream

The assumption was made that the mg of nitrogen per 100 g of buttermilk was equivalent to the mg of nitrogen per 100 g of cream serum.

Color changes among ultracentrifuge precipitates The smaller amount of caseinate in the 0UPC sample (Figure 4, D) agreed with the observation made in the previous comparison (Figure 4, B); the smaller weight of freeze dried 0UPC obtained (Table 3) confirmed this observation. By the end of the first wash almost twice as much precipitate was obtained in 1UPC than in 1UPS which was confirmed by the weights of freeze dried material obtained; similar differences were obtained between the 2UPC and 2UPS samples. Beyond the second wash the observed sizes of the precipitates were relatively uniform within each series. The 3UPC - 6UPC precipitates looked

larger, and the final weights of freeze dried material (Table 3) were larger than those of the 3, 4, 5 and 6UPS samples. The sizes of the darker colored portions of the precipitates appear uniform within each series. However, 3UPS through 6UPS showed a gradual loss of red color accompanied by the appearance of a greenish-colored material, (Figure 4, C). The 3UPC through 6UPC samples appear to retain a reasonably uniform amount of the red-colored material, and also contain more of the white background fraction. However, the ratio of red to white material seems actually higher in 3UPC through 6UPC than in 3UPS through 6UPS because of the progressive loss of red material in the latter series. The appearance of a "reddish-brown" precipitate following ultracentrifugation of washed buttermilks from separator-prepared, washed creams has been reported (37, 67, 112); the appearance of a greenish fraction has never been reported, as far as is known.

The comparisons proved to be interesting. On the basis of the apparent high concentration of red material in 2 and 3UPS (Figure 4, C) and its gradual loss during subsequent washes, the use of the cream separator to wash cream must have influenced the types of IM retained at the surfaces of the fat globules to a greater degree than did the use of the centrifuge.

Total nitrogen in buttermilk: cream lipid ratios The TNB:cream lipid ratios plotted in Figure 5 were higher in 1, 2 and 3WBC buttermilks which agreed with the higher amounts of freeze dried ultracentrifuge precipitates obtained (Table 3). Beyond the third wash the ratios were lower than the corresponding TNB:cream lipid ratios obtained on the 4, 5 and 6WBS

Table 3. Approximate yields of freeze dried ultracentrifuge precipitates from cream separator- and centrifuge-prepared, standardized, churned creams

Wash	Yield of interphase materials	
	separator (g)	centrifuge (g)
WOB	1.9845	1.3532
1WB	0.2606	0.5527
2WB	0.1522	0.2606
3WB	0.1469	0.1770
4WB	0.1276	0.1649
5WB	0.1073	0.1305
6WB	0.1120	0.1476

buttermilks; in both series the ratios continued to decrease beyond the third wash. The TNB:cream lipid ratios do not agree with the final amounts of freeze dried precipitates obtained. Beyond the third wash the TNB: cream lipid ratio varied inversely with the amount of freeze dried ultracentrifuge precipitate obtained. The question raised by this situation is: "Are glyco- or lipoproteins lost more rapidly in separator- than in centrifuge-washing?" Furthermore, the visual color changes among the photographed ultracentrifuge precipitates suggest that the use of a nitrogen to lipid ratio as the sole criterion to determine the number of washes required may not represent true changes occurring among the more and less labile IM components, as would be indicated by the change in the ratios among colored and uncolored components.

Churning times of standardized unwashed and washed creams If the fat globules in cream are equilibrated at the correct temperature, the churning time, in a commercial operation, will be from 35 - 50 min and the

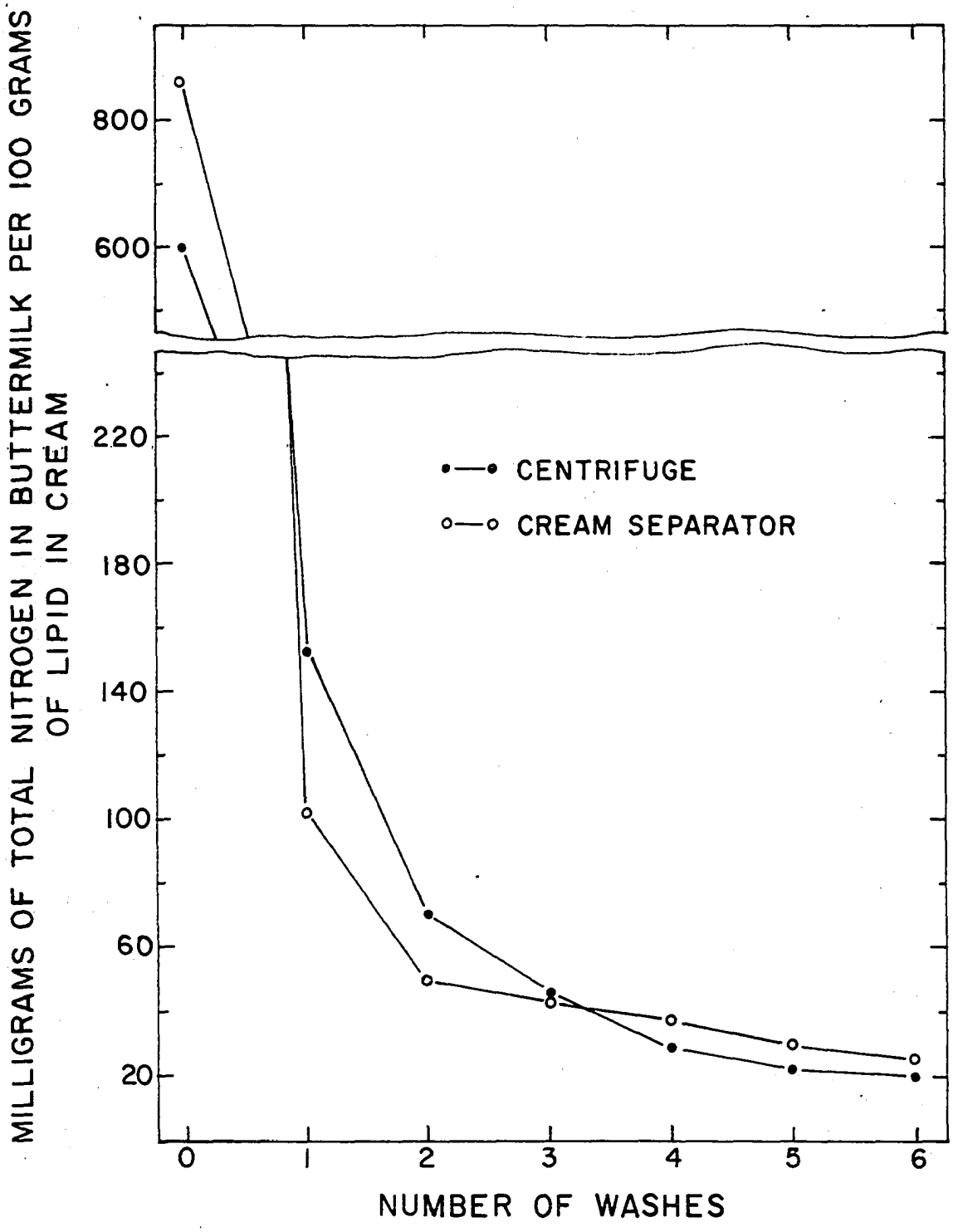

Figure 5. Total nitrogen in the buttermilk to lipid in cream ratio among buttermilks from standardized creams

churning loss approximately 1% of the total lipid placed in the churn. Churning temperatures vary inversely with the degree of unsaturation of the lipid, which in turn is dependent upon the feeding regime (pasture vs. dry feed). During many years experience with the small churning unit employed, it has been learned that, if the creams to be churned are held overnight at 4.4 C, churning times will be reasonably normal and churning losses lowest (approximately 2%).

It was considered that one measure of the "normalness" of the IM at the fat globule surfaces in zero-to six-wash creams, would be the churning time. The lipid contents of all the creams were standardized to 30% lipid; unfortunately there was some variation in cream weights. The weights were not standardized because it was desired to obtain all of the IM possible. Table 4 shows that, with the exception of WOCS and 1WCS, it is felt that the difference in weights did not invalidate the results - and with the pair cited possibly only WOCS was affected.

Disregarding WOCS because of the low sample weight, all of the churning times for the separator creams were lower than one would expect. They decreased considerably from 1WCS through 4WCS and then tended to increase. The churning time of the WOCC sample was normal. The removal of the bulk of the skimmilk constituents lowered the churning time considerably. However, the churning times of all of the centrifuge-washed creams are in close approximation of "normal" churning times. In both series, the tendency to rise beyond 4WC may result from the loss of the euglobulin fraction which Dunkley and Sommer (31) called "agglutenins," because of their apparent involvement in natural fat globule clumping. Slow loss of

Table 4. Weights of samples churned and churning times of standardized, unwashed and washed creams prepared using the cream separator and refrigerated centrifuge.

Wash	Separator		Centrifuge	
	weight churned (g)	churning time (min)	weight churned (g)	churning time (min)
WOC	257	29	375	46
1WC	430	29	369	34
2WC	358	23	364	35
3WC	354	19	414	33
4WC	351	21	392	34
5WC	357	25	399	35
6WC	359	20	378	41
6WC[a]	322	24.5	---	--

[a]Cream sample held at 4.4 C for 56 hr prior to churning

this fraction during several washes could logically be correlated with the increase in churning time.

The churning times presented suggest that the materials held at the fat globule interphase in centrifuge-washed creams more closely resemble those in the original milk than do those in the separator-washed creams. It was considered, however, that the churning time data might not be representative for the separator-washed creams because the milk or diluted creams were warmed to about 35 C for efficient separation; for this reason the lipid components might not have been in temperature equilibrium although the sample was placed in a large tray of water in the walk-in cooler (4.4 C) during the 12-hr holding period. The sample 6WCS was of sufficiently large quantity that a second churning was held for 56 hr before it

was churned. The difference in churning times of the two 6WCS samples would not account for the generally steady, larger difference between the runs.

It is considered that the churning time data indicate that the IM in the 1WCC through 6WCC samples are more like those of unwashed creams and that the skimmilk components in normal creams, either by exerting protective action on the fat globule, or by stabilizing the foam-stage in the churning process, exert an influence in the churning mechanism.

Reflectance measurements of freeze dried ultracentrifuge precipitates

Measurement of the variations of the colored material among the ultracentrifuge precipitates (UPS) from separator-prepared buttermilks was of interest. Not enough of the UPS was available from the photographed series. However, large quantities of bulk UPS from each wash had been stockpiled so that it would be available for exploratory experiments. Reflectance measurements were made on the freeze dried bulk UPS both before and after Soxhlet extraction. Both extracted and non-extracted materials were used to evaluate any role of carotenes in the results obtained. Some of the typical spectra obtained are plotted in Figure 6. The shaded area covers the spectra which were not plotted. A broad reflectance minimum, corresponding to an adsorption maximum, was observed between 420 and 480 mμ; a smaller minimum was observed between 350 and 370 mμ; little change was observed above 600 mμ and consequently the values obtained up to 1000 mμ were not plotted.

The lipids that were Soxhlet extractable did not influence the spectral characteristics of IM from the several UPS preparations; 3UPS before and after Soxhlet extraction showed the lowest reflectance mini-

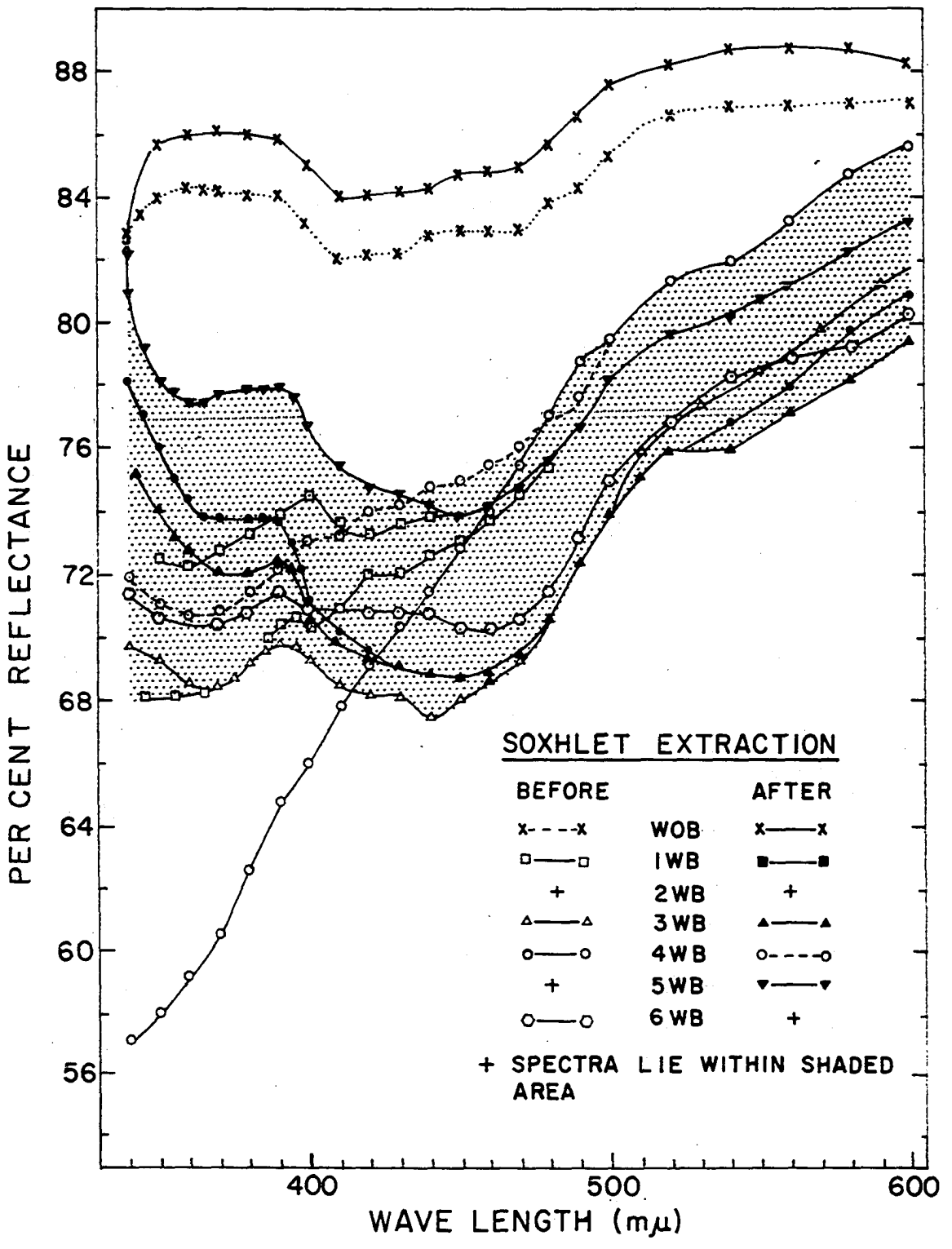

Figure 6. Reflectance spectra of freeze dried ultracentrifuge buttermilk precipitates

mum at 440 - 460 mμ and 0UPS, the highest. The 4UPS sample before and after Soxhlet extraction did not show a well defined minimum at 440 - 460 mμ; at 350 - 370 mμ the latter did show a minimum, whereas the former continued to decrease through 340 mμ. The 4UPS samples, before and after Soxhlet extraction, were yellow in color, whereas the 1, 2, 3, 5 and 6UPS samples were reddish-brown.

The changes among the minimum reflectance values at 440 to 460 mμ were believed to result from color changes among the non-lipid portions of the UPS samples.

Groves (34) studied the absorption spectra of his red protein in NaOH-glycine buffer (pH 9.9). He obtained a broad absorption band with a maximum at 470 mμ and minimum at 400 - 410 mμ. Blanc and Isliker (13) reported a maximum at 455 and a minimum at 400 mμ for their red protein preparation. The findings of these investigators suggested that the concentration of observed red material in the UPS samples (Figure 4, C and D) influenced the characteristics of the measured reflectance spectra (Figure 6). Consequently, the average reflectance value obtained from the readings at 440, 450 and 460 mμ from all of the samples was used to plot the curves in Figure 7. Regardless of the presence of Soxhlet extractable lipid, the lowest reflectance values at 440 - 460 mμ were obtained in the 2UPS and 3UPS samples, a fact which supports the visual interpretation that the red color was concentrated in the buttermilks from two- and three washed centrifuge- and separator-prepared creams samples (Figure 4, A - D). Beyond three washes the minimum reflectance values increased to 4UPS, then decreased through 6UPS; color differences occurred among the

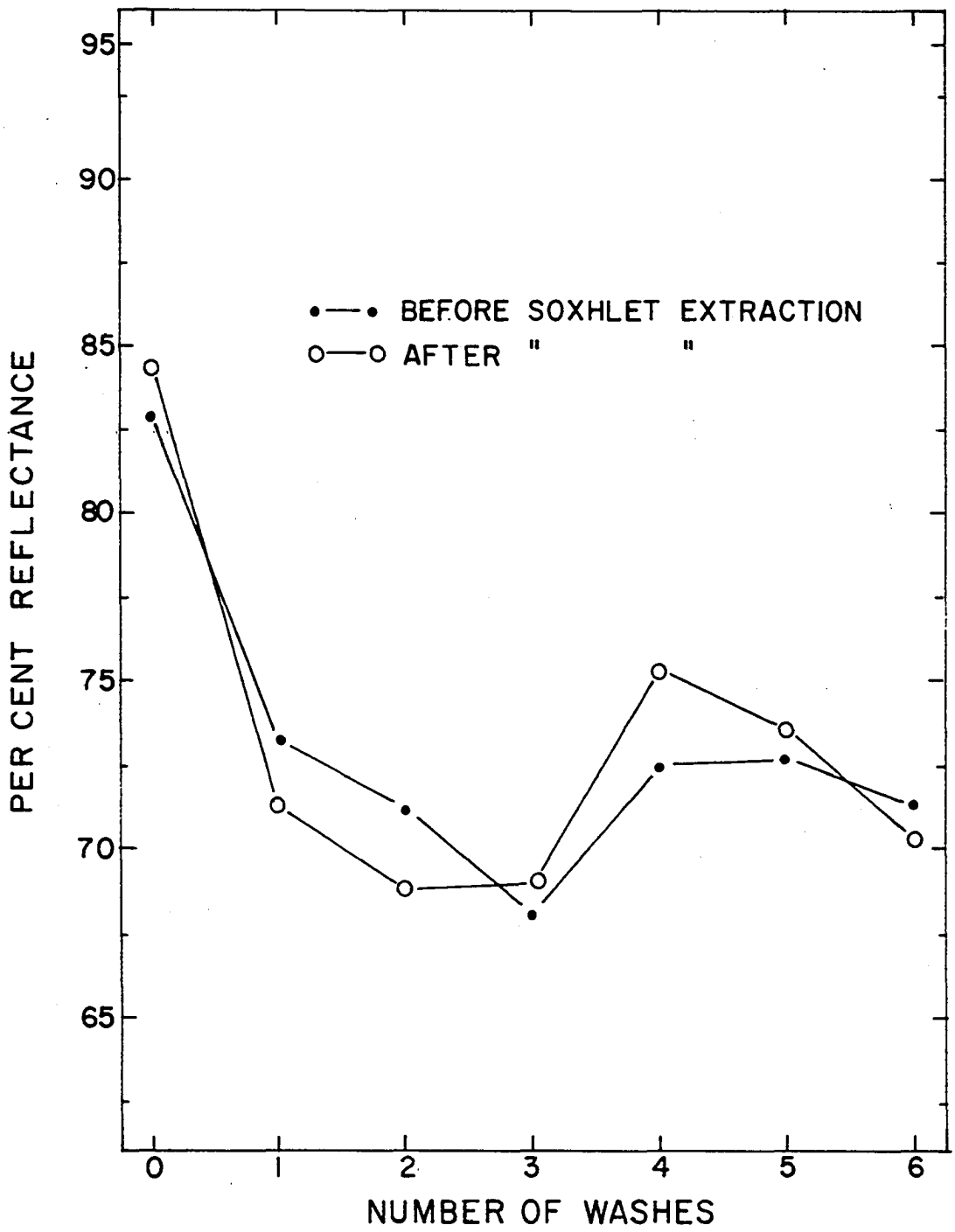

Figure 7. Reflectance values of ultracentrifuge buttermilk precipitates at 440 - 460 millimicrons

4UPS - 6UPS samples but the decreasing reflectance values did not agree with the visual interpretation that the red color continued to decrease with progressive washing by means of the cream separator.

Up to this point the reflectance minimums at 350 - 370 mμ were ignored. However, when the average reflectance value obtained from the readings at 360 and 370 mμ from all of the samples were plotted against the individual washes, the curves presented in Figure 8 were obtained. With the exception of the 2UPS sample Soxhlet extraction appeared to influence the minimum reflectance values at 360 - 370 mμ to a greater degree than at 440 - 460 mμ (Figure 7). This was especially true of the 1UPS and 4UPS samples. The changes in minimum reflectance values at 360 - 370 mμ with the number of washes employed were not in agreement with the color changes observed among the UPS samples (Figure 4, C). The measurement of the reflectances of these heterogeneous samples appeared to be sensitive to materials other than the red colored components.

It was interesting to find that the reflectance spectra in Figure 6 resembled the characteristic absorption spectra of metalloflavoproteins (64). Theorell (100) pointed out that riboflavin and flavin adenine dinucleotide (FAD) show characteristic absorption maxima at 260, 375 and 450 mμ, respectively; the absorption maxima at 375 and 450 mμ were about equal. Mahler (64), in his review, presented the spectra of butyryl coenzyme A dehydrogenase, a copper-containing metalloflavoprotein, which showed absorption maxima at 375 and 450 mμ; absorption at 450 mμ was stronger than at 375 mμ. It was interesting to compare the minimum reflectance values at 360 - 370 and 440 - 460 mμ; beyond the first wash the minimum reflectance values among the Soxhlet extracted samples were lower

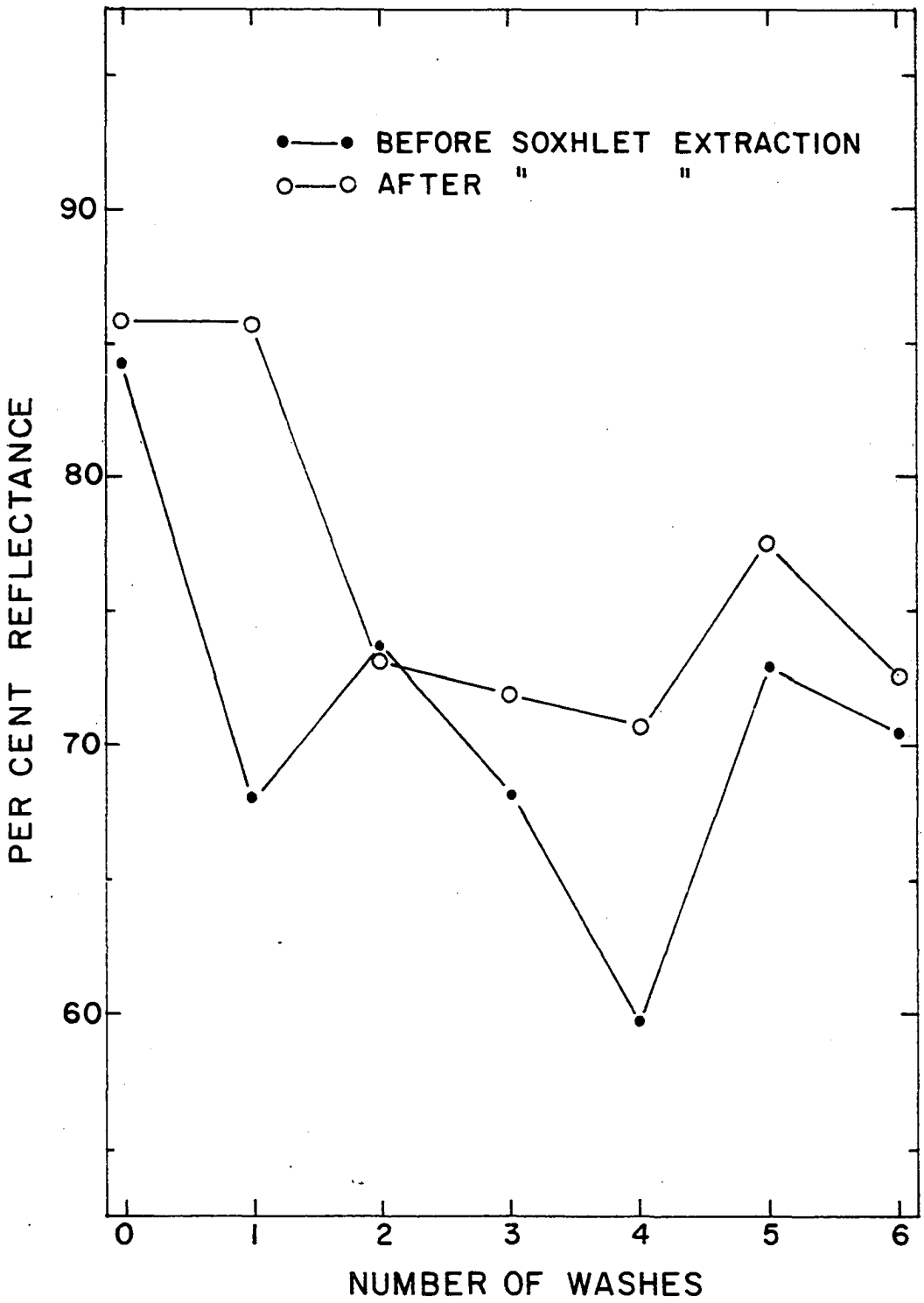

Figure 8. Reflectance values of ultracentrifuge buttermilk precipitates at 360 - 370 millimicrons

at 440 - 460 mμ, but the unextracted samples were higher than the extracted ones, with 4UPS the only exception. The heterogeneous nature of all the UPS samples prevents assignment of the spectral characteristics observed in this study to any one metal-containing protein on the basis of the differences between the reflectance values at 360 - 370 and 440 - 460 mμ.

Richert and Westerfeld (81) studied the absorption spectra of milk xanthine oxidase and tried to determine the influence of iron on the absorption maxima at 375 and 450 mμ; they were unable to show that iron exerted any influence on the absorption spectrum. The "flavin" group of the molecule apparently determines the spectral characteristics of metalloflavoproteins. Other investigators (2, 37, 67, 112) indicated that the reddish-brown ultracentrifuge precipitate from buttermilk contained xanthine oxidase. The close agreement between the reflectance minima at 440 - 460 and 360 - 370 mμ and the absorption maxima at 450 and 375 mμ of metalloflavoproteins (64), as well as the absorption maxima at 455 and 470 mμ of the iron-containing proteins (13, 34) suggested that the UPS samples contained iron.

Trace metal analysis of ultracentrifuge precipitates from centrifuge- and cream-separator prepared buttermilks

The color aspects of the centrifugal precipitates and the reflectance spectra, together with reports in the literature concerning metals in enzymes and IM, and the presence of enzymes in IM, indicated that an investigation of the presence of and variations in the quantities of trace metals might be of use in interperting changes in IM in going from zero

to six washes. It was considered that a preliminary emission spectrographic investigation should at least give a qualitative and ultimately a quantitative estimate of the metals present.

Spectrographic analysis Soxhlet-extracted and unextracted bulk 4UPS and its nitric-perchloric acid digest were analyzed spectrographically. The presence of lipids in the freeze dried sample and perchloric acid in the wet ash digests prevented successful quantitative determination of the trace metals present. The presence of iron, cobalt, zinc and copper was confirmed. Molybdenum was not detected in the sample; a molybdenum standard added to the sample could not be detected by the method employed. Spectrographic analysis of the wet ash digest looked promising. The use of nitric acid and hydrogen peroxide to wet ash samples would have eliminated interference from perchloric acid. Subsequent removal of interfering phosphorus on ion exchange columns theoretically would have solved the problem. However, the use of an acetic acid-acetate buffer to elute the desired ions from the column would have contributed new interference in the form of carbonyl groups. Dry ashing the samples and dissolving the resulting ash in hydrochloric acid was also considered. However, the presence of chlorides would have prevented the quantitative determination of copper.

Although only a small amount of sample (1 drop) was required for spectrographic analysis, the minimum concentration required for accurate analysis was 10 μg of each metal per ml. Calculations of the expected concentrations of metals in the final wet ash digests of the amounts of samples on hand indicated that the concentration requirement for spectro-

graphic analysis could not be achieved for some of the metals present. As a result colorimetric methods for trace metal analysis were considered.

Colorimetric analysis The use of colorimetric methods for quantitative determination of iron, copper and zinc offered several advantages: nitric-perchloric acid digests containing lower concentrations of metals could be analyzed directly; interference from phosphorus was of no concern. Cation interferences could be prevented by the addition of chemical binding agents or by control of pH.

The colorimetric methods described earlier were used to determine the iron, copper, zinc and phosphorus contents of the bulk 0UPS - 6UPS samples. Calcium and magnesium were also determined.

During the development and subsequent application of the zinc and copper methods to the analysis of bulk UPS samples, cobalt was not detected. Possible explanations have been presented in the discussion under "Evaluation of the accuracy of trace metal methods."

Trace metal analysis of ultracentrifuge precipitates from buttermilks from standardized, centrifuge- and cream separator-prepared creams On the basis of the metal contents of the bulk UPS samples, appropriate amounts of the freeze dried precipitates, 0.500 - 0.520 g of 0UP, 0.200 - 0.220 g of 1UP and 0.100 - 0.120 g of 2UP - 6UP, from the standardized creams were wet ashed. The amounts of calcium, copper, iron, magnesium, phosphorus and zinc found in the UPS and UPC series are presented in Figures 9 and 10, respectively.

By the end of two washes the iron contents of 2UPS and 2UPC were

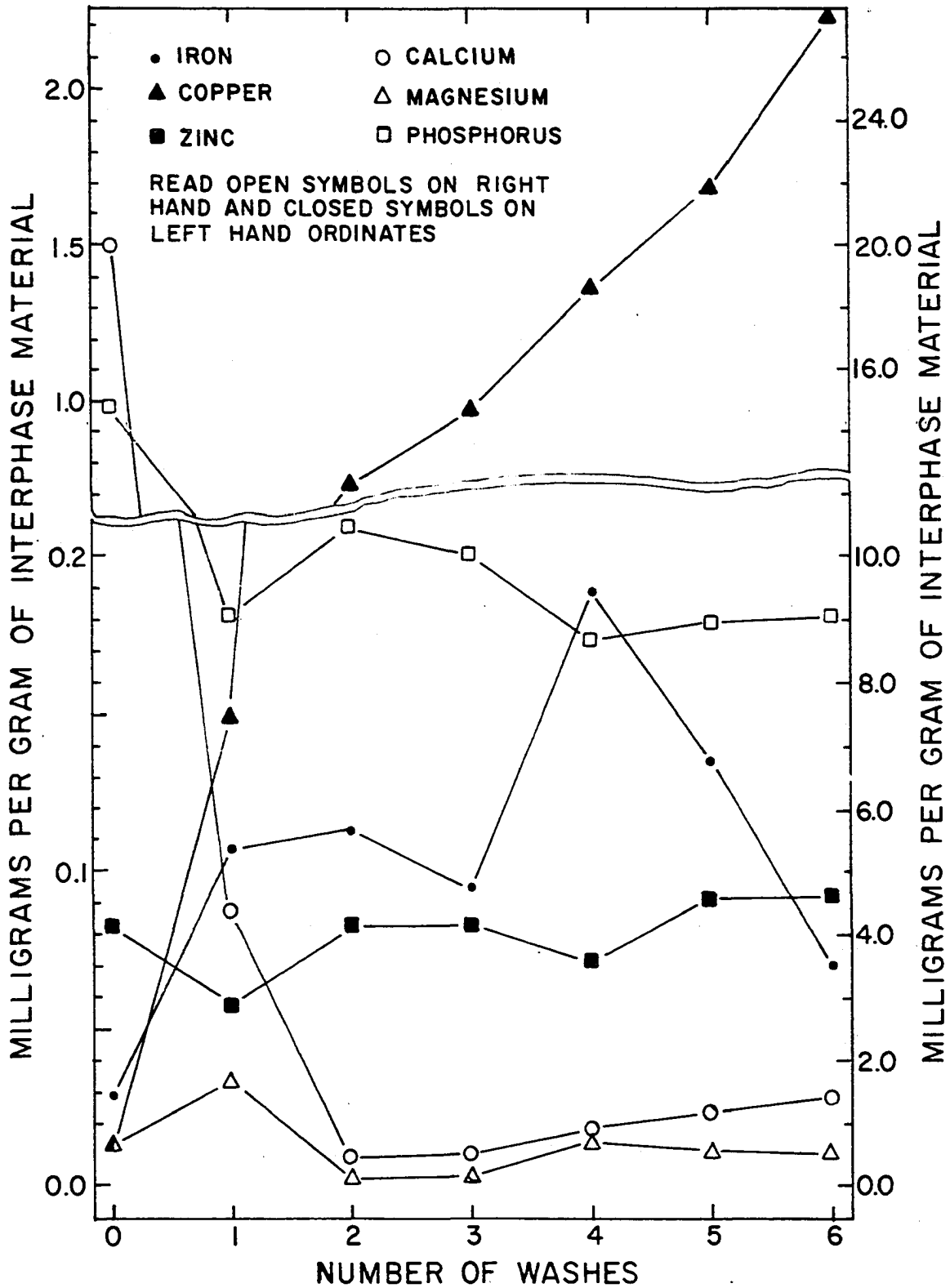

Figure 9. Trace metal contents of ultracentrifuge precipitates from buttermilks from standardized, separator-prepared creams

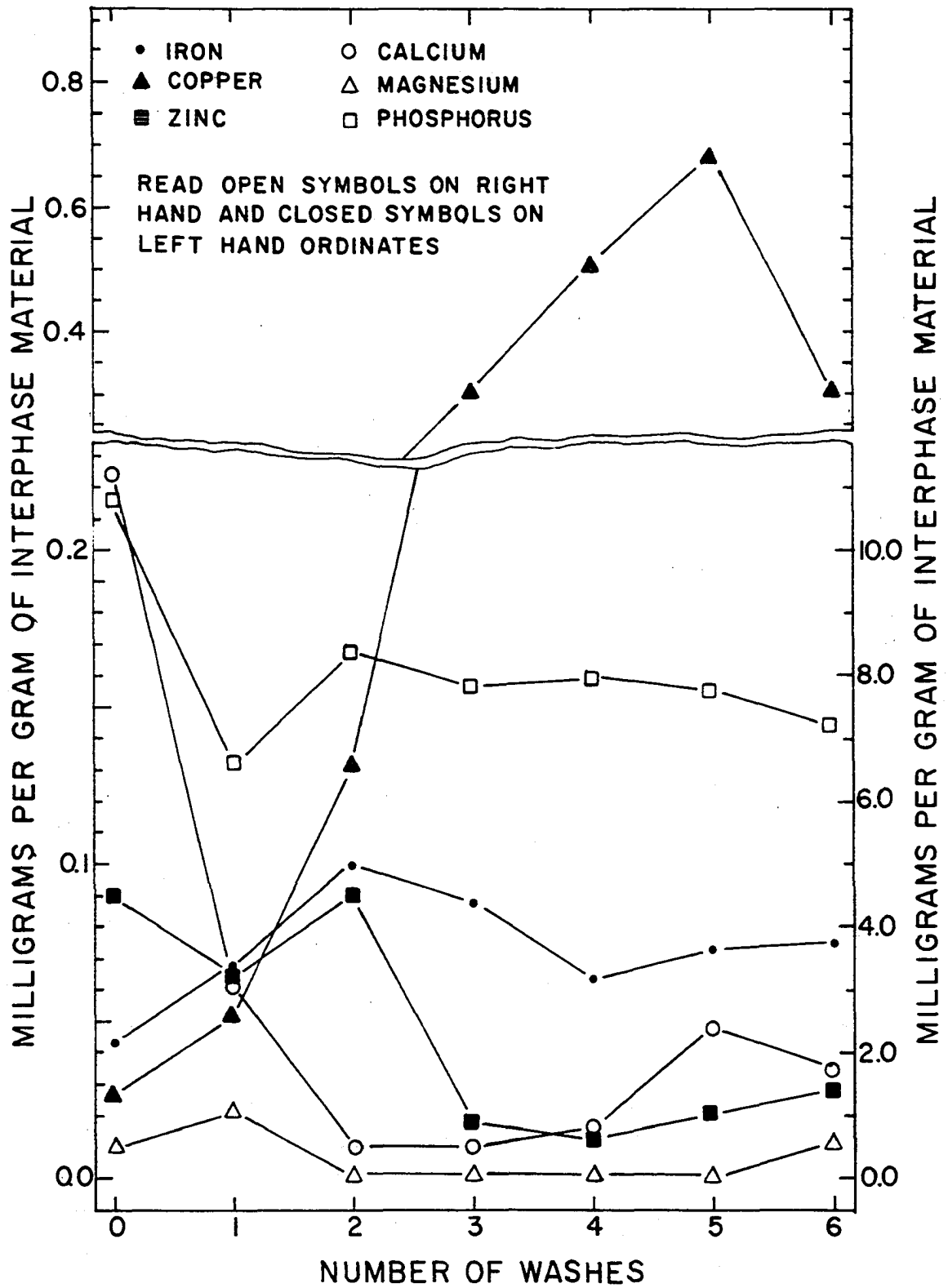

Figure 10. Trace metal contents of ultracentrifuge precipitates from buttermilks from standardized, centrifuge-prepared creams

0.114 and 0.099 mg/g, respectively. As washing progressed the iron content decreased through 3UPC - 4UPC and leveled off at 0.073 - 0.075 mg/g through 5UPC and 6UPC, respectively. The decrease in iron content going from 2UPC - 4UPC followed the decrease in red color observed in the photographs (Figure 4, D); the small changes in iron content of 4UPC through 6UPC were not recognizable in the photographs. The iron contents of 1UPS and 2UPS followed the color changes among the photographed precipitates (Figure 4, C). The small decrease in iron content, 0.114 - 0.095 mg/g, to the third wash could not be distinguished among the colors of the photographed precipitates. The large increase in iron content in the fourth and fifth washes, 0.189 and 0.136 mg/g, respectively, was not in agreement with the obvious loss of red color among the precipitates or the reflectance measurements at 440 - 460 mμ. The sharp decrease, 0.189 - 0.071 mg/g, in iron contents in 4UPS through 6UPS did agree with the observed loss of red color. It was interesting to compare the highest iron content in the 4UPS sample with the low reflectance minimum at 360 - 370 mμ obtained with the non-Soxhlet extracted 4UPS sample in Figure 8. If there was correlation between the two values, certainly the 5UPS sample should have had a lower minimum reflectance value at 360 - 370 mμ than the one reported in Figure 8 since the iron content of 5UPS was second only to 4UPS. These data suggested that the changes in red color among the UPS series did not necessarily reflect the loss of all iron-containing components of IM. On the other hand, there appeared to be some correlation between the red color and iron contents of the UPC series.

Iron and copper contents were higher in the precipitates which had the greater amounts of red color evidenced in the photographs of Figure 4,

C and D. 0UPC precipitates were evaluated as having a greater amount of red-colored component than 0UPS precipitates. The former contained 0.047 and 0.027 mg/g of iron and copper, respectively, compared to 0.029 mg of iron and 0.013 mg of copper/g. Figures 9 and 10, however, indicate that the iron and copper contents of the total UPC and UPS vary independently of each other beyond the second wash. In the UPC series the iron decreased to the fourth wash and increased slightly through the sixth. In the UPS series the iron decreased to the third wash, increased markedly to the fourth and decreased rapidly to the sixth wash. On the other hand the copper continued to increase rapidly from the second to the fifth wash in the UPC. The copper contents reached surprisingly high values, 1.687 and 2.240 mg/g, in 5 and 6UPS samples, respectively.

In UPS a greenish-gray color began to show in the 4UPS sample (Figure 4, C), whereas, in the UPC sample the reddish-brown colored component had not been eroded sufficiently by the end of the sixth wash for the greenish-gray component to become discernable in the photographs (Figure 4, C and D). If the greenish-gray color is associated with a copper-bearing component, the drop in copper content from 5UPC - 6UPC does not appear logical and possibly may have resulted from an analytical error.

The differences in zinc contents were very small, 0.089 mg/g in 0UPC and 0.082 mg/g in 0UPS. The zinc contents were in good agreement in 1UPS (0.065 mg/g) and 1UPC (0.058 mg/g), and in 2UPS (0.090 mg/g) and 2UPC (0.083 mg/g). The decrease of zinc during the first wash by both methods as compared to increase in the iron and copper contents suggest that a sizable quantity of the zinc in the 0UPC (about 27%) and 0UPS (about 29%) samples was associated either with the milk serum proteins which were al-

most completely removed by one wash or that the zinc-containing component lost is extremely labile. It would appear that there are two zinc-containing components, one of which is fairly tightly bound, and one that is labile. The former seems to occur to a greater degree in UPS than in UPC. The latter is lost more slowly in UPS than in UPC.

Zittle, et al. (112) reported that 60% of the alkaline phosphatase activity in milk was associated with the skimmilk. If zinc is a constituent (106) in milk phosphatases, the decrease of zinc after one wash would be in agreement with the removal of phosphatases with the skimmilk. Subsequent increase of the zinc content at two washes could indicate an increased concentration of a zinc-containing protein in IM following removal of more labile IM components or traces of milk serum constituents. Beyond two washes the lability of the zinc-containing protein was increased relative to other components, using the centrifuge, whereas the cream separator had no effect. No explanation can be offered for the differences between the two types of precipitates.

In the above discussion of the changes in iron, copper and zinc contents, their possible relationship to the color changes observed among the UPS and UPC samples was considered. Metal containing proteins, chiefly enzymes, are known to be present in milk and in some cases are associated with IM.

It was interesting to consider a possible relationship between the progressive loss of xanthine oxidase activity reported by Zittle et al. (112) and the loss of red color observed in the UPS samples since Zittle employed the cream separator for preparing washed creams. Although xanthine oxidase contains iron, the loss of red color does not agree

with the high amounts of iron remaining in the 4 and 5UPS samples. Tauber (99) indicated that milk lactoperoxidase contains iron and was brownish-green in color. This suggested that more than one iron-containing protein was present in IM, and the observed green color in the 5 and 6UPS samples may be partially representative of a second less labile iron-containing protein. Zittle, et al. (112) also attributed lability of alkaline phosphatase to repeated washing. Although the metallic constituents associated with milk phosphatases have not been investigated, zinc has been considered to be present in alkaline phosphatases from other sources (106). Groves (34) isolated a greenish-yellow protein which he found to contain a high amount of acid phosphatase activity. Assuming that milk acid- and alkaline phosphatases do contain zinc, the green color of the 5 and 6UPS samples may also be partially represented by a less labile phosphatase. Aside from the red-colored, copper-containing protein isolated from milk by Dills and Nelson (30), ascorbic acid oxidase is the only other copper-containing protein found in milk to date. Its association with the IM has not been confirmed. Pure preparations of the enzyme are greenish-blue (99). It was conceivable that the green color in 5 and 6UPS samples might have resulted from the presence of a copper-containing protein and possibly an iron-containing protein. The almost linear increase in copper contents from 0 to 6UPS and 0 to 5UPC suggested that such a possible green-colored, copper-containing protein was concentrated in IM as washing progressed, and its color finally dominated the precipitate, after the masking red color of the more labile iron-containing protein had been lost. Since the red color remained through 6UPC it appeared that the centrifugal washing method

employed recovered more of the labile IM proteins, as compared with separator washing, although this was not true with the zinc-containing component.

From previous interpretations of the photographs it was concluded that almost complete removal of skimmilk casein was achieved by the end of the second wash. The rapid decrease in calcium from 0UP - 2UP observed in Figures 9 and 10, the rapid decrease in phosphorus from 0UP - 1UP and the loss of magnesium from 1UP - 2UP, regardless of the washing method, supports this conclusion. The gradual increases in calcium contents from the third through sixth washes, with both washing methods, may have resulted from a proportionate increase of a calcium-binding protein in the IM as a result of the losses of other metal binding proteins. The phosphorus content increased sharply in 2UPC and 2UPS. Following a decrease to the third wash, the phosphorus content remained fairly constant from 3 through 6UPC. The phosphorus content continued to decrease from 3 - 4UPS then increased slightly to 6UPS. The more rapid changes between 2UPS and 4UPS may result from destabilization during washing resulting in the loss of IM phospholipids. The absence of severe oiling off in the centrifuge-prepared creams may have minimized the loss of IM phospholipids. Changes in the phosphorus contents among the UPS and UPC samples may have also resulted from changes in phosphorus-containing protein contents in the IM.

The presence of phosphorus in the non-lipid portion of IM has been reported by several investigators (41, 80, 82, 105, 111). Phosphorus contents of Soxhlet residues from bulk UPS preparations were known. Based on the amount of Mojonnier-extractable lipid remaining in the Soxhlet resi-

dues, the contribution to the total phosphorus contents by the presence of phospholipids was calculated. These calculations are discussed in a later section. Among all of the UPS samples analyzed, the calculated phospholipid phosphorus did not account for the total phosphorus content of the Soxhlet residues. Consequently, the changes in the phosphorus contents among the UPC and UPS samples beyond the second wash indicated changes occurring among phosphorus-containing proteins as well as in phospholipids.

Assuming the variations in metal contents among the UPS and UPC samples reflect the degree of lability of metal-containing components in the IM to repeated washing, the data presented in Figures 9 and 10 would be helpful in establishing the number of washes to use and the method of washing cream. Regardless of the method used, the iron, zinc and phosphorus (after the initial sharp phosphorus loss) contents reached a maximum after two washes. Since the calcium and magnesium contents were negligible after two washes by either method, virtually complete removal of the skimmilk caseinates appears to have been achieved. On the basis of these observations, two washes by either method would be recommended for the preparation of IM.

The iron and phosphorus contents did not fluctuate as rapidly beyond the second wash among the UPC samples as did the corresponding values among the UPS samples. These data supported the observations of more uniform red color among the UPC samples and the emulsion stability among the corresponding washed creams. Consequently, the use of the refrigerated centrifuge would appear, on this basis, to be preferred to the cream separator. The differences in zinc contents between UPC and UPS samples beyond the

second wash would not support this recommendation, if more than two washes were employed. The rates of change of the copper contents would indicate a loss of components beyond one wash that was greater by separator than by centrifuge washing; a comparison of the copper contents of OUPS and 1UPS with OUPC and 1UPC indicates that there is a greater total loss of copper or a greater inclusion of non-copper-bearing components in IM from the centrifuge than from the separator. A similar statement can be made concerning calcium, magnesium and phosphorus but not of iron and zinc.

It would appear that neither method retains all of the original IM and that it will be necessary to study this subject further before a final conclusion can be reached.

Additional Characteristics of Isolated Interphase Materials

Quantitative and qualitative estimation of gross lipid fractions

Interest in the lipid portion of the IM resulted from a desire to completely remove IM lipids and obtain quantitative yields of lipid-free IM by the isolation procedures employed, and to determine whether the total phosphorus content of crude IM resulted from the presence of phospholipids or a combination of these and a phosphorus-containing protein.

Separation of lipids from non-lipid IM was attempted. Soxhlet extraction with ethyl ether of freeze dried, acid-precipitable and ultracentrifuge-precipitable IM was used to remove lipids "loosely associated" with the IM. The resulting Soxhlet residues were then treated by either the Mojonnier (5) or Bloor (14) extraction procedure to remove IM lipids more "closely associated" with the IM.

The amounts of neutral lipid and phospholipid contained in the Soxhlet extract were estimated on the basis of the solubility of the petroleum ether- or ethyl ether-soluble fraction from the Soxhlet extract in acetone; acetone-soluble phospholipids were determined by precipitation with magnesium chloride.

The amounts of neutral lipid, phospholipid and free fatty acids contained in the Bloor extract of the Soxhlet residue were estimated by the fractionation procedure described by Bloor (14).

Lipid content of acid-precipitable interphase materials from combined buttermilk and butterserum from centrifugally-prepared washed cream Soxhlet extraction of a freeze dried sample of acid-precipitable, pH 4.6 - 4.7, buttermilk plus butterserum (APBB) from six-times washed cream (6WC) was carried out. The Soxhlet-extractable material represented 61.15% of the freeze dried APBB; increasing the Soxhlet extraction time from 24 - 36 hr increased the yield to 61.43%. This increase was considered negligible, and 24 hr was employed throughout this study.

During the Soxhlet extraction of APBB an interesting observation was made. Considerable cloudiness appeared in the ethyl ether on its first contact with the APBB contained in the extraction thimble. As extraction progressed, the concentration of insoluble material increased in the solvent reservoir. Possible contamination from the extraction thimble and cotton plug was considered. However, extraction of an empty thimble and cotton plug with ethyl ether did not yield any solvent-insoluble residues. Chemical characteristics of this material will be discussed in a later section.

After weighing the total Soxhlet extract in a tared Mojonnier dish, the crude lipids were dissolved in petroleum ether in an attempt to solublize a larger portion of the crude lipid fraction. The petroleum ether-insoluble and -soluble fractions were separated according to the previously described procedure. The petroleum ether-insoluble fraction represented 4.03% of the total Soxhlet extract and 2.46% of the original APBB sample. The petroleum ether-soluble fraction was redissolved in acetone after its weight had been determined. Acetone-soluble, neutral lipids and phospholipids, and acetone-insoluble phospholipids were separated according to the procedure described under "Methods". Acetone-soluble, neutral lipids represented 88.84% of the total Soxhlet extract and 54.59% of the original APBB sample (Table 5); the combined weights of the acetone-soluble and -insoluble phospholipids represented 6.99 and 4.36% of the total Soxhlet extract and original APBB, respectively.

Quantitative and qualitative estimation of the "more firmly bound" lipids remaining in the Soxhlet residue was achieved using the modified Bloor extraction and fractionation procedures (14). The total Bloor extract represented 22.39% of the weight of Soxhlet residue used and 8.70% of the original weight of APBB. Upon fractionation, the Bloor extract was found to contain 78.50% phospholipid, 14.03% free fatty acids and 7.47% neutral lipids; calculated as a percentage of the original weight of APBB, the corresponding values were 6.71% phospholipid, 1.20% free fatty acids and 0.62% neutral lipid. A summary of the lipid composition of the Soxhlet extract and residue is presented in Table 5. By estimating the total lipid and individual lipid fractions contained in the Soxhlet extract and residue as percentages of the total weight of APBB

Table 5. Approximate gross lipid composition of acid-precipitable buttermilk plus butterserum (APBB) from centrifuge-prepared, six-times washed cream

Fractions	Freeze dried buttermilk-butterserum			Thompson et al. (104)	
	Soxhlet extract (%)	Bloor extract[a] (%)	Total (%)	Scheme I[b] (%)	Scheme II[c] (%)
Total lipid	61.15	8.70	69.85[d]	67.51	43.76
Neutral lipid	54.59	0.62	55.10	49.49	31.19
Phospholipid	4.36	6.71	11.07	13.76	12.57
Free fatty acids	-----	1.20	1.20	4.26	-----

[a]Total lipid and lipid fractions obtained by Bloor extraction (14) and fractionation of Soxhlet residue

[b]Scheme I: BMBS from four-times washed cream, pervaporated and freeze dried; lipids extracted with 1:2 (v:v) ethanol in ether and fractionated on a silicic acid column

[c]Scheme II: Started with uncooled raw milk; final BMBS from four-times washed cream was extracted with ether to remove free fat prior to freeze drying. BM lipids extracted and fractionated as in Scheme I

[d]Contained 2.46% of the weight of freeze dried buttermilk butterserum; was ethyl ether, petroleum ether-insoluble material

used, the resulting values could be added together to obtain the total lipid and individual lipid contents of the whole APBB sample. These values are compared in Table 5 with the data reported by Thompson et al. (104).

The total lipid and neutral lipid fractions from APBB were higher, 69.85 and 55.10%, respectively, than the corresponding values obtained by Thompson et al. (104) of 67.51 and 49.49%, respectively. The neutral lipid fraction reported by these investigators is represented by triglycerides and other minor lipid fractions eluted from the silicic acid

column. Also their free fatty acid fraction included some triglycerides which may account for its higher value of 4.26% compared to 1.20% for the free fatty acids from APBB. Only the phospholipid fraction, 11.07%, was lower than the value, 13.76%, of Thompson et al. (104). By extracting the BMBS with ethyl ether prior to freeze drying (Scheme II), they were able to reduce the final lipid content to 43.76%. The decrease resulted from removal of neutral lipids, whereas the phospholipid fraction was virtually unchanged. These findings confirmed our earlier suggestion that acid precipitation of BMBS included considerable amounts of lipid material which were not representative of the real IM lipid. Rimpila and Palmer (82) reported a range of 17.53 to 19.46% phospholipids and 38.52 to 40.42% ether-soluble non-phospholipids estimated from the lipid phosphorus content of ethanol-ether extracts of dried BMBS from two lots of cream washed four and eight times, respectively, using a Sharples centrifuge. The liquid BMBS preparations were "supercentrifuged" to remove free lipid which would account for the low ether-soluble, non-phospholipid content of the final freeze dried BMBS. Palmer and Wiese (76) reported that the percentage of phospholipids, with an average phosphorus content of 3.85%, contained in a crude IM preparation from six-times washed BMBS was 17.5 - 20.0%. The phospholipid content of the Soxhlet residue from our APBB preparation was found to be 17.28%, which was in good agreement with the lower values of Palmer and Wiese (76) and Rimpila and Palmer (82).

The total neutral lipid extracted with ethyl ether from APBB, 55.10%, was higher than the Scheme II, combined neutral lipid, 31.19%, of Thompson et al. (104) and the ether-soluble, non-phospholipid fraction, 38.52 - 40.42%, of Rimpila and Palmer (82). Since no effort was made to remove

undesirable lipids from the BMBS prior to precipitation with acid, these results seem logical.

Based on the above findings, Soxhlet extraction of acid-precipitated IM with ethyl ether, was an essential step for removing contaminating lipids. However, the data in Table 5 suggested that Soxhlet extraction with ether removed some of the phospholipids considered to be a part of the total IM lipid by Thompson et al. (100). These data indicated that although the binding forces between lipid and protein are not broken by extraction of the original aqueous suspension of IM, the bonds are not all of equal strength. Freeze drying apparently ruptures a proportion of these bonds causing approximately 46% of the total phospholipid in the buttermilk to become Soxhlet (ethyl ether) extractable. However, the amount of phospholipids retained after Soxhlet extraction was in good agreement with values reported by Palmer and Wiese (76).

Soxhlet-extractable lipid content of ultracentrifuge precipitates from buttermilk from cream separator-prepared creams A series of bulk UPS preparations were Soxhlet extracted with ethyl ether and the amounts of ethyl ether-soluble and -insoluble material present in the resulting extracts was determined. These findings are summarized in Table 6.

As washing progressed the amount of Soxhlet-extractable material contained in the UPS samples from buttermilk increased to a maximum, 49.50%, by the end of four washes and decreased thereafter. The amount of ethyl ether-insoluble material contained in the Soxhlet extract reached a maximum, 3.57%, by the end of three washes and was variable but lower than the 3W value thereafter. The amount of this material appeared to be af-

Table 6. Ethyl ether-soluble and -insoluble fractions contained in the Soxhlet (ethyl ether) extracts of freeze dried, ultracentrifuge buttermilk precipitates (separator)

Sample	Freeze dried UPS			Soxhlet extract
	Total Soxhlet extract (%)	ether soluble fraction (%)	ether insoluble fraction (%)	ether soluble fraction (%)
0UPS[a]	0.37	0.37	0.00	100.00
1UPS	7.89	5.77	2.11	73.13
2UPS	34.38	30.98	3.50	90.11
3UPS	43.25[b]	39.68	3.57	91.74
4UPS	49.50	47.75	1.74	96.46
5UPS	48.55	47.02	1.53	96.85
6UPS	43.85	41.83	2.02	95.39
6UPS	50.49[b]	42.00	8.75	83.30

[a]Increased to 43.50% by increasing the extraction time from 24 - 40 hr

[b]6UPS extracted with petroleum ether

fected by the number of washes; higher amounts (2.11 and 3.50%) were present in 2UPS and 3UPS samples than in 4UPS and 5UPS samples (1.74 and 1.53%).

The presence of Soxhlet (ethyl ether) -extractable lipid, up to 49.50% in 4UPS in these ultracentrifuge precipitates was not anticipated. Following ultracentrifugation of the buttermilks a sizable lipid plug was obtained in the top of the tube. This plug presumably contained all the free lipid (possibly as unchurned fat globules) present. However, the presence of ethyl ether-extractable lipids in UPS samples suggested that a sizable quantity of "loosely bound" lipids was associated with the IM.

Possible inclusion of some free lipid and unchurned fat globules in the final UPS samples may have occurred during ultracentrifugation. Inclusion of the butterserum in the buttermilk in this study was avoided since most of the solid material contributed by the butterserum was lipid. Soxhlet extraction of ultracentrifuged butterserum precipitate from 6WC yielded a lipid fraction representing 70% of the total precipitate.

The solvent-insoluble material contained in the Soxhlet extract was of interest. Substitution of petroleum ether for ethyl ether during the Soxhlet extraction of 6UPS increased the yield of insoluble material from 2.02 to 8.75% while the soluble lipids extracted remained unchanged, 41.83 vs. 42.00%. This increase suggested that the insoluble material was non-polar, and consequently more soluble in petroleum ether than in ethyl ether. More efficient extraction of the insoluble material by the petroleum ether was also considered. The higher boiling point of petroleum ether (60 - 70 C vs. 35 C for ethyl ether) resulted in the conduction of more heat to the extraction chamber via the connection to the solvent reservoir perhaps causing greater solubility of this insoluble material. The insoluble material was suspended in sodium hydroxide-glycine buffer, pH 12.23, and the resulting suspension was subjected to Mojonnier extraction. Mojonnier-extractable lipid was not obtained. The remaining aqueous suspension yielded a Molisch test of $+1$[4] and contained 9.8 mg of nitrogen per g of solid material. These findings suggested that the

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[4]Definite differences of color intensity and the depth of the purple ring were observed among various samples; the results of the Molisch tests were classified as positive (+ 1), strong (+ 2) and very strong (+ 4).

insoluble material may be a glycolipid or glycoprotein fraction associated with IM. Extraction by ethyl ether or petroleum ether without sustained solution in the solvent even when hot (in the Soxhlet solvent reservoir) is its most unique property.

Mojonnier-extractable lipids contained in the Soxhlet residues of ultracentrifuge precipitate preparations In order to obtain a lipid-free IM preparation the Soxhlet residues were subjected to Mojonnier extraction following peptization in sodium hydroxide-glycine buffer, pH 12.23. The amounts of residual lipids removed from Soxhlet residues of OUPS - 6UPS samples are reported in Table 7. Beyond the first wash 13.05% - 15.72% of the Soxhlet residue was Mojonnier-extractable lipid. The Mojonnier-extractable lipid retained by the Soxhlet residue varied directly as the Soxhlet-extractable lipid content of the crude UPS samples. Beyond the second wash the Mojonnier-extractable lipid retained by the Soxhlet residue showed only a small increase, 14.09 - 15.15%, to the fourth wash; the Soxhlet-extractable lipid of crude UPS increased at a faster rate, 34.38 - 49.50%, by the fourth wash. When petroleum ether replaced ethyl ether in the Soxhlet extraction of 6UPS, a Soxhlet residue containing 12.04% Mojonnier-extractable lipid was obtained, instead of the 13.05% in the ethyl ether residue.

Assuming that the nitrogen present in the Soxhlet residues represented IM proteins, the nitrogen content was calculated on a lipid-free basis and compared to the determined nitrogen present in the aqueous layer following the modified Mojonnier extraction. The data in Table 7 (which are corrected by a glycine-NaOH buffer blank) show that beyond the initial

Table 7. Total nitrogen and Mojonnier-extractable lipid contents of Soxhlet-extracted ultracentrifuge precipitates (separator) and nitrogen contents of these precipitates after Mojonnier extraction

Sample	Soxhlet residue		Lipid-free Soxhlet residue[a]	
	lipid (%)	nitrogen (%)	nitrogen calculated[b] (%)	nitrogen determined (%)
0UPS	0.37	9.01	9.04	9.31
1UPS	2.20	12.46	12.74	12.45
2UPS	14.09	12.32	14.34	12.19
3UPS	15.13	11.98	14.12	13.00
4UPS	15.15	11.59	13.66	11.40
5UPS	15.72	11.89	14.11	11.11
6UPS	13.05	12.21	14.03	10.41

[a] Contained in aqueous layer following Mojonnier extraction

[b] $\frac{\% \text{ nitrogen in Soxhlet residue } (100)}{100 - \% \text{ Mojonnier-extractable lipid}} = \%$ nitrogen in lipid-free residue

separation the calculated nitrogen was consistently higher than the determined nitrogen content of the lipid-free UPS. These data suggest that the Mojonnier extraction procedure removed a portion of the nitrogen from the Soxhlet residue, possibly as a soluble protein or complexed proteolipid. No investigation of this loss of nitrogen was made.

On the basis of these results, the final trace metal investigations were conducted using crude freeze dried UPS and UPC samples in order to avoid possible losses of metal-containing proteins during lipid extraction procedures.

Non-lipid portion of interphase materials

Solubility studies of ultracentrifuge precipitate samples One of the major problems in the study of IM proteins is their low solubility in slightly alkaline buffers. Isolated IM protein fractions have been successfully dissolved in buffers with the help of reagents such as peracetic acid, sodium sulfide or non-ionic detergents (18, 37, 40). We were interested in finding a suitable buffer or combination of buffer plus a reagent which would solublize lipid-free Soxhlet residues of UPS samples without denaturing the protein and producing a final solution suitable for free boundary electrophoresis. A summary of the buffers and buffers plus reagents used is presented in Table 8.

Native caseinates, the caseinates in their natural forms in milk, are considered to be combinations of unknown composition among casein components, calcium and phosphate. In another connection, it was learned in these laboratories, that 80% of these combinations could be ultracentrifuged during 20 hr at 20,000 rev/min, and 100% at about 70 hr at 40,000 rev/min. The 80%, precipitated during 20 hr cannot readily be peptized either mechanically or in usual alkaline buffers. It has been reasoned that if the hypothesis that casein and phosphate are linked through a calcium bridge, and if the solubility of this complex were greater than that of calcium oxalate, the addition of 0.01 - 0.05 M ammonium oxalate to a normal buffer should precipitate calcium oxalate and allow normal peptization of the casein. This was found to be true and free electrophoresis of the native caseinates so treated yielded essentially normal patterns.

Consequently ammonium oxalate was employed in these studies because

Table 8. Buffers and reagents used in an attempt to solubilize non-lipid interphase materials

Buffer	Reagent added to buffer	Final pH
NaOH-glycine-NaCl	-----	9.20
NaOH-glycine-NaCl	-----	11.75
NaOH-glycine-NaCl	0.01M $(NH_4)_2\ C_2O_4$	11.96
NaOH-glycine-NaCl	0.015M $(NH_4)_2\ C_2O_4$	12.09
NaOH-glycine-NaCl	0.070M $(NH_4)_2\ C_2O_4$	11.93
NaOH-glycine-NaCl	-----	12.23
NaOH-glycine-NaCl	0.5% Triton - x - 100[a]	12.02
NaOH-glycine-NaCl	0.01M $Na_5P_3O_{10}$	12.00
NaOH-glycine-NaCl	0.01M $(Na_3PO_4)_6$	12.02
Na-Veronal-Veronal	-----	8.43
Na-Veronal-Veronal	0.01M $(NH_4)_2\ C_2O_4$	8.40
Na-Veronal-Veronal	0.5% Triton - x - 100[a]	8.42
Na-Veronal-Veronal	0.01M $Na_5P_3O_{10}$	8.32
Na_2HPO_4-NaH_2PO_4-NaCl	-----	7.52
Na_2HPO_4 citric acid	-----	2.60
KCl - HCl	-----	1.80

[a]Non-ionic detergent; courtesy Rohm and Haas Co., Philadelphia, Pa.

it had been thought that part of IM might be a "more soluble" caseinate or other calcium phosphate-complexed protein. Regardless of the presence of non-ionic detergents, polyphosphates or ammonium oxalate, complete dispersion of the material was never achieved; the use of highly acid buffers (pH 1.8 - 2.6) was also unsuccessful. In many cases the aqueous layer of a peptized sample in buffer following Mojonnier extraction of the residual lipids looked translucent at room temperature but became opaque while standing for 12 - 24 hr at 4.4 C, and also during dialysis at 4.4 C. A

sodium hydroxide-glycine buffer, pH 12.23, was the best peptizing agent studied. The peptized samples retained enough translucency to permit electrophoretic studies.

Electrophoretic behavior of ultracentrifuge precipitate samples

Soxhlet residues of 0UPS - 6UPS samples were peptized in sodium hydroxide-glycine buffer, pH 12.23, subjected to Soxhlet extraction, and dialyzed for 24 hr against three changes of buffer following attempts to evaporate excess Mojonnier solvents. Samples of 0UPS and native caseinate from the same milk source were treated in the same manner, but the Mojonnier extraction step was avoided. The resulting electrophoretic patterns are presented in Figure 11.

The pattern of native casein in sodium hydroxide-glycine buffer, pH 12.23, was different from patterns obtained using lower pH buffers. Four distinct peaks were observed in contrast to the usual α, β and γ peaks in lower pH buffers. In addition a large false boundary was obtained which suggested that a large portion of the casein did not migrate. The patterns obtained with 0UPS samples before and after extraction suggested that Mojonnier extraction of residual lipids did not affect the electrophoretic pattern in sodium hydroxide-glycine buffer except for the shoulder on the peak of the most rapidly moving component.

The pattern of 1UPS closely resembled the pattern of 0UPS; the shoulder on the most rapidly moving peak was not apparent. The pattern of 2UPS was completely different from 1UPS which indicated the lack of casein in the 2UPS samples. These observations were in agreement with visual observations (Figure 4, A - D) of the photographed UPS samples and subsequent

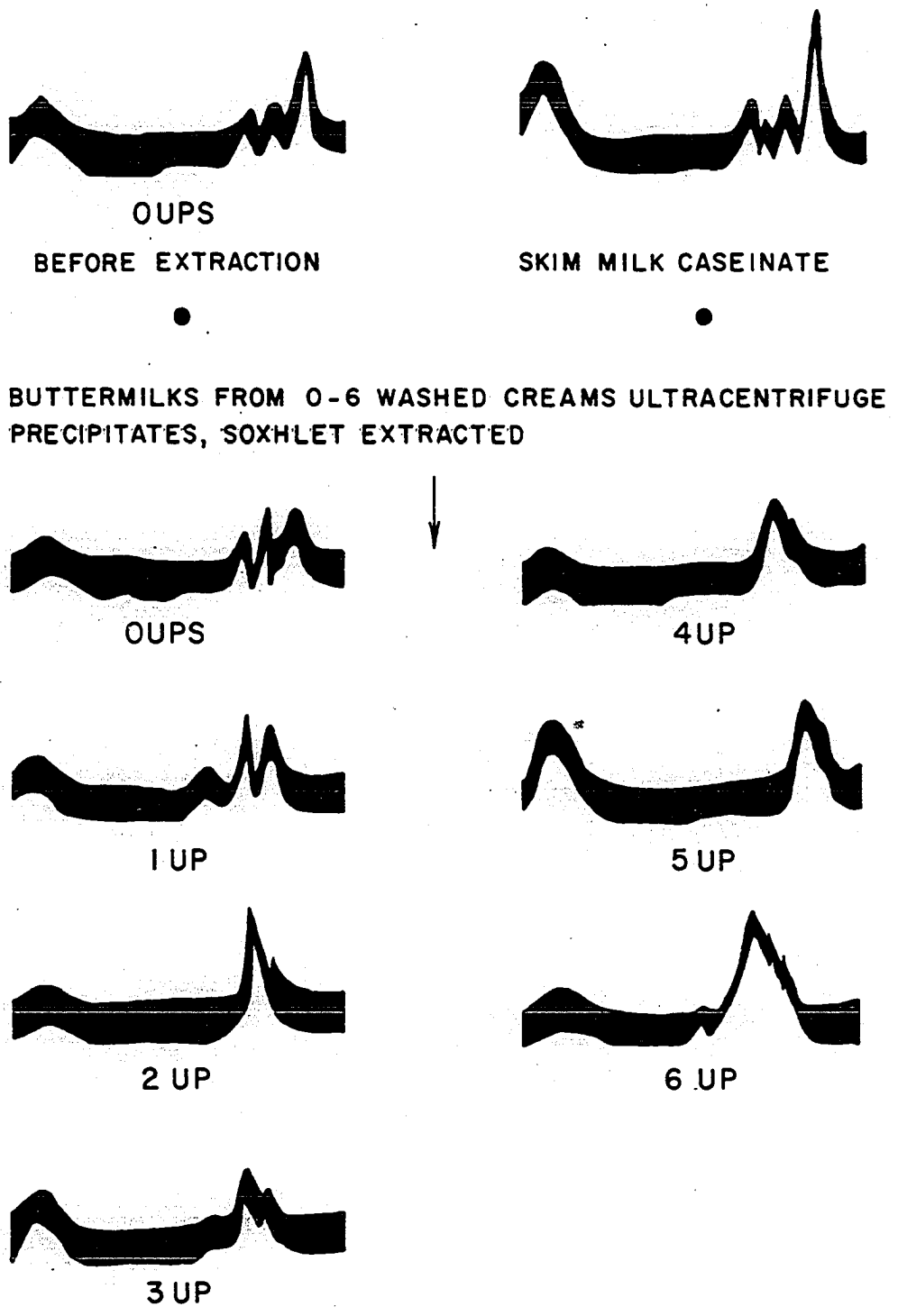

Figure 11. Electrophoretic behavior of the non-lipid fractions from ultracentrifuge precipitates (separator) in sodium hydroxide-glycine buffer, pH 12.23

calcium, magnesium and phosphorus analyses (Figures 9 and 10), which suggested that caseinates as well as other milk proteins were removed by the end of two washes. In general, the patterns of 2UP, 3UP, 4UP, 5UP and 6UP showed one distinct peak with one or two less distinct faster moving peaks; in addition 3UP and 6UPS patterns suggested the presence of a slower moving component.

One interesting observation made during the dialysis of all the samples presented in Figure 11 was the increased volume of the dialysate following dialysis. Samples of 0UPS - 5UPS showed a volume increase of from 68.0 - 111.0%. When ethanol was omitted from the third step of the Mojonnier extraction precedure, the dialysate volume increase was reduced from 111.0 and 100.0% in 3UP and 4UPS, respectively, to 68.0, 74.3 and 67.3% in 0UP, 1UP and 2UPS, respectively. Increasing the solvent evaporation time from 2 - 4 hr following Mojonnier extraction limited the volume increase to 74% in 5UPS while decreasing the dialysis time from 24 - 12 hr reduced the volume increase to 47.0% in 6UPS. A sample of buffer alone was subjected to Mojonnier extraction followed by removal of excess solvent and then dialysis. The volume of dialysate increased 16.2%.

These observations suggested that the presence of ethanol in the peptized sample was responsible for the volume change. However, a series of peptized native caseinate samples in which the caseinate concentrations were 1.0, 2.0 and 2.5% showed volume increases of 24.0, 42.5 and 62.8%, respectively, during dialysis. These samples were not subjected to Mojonnier extraction. These results indicated that the concentration of protein as well as the presence of residual ethanol in the peptized sample

influenced the volume change during dialysis. As a result of volume changes during dialysis, the estimated protein concentrations in the final dialysate ranged from 0.38% in 3UPS up to 0.70% in 5UPS. Consequently, studies on electrophoretic behavior of UPS samples were limited to qualitative interpretations of the effect of repeated washing on IM.

Phosphorus present in non-lipid portion of Soxhlet residues from bulk separator-prepared ultracentrifuge precipitate samples Previous analysis of the Soxhlet residue from APBB revealed that it contained 8.70% of residual lipids, of which approximately 78% was phospholipid estimated by the Bloor fractionation procedure (14). The phosphorus contents of the Soxhlet residues of bulk UPS samples may have resulted in part from the presence of a phosphorus-containing protein. In order to estimate the possible contribution of phospholipids to the total phosphorus content of UPS, Soxhlet residues, the following assumptions and calculations were made.

The assumption was made that the Mojonnier-extractable, residual lipid in the Soxhlet was 100% phospholipid. The phospholipids were represented by a model of lecithin (stearyl-oleyl type) with a molecular weight of 790 and containing one atom of phosphorus. The percent phosphorus in the lecithin model was 3.921%. Finally, 100%/3.921% = 25.5, the factor used to convert percent lipid phosphorus to percent phospholipids (11). Knowing the residual lipid content of the Soxhlet residues, the factor 25.5 can be used in reverse to convert percent phospholipid (residual lipid) to percent phosphorus:

$$\frac{\%\ \text{residual lipid in Soxhlet residue}}{25.5} = \%\ \text{phospholipid phosphorus in the Soxhlet residue}$$

The phospholipid phosphorus was subtracted from the total phosphorus giving the non-lipid phosphorus content of the Soxhlet residues. The results of these calculations as well as the non-lipid:total phosphorus ratios (NLP:TP) are presented in Table 9.

Table 9. Total lipid, total phospholipid and non-lipid phosphorus contents, and non-lipid phosphorus:total phosphorus ratios (NLP:TP) among Soxhlet-extracted ultracentrifuge precipitates (separator)

Sample	Soxhlet residue				
	total lipid (mg/g)	total phosphorus (mg/g)	phospholipid phosphorus (mg/g)	non-lipid phosphorus[a] (mg/g)	ratio NLP:TP
0UPS	3.7	16.36	0.14	16.22	0.992
1	22.0	13.66	0.86	17.80	0.938
2	140.9	8.98	5.52	3.45	0.384
3	151.3	8.73	5.93	2.80	0.321
4	151.5	9.84	5.94	3.90	0.396
5	151.2	11.56	6.16	5.40	0.468
6	130.5	13.17	5.98	7.19	0.546

[a](total phosphorus) - (phospholipid phosphorus) = non-lipid phosphorus

The NLP:TP ratio drops sharply, 0.938 - 0.384, from 1UPS to 2UPS, which would follow the removal of a large quantity of phosphorus-containing milk proteins and the soluble and colloidal phosphates. After reaching a minimum of 0.321 in 3UPS, the NLP:TP ratio increased to 0.546 in 6UPS. A plot of the ratio data (not shown) indicates that the ratio drops exponentially from 1W to 3W and increases linearly from 3W to 6W. This progressive increase suggested that the concentration of phosphorus-containing proteins in IM was increasing as a result of a progressive loss

of other proteins for the estimated phospholipid content of the Soxhlet residue did not change significantly from 3UPS - 5UPS. The changes in the calculated NLP:TP ratios with washing suggest that a phosphorus-containing protein is present in IM, and that it increases as the number of washes (separator) increases.

The NLP:TP ratio drops sharply, 0.938 - 0.384, from 1UPS to 2UPS, which would result from removal of the bulk of phosphorus-containing milk proteins and soluble and colloidal phosphates. A plot of the ratio data (not presented) indicates that the ratio drops exponentially from 1W (0.938) to 3W (0.321) and then increases linearly to 6W (0.546). During this ratio increase (3W to 6W), the phospholipid content, except for 6W, was essentially constant (15.13 - 15.72% of the Soxhlet residue; Table 7). This would indicate that a lipid-containing component decreased at the same rate as total UIM decreased. Because the ratio and the total phosphorus increased, the non-lipid, phosphorus-containing component must be released, by the washing, at a much slower rate than is the lipid component containing phosphorus. In as much as there is a non-lipid component, which varies independently of the phosphorus-containing lipid in IM, it is possible that a phosphoprotein, which is one of the strongly irreversible components, may exist at the fat globule interphase.

Acid-precipitable nitrogen and carbohydrates contained in the ultracentrifuge precipitates Following peptization of the Soxhlet residue from a 6UPS sample in sodium hydroxide-glycine buffer (pH 12.23), and subsequent Mojonnier extraction, an aliquot of the remaining aqueous layer gave a + 2 Molisch test. After removal of a second aliquot for

total nitrogen determination, the pH of the aqueous layer was adjusted to 4.6 - 4.7 with 0.01 N hydrochloric acid. The resulting supernatant gave a + 4 Molisch test and contained only 5% of the total nitrogen in the initial aqueous layer. Peptization of the acid precipitate in sodium hydroxide-glycine buffer, pH 9.2, resulted in a cloudy suspension. By adjusting the pH up to 12.23 with 0.01 N sodium hydroxide, the precipitate went into solution; a + 1 Molisch test was obtained with the final peptized precipitate. The acid-precipitability (pH 4.6 - 4.7) of the non-lipid material contained in the aqueous layer resembled that of crude APBB under similar acid conditions. The strong Molisch test obtained on the acid supernatant suggested the occurrance of acid hydrolysis of possible glycoproteins contained in the 6UPS sample. The concentration of 95% of the total nitrogen in the acid precipitate suggested that the IM non-lipid fraction behaves much like casein at pH 4.6 - 4.7.

SUMMARY AND CONCLUSIONS

The materials at the fat globule interphase, interphase materials (IM), can be divided into two classes, labile and non-labile. The former are easily removed from the interphase by physical means, whereas the latter resist removal to a greater degree. The labile IM have been considered to be constituents of skimmilk, while partially classified lipoproteins and glycoprotein complexes, together with other lipid constituents, have been considered to be among the non-labile IM.

Cream was allowed to rise (gravity) through columns of water in early attempts to "wash" fat globules. Use of a cream separator replaced this method because the fat globules could be washed "more efficiently" by repeated dilution and separation of washed cream; another advantage claimed by this method was the higher yield of IM obtained.

We considered that the gravity washing procedure might yield washed fat globules retaining IM more nearly representative of their natural state than would the cream separator. Separator washing was considered to be the more rigorous of the two methods and to involve greater shear-forces at fat globule surfaces; thus IM obtained should not be representative of the natural IM (71). Attempts to repeatedly wash cream by the gravity technique failed due to inability to overcome a strong cream-water interfacial tension in the columns and to the occurrance of bacterial decomposition when repeated washing was attempted. Consequently, an accelerated gravity washing technique was developed in order to reduce the time required and minimize bacterial decomposition while essentially retaining the gravity washing principle. A refrigerated Model PR 2

International centrifuge was employed at 4.4 ± 1 C. Cream was washed from zero to six times by redispersing the original and subsequent washed creams in 4.4 C redistilled water and centrifuging at 2250 rev/min for 90 min. Macroscopic and microscopic appearances of the cream layers and fat globules, respectively, suggested that the proposed method was effective in washing fat globules without causing the formation of fat-globule clumps or oiling off; emulsion stability of the washed cream was an advantageous property.

A farm cream separator was employed to repeatedly wash cream according to procedures described by others (43, 82). Essentially the method involved dilution of the original and subsequent washed creams to the original milk volume with water and separation at temperatures of 37.7 C (original and first three washes) and 34.9 C (fourth through sixth washes). Although emulsion stability of the washed cream was claimed for this method, oiling off of washed cream was repeatedly encountered. Destabilization of the washed cream suggested that considerable quantities of labile and possibly some non-labile IM were lost as a result of this more rigorous washing procedure.

The cream separator and refrigerated centrifuge were used to wash cream up to six times. Samples of the original cream (WOC) and subsequent washed creams (1WC - 6WC) prepared by both methods were compared regarding emulsion stability of the washed creams, the calculated interphase materials nitrogen (IMN):lipid ratio, the calculated IMN:total nitrogen (TN) ratio and the churning times of WOC - 6WC samples standardized to a 30% lipid content. Regardless of the number of washes

applied emulsion stability was a property possessed only by centrifuge-prepared creams; severe oiling off was an unavoidable property of separator-prepared creams. Emulsion stability of the centrifuge-prepared creams appeared to be correlated with the more normal, longer churning times resembling those of unwashed creams. Centrifuge-prepared washed creams had higher IMN:lipid and higher IMN:TN ratios (Figure 2) through three washes, suggesting that a higher proportion of the more labile IM were retained; beyond three washes the lower IMN:lipid ratios suggested increased lability of IM to repeated washing by the centrifugal method. The higher IM:lipid ratio among the separator creams beyond four washes may have resulted from the inclusion of some types of milk serum constituents through the sixth wash.

During the churning of cream IM are released to the resulting buttermilk and butterserum. Subsequent ultracentrifugation (67, 68, 89) or acid precipitation (37, 40, 76) of buttermilk and/or butterserum have been used to concentrate IM. A series of buttermilk-butterserum combinations (BMBS) were prepared from WOC - SWC separator-prepared creams. By adjusting the pH to 4.6 - 4.7 a casein-like precipitate was obtained. The clear supernatant that resulted gave a negative Biuret and Fehlings test beyond the first wash, whereas a positive Molisch test was obtained through the fifth wash. These results suggested that milk serum proteins and lactose were removed by the end of the first wash; the positive Molisch test through the fifth wash suggested that acid-soluble IM glycoproteins were present in the supernatant or mild acid hydrolysis of precipitated IM glycoproteins, during acidification, released

carbohydrates to the supernatant. Beyond the fourth wash 95 to 98% of the total buttermilk nitrogen was concentrated in the precipitate (Figure 3).

The acid-precipitable (pH 4.6 - 4.7) nitrogen (APN) contained in buttermilks from centrifuge- and separator-prepared WOC - 6WC samples was determined from the differences between total nitrogen (TNB) and "non casein" nitrogen (acid precipitable at pH 4.6 - 4.7) contents of the buttermilks.

APN:TNB ratios in the buttermilks from centrifuge-prepared 2WC - 4WC (0.92 - 0.97) were higher than the corresponding values from separator-prepared 2WC - 4WC (0.91 - 0.92). The higher amounts of APN among the former samples suggested that labile APN was removed from fat globules at a slower rate when the centrifuge was employed (Figure 3). If the changes among the APN:TN ratios were representative of the lability of a major portion of the IM, three washes should not be exceeded by either method; the centrifuge would be preferred since the APN:TNB, as well as the IMN:lipid and IMN:TN ratios in the washed creams, were all higher than the corresponding ratios among separator-prepared creams and subsequent buttermilks.

The behavior of IM from EMBS under acid conditions (pH 4.6 - 4.7), suggested that the IM could be concentrated by ultracentrifugation under conditions similar to those used to precipitate natural caseinates from skimmilk (20,000 rev/min for 20 hr at 0 C). Ultracentrifugation of buttermilk from separator-prepared WOC - 6WC concentrated up to 86.7% of the total nitrogen in the resulting precipitate (Figure 3) after

two washes. Beyond two washes only 82.5 ± 1.5% of the total buttermilk nitrogen was concentrated in the resulting precipitates. These results suggested that some of the IM nitrogen may be low-density lipoproteins or be associated with unchurned fat globules which are not sedimented during ultracentrifugation.

A comparison of the resulting ultracentrifuge precipitates from buttermilk from separator- and centrifuge-prepared WOC - 6WC was made. The red material distributed among the OUPS - 6UPS and OUPC - 6UPC samples is associated with IM concentrated in the buttermilk. Only a trace of red material is present in the natural caseinates from skimmilk compared to its concentration (two distinct layers) in OUPC and 1UPC (Figure 4 B,b). Photographs (Figure 4, A and B) revealed that the amount of precipitate obtained and the ratio of red to white material within the precipitate decreased rapidly as separator washing progressed. Comparative precipitates obtained from centrifuge-prepared buttermilks were larger and showed a higher red to white material ratio to six washes. Progressive dilution of the separator-washed cream was evident from the variation in cream lipid content, 22.8% in WOC to 4.3% in 6WC; by contrast the centrifuge-prepared creams varied in lipid content from 38% in WOC to 30% in 6WC. Consequently, the amount of ultracentrifuge-precipitable IM present in the corresponding buttermilks varied directly with the lipid content of the cream. These results suggested that the effect of the washing method used might be compared using the ultracentrifuge-precipitable IM from buttermilk.

A second comparative series of runs were made using the same raw milk source for the preparation of the centrifuge- and separator-washed WOC - 6WC samples. Dilution of the washed cream with progressive separator washing was prevented by adjusting the cream screw on the cream separator, two full turns in, following the separation of WOC. The resulting creams from both series were all standardized to a 30% lipid content prior to churning. The resulting ultracentrifuge buttermilk precipitates OUPC - 6UPC and OUPS - 6UPS, from centrifuge-prepared WOC - 6WC and separator-prepared WOC - 6WC creams were photographed (Figure 4, C and D); yields of freeze dried OUPC - 6UPC and OUPS - 6UPS were determined and compared (Table 3); the total nitrogen content of the buttermilk was determined and the total buttermilk nitrogen (TNB) to cream lipid ratios were calculated and compared for both washing methods (Figure 5); and the calcium, magnesium, phosphorus, iron, copper and zinc contents of the OUPC - 6UPC and OUPS - 6UPS samples were determined (Figures 9 and 10).

The TNB:cream lipid ratios were higher among 1UPC - 3UPC but lower among 4UPC - 6UPC than corresponding 1UPS - 6UPS samples; these ratios varied inversely with the yields of freeze dried 4UPC - 6UPC and 4UPS - 6UPS samples which suggested that nitrogen to lipid ratio does not necessarily reflect the changes occurring among IM during repeated washing of cream by either method. Color changes among the UPS and UPC samples support this suggestion.

The precipitates from 1UPC - 6UPC were slightly larger in appearance than corresponding 1UPS - 6UPS; higher yields of freeze dried

precipitates supported this observation. The ratio of red to white material was higher and more uniform among 4UPC - 6UPC compared to the progressive loss of red material and the appearance of a greenish-brown material among the 4UPS - 6UPS. Color changes among the 4UPS - 6UPS samples suggested that a labile red material was lost more rapidly when the cream separator was used to wash cream.

An attempt was made to confirm the appearance of more red material among 1UPS, 2UPS and 3UPS and its subsequent loss in 4UPS - 6UPS by measuring the reflectance spectra of previously-prepared, freeze dried, bulk 0UPS - 6UPS samples (Figure 6). The lowest reflectance value at 440 - 460 mμ (Figure 7) agreed with the highest observed concentration of red color in 2UPS and 3UPS (Figure 4, A and C). Reflectance spectra of 1UPS - 6UPS samples (Figure 6) resembled the characteristic absorption spectra of metalloflavoproteins (65).

The observed color changes among the photographed 0UPS - 6UPS samples (Figure 4, A and C) and the reflectance spectra of related freeze dried samples suggested that metal-containing materials were present in the ultracentrifuge precipitates.

Trace metal analysis of the freeze dried UPS and UPC samples from standardized, unwashed and washed creams suggested: 1) color changes among the UPS and UPC samples correspond to changes in iron and copper contents, 2) lowest reflectance values at 440 - 460 mμ were not associated with the highest concentration of iron, 3) the highest iron concentration in 4UPS may be related to its lowest minimum reflectance value at 360 - 370 mμ, 4) the progressive increase in copper contents

with repeated washing among the UPC and UPS samples indicated that a copper-bearing component was among the least labile IM components, 5) the rapid increase in copper reflected the loss of more labile IM at a faster rate among cream-separator prepared samples, 6) a portion of the zinc was lost together with milk serum constituents, and beyond the second wash zinc-containing components of IM were removed at a faster rate when the centrifuge was used, 7) sharp decreases in phosphorus, calcium and magnesium contents by the end of the first wash agree with the observed loss of caseinates from the photographed precipitates, 8) phosphorus increases in the second wash reflect the association of phosphorus-containing components with IM, and 9) changes in phosphorus contents beyond the first wash were represented by changes in phospholipid as well as non-lipid phosphorus.

Beyond three washes iron, copper and phosphorus contents of UPS samples showed greater rates of change than did corresponding iron, copper and phosphorus contents of UPC samples. These changes together with the noticable color changes among the UPS samples and the lower IMN:lipid ratios beyond the third wash suggest that three washes should not be exceeded using the cream separator. The rapid decrease in zinc using the centrifuge suggested that two washes should not be exceeded.

The higher IMN:lipid and IMN:TN ratios, the emulsion stability and more normal churning times of the centrifuge-prepared washed creams, together with the higher yields of freeze dried UPC samples suggested that the use of the refrigerated centrifuge would produce materials more nearly representative of the natural IM.

Interest in the lipid portion of the IM resulted from a desire to completely remove IM lipids and obtain quantitative yields of lipid-free IM by the isolation procedures employed, and to determine whether the total phosphorus content of crude IM resulted from the presence of phospholipids only or a combination of these and a phosphorus-containing protein. Soxhlet extraction with ethyl ether was used to remove lipids loosely associated with freeze dried IM. Resulting Soxhlet residues were then treated by Mojonnier (5) or Bloor (14) extraction procedures to remove IM lipids more "closely associated" with the IM.

The amounts of neutral lipid and phospholipid contained in the Soxhlet extract were estimated on the basis of the solubility of the petroleum ether- or ethyl ether-soluble fraction from the Soxhlet extract in acetone; acetone-soluble phospholipids were determined by precipitation with magnesium chloride. The amounts of neutral lipid, phospholipid and free fatty acids contained in the Bloor extract of the Soxhlet residue were estimated by the fractionation procedure described by Bloor (14).

Lipids contained in acid-precipitable (pH 4.6 - 4.7) IM from a buttermilk-butterserum mixture (APBB) from centrifuge-prepared, six-times washed cream were studied. Of the initial weight of APBB used, 61.15% was Soxhlet-extractable lipid and 8.70% was Bloor-extractable lipid; the fractionated Bloor extract contained 78.50% phospholipids, 7.47% neutral lipids and 14.03% free fatty acids. The Soxhlet extract contained 88.84% acetone-soluble neutral lipids, 6.99% acetone-soluble and -insoluble phospholipids and 4.17% of ethyl ether, petroleum ether insoluble material (Table 5). The high phospholipid content of the

Bloor extract indicated that most of the IM phospholipids are closely associated with the non-lipid IM. The high neutral lipid content of the Soxhlet extract suggested that large amounts of lipid (free lipid and unchurned fat globules) were occluded during acid precipitation of IM. Neutral lipids, chiefly triglycerides, are known (43, 77, 102) to be associated with IM, but appear to be easily removed by ethyl ether (Soxhlet) extraction.

A series of bulk 0UPS - 6UPS preparations were Soxhlet extracted (ethyl ether) and the resulting ethyl ether-soluble and -insoluble fractions were determined quantitatively. Some chemical properties of the insoluble fraction were studied. The remaining Soxhlet residues from 0UPS - 6UPS were peptized in sodium hydroxide-glycine buffer, pH 12.23, and residual, "closely associated" lipids were removed by Mojonnier extraction and determined quantitatively. Aliquots of the peptized Soxhlet residue before and after Mojonnier extraction were removed for the determination of total nitrogen. Aliquots of the Mojonnier-extracted Soxhlet residue were subjected to free electrophoretic studies.

The amount of Soxhlet-extractable lipid contained in the freeze dried UPS samples reached a maximum of 49.50% in 4UPS (Table 6). The high lipid content of these samples suggested that the non-lipid portion influences the density of the IM to a greater degree during ultracentrifugation than does the presence of lipids. An ethyl ether-insoluble material present in the Soxhlet extract represented as much as 3.57% of the original 3UPS sample and 8.26% of the total Soxhlet extract (Table 6). The low nitrogen content, positive Molisch test, and absence of Mojonnier

-extractable lipid were not helpful in attempting to characterize this material. It possessed the unique property of being Soxhlet-extractable (ethyl ether) while remaining insoluble in the chosen solvent.

Mojonnier-extractable lipid present in the Soxhlet extracts from 0UPS - 6UPS did not change as much, 13.05 - 15.72% (Table 7), from 2UPS - 6UPS as did the Soxhlet-extractable lipid, 34.38 - 49.50%. The latter change reflected the occlusion of varying amounts of free fat and unchurned fat globules among the UPS samples during sedimentation of the micellar IM. Failure of the nitrogen content to increase (Table 7) following Mojonnier extraction of the 0UPS - 6UPS Soxhlet residues indicated that Mojonnier extraction removed a portion of the non-lipid nitrogen from IM.

One of the major problems in a study of non-lipid IM is its relative insolubility. Several buffers and buffer plus chemical agents (Table 9) were used in an attempt to solublize lipid-free IM. Sodium hydroxide-glycine buffer pH 12.23 was the best peptizing agent studied. Soxhlet extracts of 0UPS - 6UPS samples were peptized in sodium hydroxide- glycine buffer, pH 12.23 and the residual Mojonnier-extractable lipids were removed. The resulting aqueous samples were dialyzed and subjected to free electrophoresis (Figure 11). The 0UPS and 1UPS patterns closely resembled the pattern obtained from natural skimmilk caseinates under identical electrophoretic conditions. The pattern obtained from 2UPS was completely different, suggesting that caseinates were completely removed by the end of two washes. Electrophoretic patterns of 2UPS - 6UPS each showed a major peak together with one or two

slower or faster migrating components. These minor peaks were not well defined and appeared as shoulders on the major peak.

The total phosphorus content of the Soxhlet-extracted 0UPS - 6UPS samples were determined. Assuming the corresponding Mojonnier-extractable lipid was all phospholipid, the phospholipid phosphorus was calculated (11). The phosphorus contents of the non-lipid fractions from 0UPS - 6UPS were then estimated from the difference between the total and phospholipid phosphorus. The calculated non-lipid phosphorus (NLP) to total phosphorus (TP) ratio was calculated (Table 9). A plot of NLP:TP ratio vs. the number of washes revealed that the ratio decreased rapidly from 1UPS to 2UPS, resulting from the removal of caseinates, followed by a linear increase to 6UPS. During this linear increase the phospholipid content, except for 6UPC, was essentially constant 15.13 - 15.72% (Table 7). These results suggested that a non-lipid phosphorus containing component was released at a much slower rate than is the phosphorus-containing lipid component during progressive washing. These results also support the theory that changes among the phosphorus contents of UPS and UPC samples, Figures 9 and 10, reflect changes in non-lipid as well as lipid phosphorus-containing components with progressive washing.

The comparison of the two methods for washing cream, using a refrigerated centrifuge and a cream separator, has provided some interesting results. The more consistent MN:lipid ratios among the cream separator washed creams, the apparent retention of more non-acid-precipitable MN as suggested by the lower APN:TNB ratios among the corresponding

buttermilks and the ability to process more milk and obtain larger quantities of IM favor the use of the cream separator. The higher iron, copper and zinc contents of the ultracentrifuge precipitates from buttermilk suggest that IM from cream separator-prepared creams contains higher amounts of these metal-containing materials. The emulsion stability and the more normal, longer churning times of the centrifuge-prepared washed creams, together with the more uniform color composition of the corresponding ultracentrifuge buttermilk precipitates and the consistently higher yields of freeze dried precipitates suggest that the use of the refrigerated centrifuge to wash cream provides IM in their most natural state. Based on these results the use of the refrigerated centrifuge is favored, but more data are needed to confirm our selection.

Regardless of which method is used to prepare washed cream and ultimately IM, changes among washed cream IMN:lipid and APN:TN ratios, as well as color and trace metal composition of corresponding ultracentrifuge buttermilk precipitates, indicate that at least two and not more than three washes would be sufficient for the preparation of IM in their most natural state.

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