SOME PROPERTIES OF THE FAT GLOBULE INTERFACE AS RELATED TO LIPOLYSIS IN MILK AND CREAM

by

SAM HORMASJI DALAL

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APPROVED:

Signature redacted for privacy.

Professor of Dairy Husbandry

In Charge of Major

Signature redacted for privacy.

Head of Department of Dairy Husbandry

Signature redacted for privacy.

Chairman of School Graduate Committee

Signature redacted for privacy.

Dean of Graduate School

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INTRODUCTION

Fifty years of research has definitely established the existence in normal milk of a fat-splitting enzyme which is the chief cause of hydrolytic rancidity in milk and milk products. With the introduction of homogenization as one of the unit processes in the dairy industry, the problem of lipase activity was intensified. Now it is an accepted fact that pasteurization, which inactivates lipase, must precede or immediately follow homogenization.

All milks will undergo lipolysis if the conditions are favorable. In some instances, such conditions occur naturally but, in the majority of cases, they have to be induced.

The recent increase in pipeline milking, farm tank storage and tank truck shipment of bulk milks increases the possibilities of large volumes of milk becoming rancid. The increase in storage time, agitation during pumping and long distance shipment, and mixing of raw milks from different sources and perhaps at different temperatures are conducive to lipase action. Hence the problem of rancidity has again assumed considerable importance.

In spite of the fact that considerable work has been done on lipolysis in milk, the mechanism of lipase action is not clearly understood. The lipase enzyme, being proteinaceous, occurs in the continuous aqueous phase but its substrate, fat, is in a dispersed phase; thus the enzyme action must take place at the interface. This work was undertaken to study some of the properties at the fat-

aqueous interface which might play an important role in promoting lipolytic action.

REVIEW OF LITERATURE

Lipase

Lipase is an esterase which preferentially hydrolyzes fats but has only slight activity for the simple esters. The enzyme has low specificity, but it has been shown to exhibit stereochemical and cis-trans specificity. The fatty acid radicals of a triglyceride are not acted upon simultaneously, but are split off successively under the influence of lipase. Thus, di- and mono-glycerides are produced as intermediate products (1, pp.402-403).

Lipases are widely distributed. They occur in the gastric, pancreatic and intestinal juices of vertebrates and many invertebrates. They also occur in plants, especially in seeds, and have been found in other organisms, such as in molds (Aspergillus) and (Pseudomonas), as well.

The most important fat-splitting enzyme in the digestive tract is the pancreatic lipase. Bile salts have been found to accelerate lipolysis largely due to their physico-chemical action in facilitating closer contact between the water-soluble lipase and the fat globule. Although an important feature of lipase is that it also catalyzes the synthesis of fat in the body, very little is known about this phase of lipase action.

Nature and Distribution of Lipase in Milk

Moro (cited from 37, p.255), as early as 1902, demonstrated

the presence of lipase in human milk. A few years later Maas (cited from 37, p.248) confirmed its presence in cow's milk. In 1922, however, Palmer (79, p.62) failed to obtain evidence of a lipase in milk. At the same time, Rice and Markley (96, p.80) found evidence to indicate the presence of lipase in milk by using sugar-saturated creams as substrates. Later, during the same year, Palmer (81, p.210) showed lipolytic activity in the milk of some cows late in lactation. It is now definitely established that at least one lipase is a natural constituent of cow's milk.

Lipase, like other enzymes, is proteinaceous in character and hence is inactivated by heat, e.g. by pasteurization. According to Gould (22, p.875) milk lipase is a non-specific fat-splitting enzyme capable of producing lipolysis upon a wide variety of "fatty" substrates under favorable conditions.

The earliest study regarding the distribution of lipase in milk (and milk products) was reported by Dorner and Widmer (12, p.561). They concluded that skim milk and not the cream, contains the rancidity agent. Later investigators have confirmed this observation (19, p.822), (21, p.787), (55, p.259), (58, p.675), (71, p.246), (74, p.71), (84, p.430), (90, p.143), (112, p.48).

Palmer and Hankinson (84, p.442) demonstrated the presence of an esterase by hydrolysis of diglycol laurate, diglycol oleate, and other esters at temperatures below 10° C. Schonheyder and Volquartz (101, p.179) obtained a highly active preparation of lipase from human milk by the concentration of rennet whey. The activity was

measured on a number of triglycerides. Contrary to these observations, Gould (21, p.786) reported that a considerable amount of lipase activity is removed with casein, because fat homogenized in raw skim milk exhibits greater lipolysis than when it is homogenized into rennet whey prepared from the same lot of skim milk.

Cream separated by gravity has higher lipase activity than either the skim milk (59, p.816) or the centrifugally separated cream from the same source (99, p.834). Kelly (58, p.676) found that samples of dried cream showed considerably higher lipase activity than did samples of whole or skim milk. Pfeffer et al. (90, p.143) reported that the slime from the separator bowl is an excellent source of lipase. The distribution of lipase in cream or in the plasma phase may be dependent on the temperature of separation (104, p.13) and the temperature history of milk (66, p.284). Sharp and de Thomasi (104, p. 13) observed more lipolysis in cream separated at 25° to 30° C. (75° to 85° F.) than in that obtained at 37° to 43° C. (100° to 116° F.). This was confirmed by Roahen and Sommer (99, p.834).

Factors Affecting Activity of Lipase in Milk

The bovine mammary gland tissue which is developed during pregnancy exhibits lipolytic activity (57, p.398). Milk is secreted continuously and stored in milk ducts and cisterns of the mammary gland between milking periods. No lipolysis occurs while the milk is in the udder (114, p.310), immediately upon drawing (33, p.132),

(56, p.88), or while maintained at or near body temperature providing it is not pre-cooled (114, p.310).

All milks exhibit rancidity when favorable conditions are induced (12, p.549), (33, p.134), (67, p.681), (118, p.317). Different treatments which induce rancidity are largely due to the change in the substrate (119, p.11) and not due to the activation of the enzyme lipase, as was erronously concluded by early investigators. That the enzyme already exists in the active form is indicated by its activity when a small amount of fresh raw milk is added to homogenized pasteurized milk (1[illegible], p.554), (70, p.777).

a) Effect of stage of lactation: Since the first observation of Maas (cited from 37, p.248), many investigators have reported increased lipolytic activity in milk obtained from cows late in lactation (6, p.524), (16, p.482), (17, p.527), (37, p.255), (56, p.88), (61, p.683), (66, p.284), (67, pp.679-680), (81, p.210), (112, p.48). On the other hand, some workers have reported that there is no relationship between stage of lactation and lipolysis (34, p.152), (53, p.67), (74, p.66), (90, p.143), (99, p.840). It must be pointed out, however, that negative results may have been obtained because of the different experimental conditions employed.

b) Effect of seasonal variation: General observations tend to indicate increased lipolytic activity during fall and winter months and reduced activity during spring and summer (16, p.483), (17, p.525), (37, p.255), (67, p.679). This may be caused by the fact that during fall and winter months a majority of the cows are late in their

lactation (104, p.14).

c) Effect of feed: A change from dry to succulent feed and vice versa appears to cause a temporary change in the lipolytic activity of milk (16, p.483), (90, p.143), (111, p.36).

d) Effect of milk constituents: Little information is available regarding the correlation between "natural" rancidity and constituents of milk. Sharp and de Thomasi (104, p.15) indicated a possible relationship between chloride concentration and lipase activity. A statistical study by Reder (95, p.377) indicated that milk which exhibits natural rancidity is higher in fat, total solids, lactalbumin, casein, total protein, chloride, titratable acidity and H ion concentration, but lower in lactose content.

Microscopic examination by Fouts and Weaver (16, p.483) showed very little difference in the size, shape or numbers of fat globules between normal and rancid samples of milk.

e) Effect of pH: Investigators agree that the optimum pH for lipase and/or tributyrinase activity is approximately 8.5 (19, p.822), (22, p.870), (47, p.270), (55, p.259), (74, p.61), (89, p.234), (99, p.837). Gould (22, p.874) showed that low pH values of four to five, adversely and permanently affect milk lipase. Thus, acidified whey failed to exhibit appreciable lipolytic activity even though the pH was subsequently raised to the optimum range.

f) Effect of bacteria: Bacteria are always present in raw milk and their metabolic activity interferes with and complicates the study of enzyme action. This is particularly true in the study of lipolysis

in raw milk. In order to minimize bacterial growth, lipolysis in raw milk is carried out at low temperatures. However, certain types of bacteria, viz. Pseudomonas, are psychrophilic and hence grow well at low temperatures. Moreover, certain species of this genus are lipolytic. Therefore, the success of the study of lipase activity depends on the control of bacterial growth without interfering with the action of the enzyme. For this reason many bacteriostatic and bacteriocidal agents have been used. Few, however, have been effective because they also interfere with the action of lipase.

Maas (cited from 37, p.248) was the first to use formaldehyde to inhibit bacterial action to demonstrate lipase activity and since then it has been used quite extensively. However, formaldehyde partially inhibits (or reduces) the action of lipase (33, p.134), (89, p.234). Tarassuk (111), however, reported a study of milk from one cow in which the formaldehyde did not influence the activity of lipase. Herrington and Krukovsky (33, p.134), (34, p.152) concluded from their study that there are two lipases, one being formaldehyde tolerant and the other formaldehyde sensitive. Peterson et al. (89, p.234) reported that a concentration of 0.2 per cent formaldehyde reduces the original activity by 75 per cent. On the other hand, Gould (21, p.782) found that formaldehyde has no influence on the lipase activity of homogenized milk.

Palmer (80, p.1527) reported that acetone, iodoform and chloroform had marked inhibitory effect on lipase activity. Peterson et al. (89, p.235) found no deleterious effect on lipase activity by using

chloral hydrate (0.166%), toluene (0.2%), merthiolate (0.25%), NaF (0.042%) and $CHCl_3$ (0.2%) as preservatives.

Rice and Markley (96, p.71) prevented bacterial growth by utilizing a substrate of boiled cream saturated with sugar. This principle was later used by others (99, p.833), (104, p.7). Roahen and Sommer (99, p.835) observed that shaking increased lipolysis in raw milk but decreased it in cream-sugar substrates inoculated into the same milk.

g) Effect of activators and inhibitors: Enzyme action appears to be activated or retarded by the presence of certain (inorganic and simple organic) salts and/or agents called activators and inhibitors.

Peterson et al. (89, p.236) studied many of the so-called activators (0.0001% of $ZnCl_2$, KCN, $MnSO_4$, $MgCl_2$, cysteine) for the lipase from other sources and found them not only ineffective but in most cases inhibitory to milk lipase. Itoh et al. (48, pp.288-289) reported that human milk esterase activity is enhanced by reducing agents such as l-ascorbic acid, KCN, pyrocatechol and hydroquinone, and is retarded by the presence of oxidizing agents such as iodine, H_2O_2, and pure oxygen. Cu, Fe, Ni, Co, Mn, and Cr act as inhibitors of lipase in butter (11, p.263). Tin and aluminum have no effect. According to Krukovsky and Sharp (68, p.1122) dissolved Cu in the absence of dissolved oxygen in normal whole milk causes no inactivation of lipase. Ramsey and Tracy (93, p.45) mentioned that there is an antagonistic reaction between the agents

of tallowiness and rancidity in raw milk containing Cu-salts. Gould (21, p.781) observed that although Cu inhibits lipolysis in raw milk, it does not influence the lipase activity of homogenized milk. He concluded (21, p.787): "Whether these variations are due to different lipases or whether merely due to physical or physico-chemical changes involving the fat globules has yet to be definitely determined."

Kay (54, p.511) first demonstrated that exposure of fresh milk in glass vessels to sunshine or to a quartz mercury vapor lamp destroys a considerable amount of lipolytic activity; this destructive activity continues for several hours even when milk is put in the dark. The rate of photodestruction of lipase is markedly increased by adding 10 mcg. of riboflavin per 100 ml. of milk, and is greatly diminished if oxygen is removed from the milk before exposure to sunlight. Krukovsky (62, p.286) confirmed that milk samples exposed to light for 30 minutes lost 50 to 80 per cent of their lipolytic activity. Kanan and Basu (53, p.72) also reported that milk tributrinase is partially inactivated by diffused daylight and completely destroyed or inactivated in some cases when exposed to ultraviolet light.

h) Inactivation of lipase by heat: The observations of different investigators are summarized in Table I.

Table I.

TIME AND TEMPERATURE REQUIRED FOR INACTIVATION OF LIPASE

Authors		Temp.	Time
Dorner and Widmer	(12, p.561)	131° F.	for 20 minutes
Gould and Trout	(26, p.102)	145° F.	for 30 minutes
Hajek, Z.	(28)	143° F. 151° F.	for 50-115 minutes instantaneously
Jack et al.	(49, p.18)	150° F. 180° F.	for 30 minutes flash
Kannan and Basu	(53, p.71)	145° F.	for 15 minutes
Krukovsky and Herrington	(64, p.234)	140° F. 155° F.	for 35 minutes instantaneously
Sandelin	(100)	176° F.	momentarily
Hetrick and Tracy	(36, p.887)	185° F. 142° F.	for 5 seconds instantaneous when heated at the rate of 5° F./minute

The discrepancy in the time-temperature of inactivation of milk lipase in milk may be due to the different times required to attain the temperatures used by different investigators.

Relation of Lipolysis to the Physical State of Milk Fat

a) Effect of Temperature:

1) Lipolysis by cooling: Milk from some cows exhibits lipase activity when cooled. This is termed "spontaneous", "natural" or

"uninduced" rancidity (67, p.681), (113, p.674), (114, p.310). Herrington and Krukovsky (35, p.247) showed that the rate of lipase action is influenced by the rate of cooling of raw milk, being retarded by sufficiently rapid cooling which hardens the fat more or less instantaneously. They (63, p.143) concluded that there is an optimum degree of solidification for lipase action, but lipolytic action is retarded when the fat is too thoroughly hardened.

According to Krukovsky and Sharp (69, p.1115) fat globules with natural membranes exhibit a negative temperature coefficient for lipolysis, whereas, a positive temperature coefficient is shown by all re-emulsified fats. Krukovsky and Herrington (63, p.142) showed that the rate of lipolysis, for temperature-activated milk, passes through a maximum at about 10° C. According to Sharp and de Thomasi (104, p.6), when sucrose-saturated skim milk, whole milk and cream were used as substrates the lipase exhibited a positive temperature coefficient.

2) Lipolysis by temperature activation: Considerable increase in lipolysis was secured by Krukovsky and Herrington (63, p.147) by pre-cooling fresh raw milk to 5° C. or lower, re-warming to about 30° C. and then re-cooling to 10° C. or lower. They termed this treatment of inducing lipolysis "temperature activation." Later, Tarassuk and Richardson (114, p.310) confirmed these results but regarded this phenomenon of temperature activation as the effect of cooling on the permeability to lipase of the adsorption "membrane" surrounding the fat globules rather than as the effect on the

physical state of the fat. Rao (94, p.75) recently found that artificial emulsions with phospholipid in the fat phase respond to the temperature activation phenomenon.

Sommer (119, p.11) explained this activation as a process of fractional melting of the fat in the solidified globules. This is substantiated by the observations of Krukovsky et al. (69, p.1115) and Rao (94, p.77) that fractions of milk fat remaining liquid at 10° C., when emulsified in artificial emulsions, do not exhibit the temperature activation phenomenon. According to Sommer (119, p.11):

> When the fat is fully liquified the millions of glyceride molecules (ca. 380 million in a fat globule with a diameter of one micron) in each globule are free to move and there is a minimum of orientation. Rapid chilling will lock the molecules into position on solidification. Fractional melting will create a condition where part of the glycerides in the globule will be liquid and this portion will represent the continuous phase within the globule, and its surface will now be typified by this liquid portion. This change in surface composition, as well as a possible orientation of the molecules at the surface, is apparently what is involved in the temperature activation phenomenon.

b) Lipolysis induced by shaking: Krukovsky and Sharp (67, p.631) showed that shaking of raw whole milk increases lipolysis if the fat is in a partially liquified state. They considered this to be due to the alteration in the surface characteristics of fat globules. Tarassuk and Jack (112, p.48) concurred with this explanation. Hlynka et al. (39, p.1118) substantiated these findings but explained the phenomenon on the basis of increase of the fat/water interface (40, pp.81-82). King (60, p.734) gave the same explanation. Lonein and Jacqmain (71, p.247) enhanced lipolysis by mechanical

agitation, and found that lipolysis was complete after three hours. They further showed that shaking produced considerably more titratable acidity in human milk than in cow's milk.

Hoadhouse (98, pp.73-74) showed that the fat globules in freshly-drawn milk exist as individuals, and that the clusters appearing on storage for 22 hours at 5° C. are broken up by shaking for two minutes at temperatures below 10° C. If shaken at 10°-15° C. for two minutes, churned areas are noticeable in photomicrographs. This indirectly substantiates the idea that disruption of the natural fat globule membrane leads to coalescence of the fat globules.

c) Lipolysis induced by homogenization: All raw milks show considerable and rapid lipolysis upon homogenization (12), (36, p.883), (26, p.102), (118, p.314). Homogenization of milk at low temperature, e.g. at 5° C., fails to develop rancidity. This is explained as being due to the fact that when fat is not in a liquid state (20, p.877), homogenization is not really accomplished (117, p.131). Microscopic observations support this point of view (12, p.546), (117, p.131). Gould (20, p.877) found a maximum amount of lipolysis when milk is homogenized between 40°-46° C. (105°-115° F.). Homogenized milk (which has "re-surfaced" fat globules) exhibits a positive temperature coefficient (21, p.784), (69, p.1117). Investigators who have studied homogenization-induced rancidity agree that most, if not all, of the increased lipolytic action as compared to non-homogenized milk, is due to the increase in the surface area of contact with the enzyme (70, p.777), (90, p.143), (26, p.103).

d) Adsorption on liquid and solid fat globules: Anomalies in surface tension (s.t.) at 20° C. led Mohr and Brockmann (75, p.93) to conclude that the material adsorbed on the surface of solid fat globules differs from that adsorbed when the fat globules are liquid. Sharp and Krukovsky (105, p.745) studied the surface tension and creaming of milk by combining skim milk and cream obtained at different temperatures. They postulated that in natural milk, agglutinin in the plasma phase apparently is associated with an increase in surface tension and the liquification of the fat; whereas, the absence of agglutinin from the plasma phase or its presence on the surface of the fat globules is associated with lower surface tension and the solidification of fat.

Lipolysis and the Fat Globule Membrane

Many investigators have associated lipase activity with changes at the fat-plasma interface, involving the so-called fat globule "membrane".

Extensive studies by Palmer and associates (83) have definitely established that the emulsion-stabilizing substances adhering to the fat globules consist of a conjugation of protein and phosphatides—lipoprotein. The percentages of these two compounds varies from one sample of natural cream to another. Evidence was obtained (Palmer et al.) that the protein-phospholipid complex is dissociable. Palmer (82) suggests that the membrane very likely is an elastic material rather than a solid film, and is capable of stretching into a net-like

or lattice structure.

Jenness and Palmer (50) found that mere aging of milk at low temperatures followed by re-warming before separation greatly decreased the retention of protein and phospholipid by the fat globules during the washing of the cream. Even agitation of aged milk at 33° C. for one hour immediately before re-warming for separation resulted in a cream that oiled off at the second washing.

Maimistova (73) studied the nitrogen-constituents of milk plasma for their capillary activity; and reported that the fat globule membrane possessed the greatest surface activity. He attributed this superior surface activity to the membrane protein or its combination with phospholipids. This was later confirmed by Palmer (83). Belousov (4) stated that the capillary activity and adsorbility of the membrane substance exceeded by 1.5 to 2.0 times that of the plasma substances. He postulated that the membrane consists of three layers: an oriented layer of glycerides, a layer of lecithin, and an outer layer of protein micelles possessing strong polar groups.

Several enzymes are associated with the substances at the fat-serum interface. Schardinger's enzyme (dehydrogenase) (102, p.559) and phosphatase (97, p.835) are retained even after the cream is washed. Other enzymes such as amylase, catalase, reductase (9, pp.172-189), and xanthine oxidase have been found in greater amounts in cream than in skim milk and may be associated with the membrane.

Phospholipids in Milk

The existence of phospholipids in milk has been definitely established by many investigators. The early literature has been reviewed by Mohr, Brockmann and Muller (1932), Horrall (1935), and Holm, Wright and Deysher (1933, 1936). Considerable variations in the phospholipid content in different milk products have been reported, perhaps largely due to different methods used for their determination. Table II summarizes the data of several investigators.

It is generally agreed that the percentage of phospholipid in the fat decreases as the percentage of fat in the milk increases (24). Tayeau (115, p.134) found that 20 per cent of the phospholipids in milk were extracted by ether alone and therefore existed in free state; 30 per cent were extracted by ether plus sodium-linoleate (soap) and hence occurred in loose combination with protein; and the remainder was extracted by hot alcohol and ether, indicating it to be chemically-bound to protein.

According to Palmer and Wiese (87, p.50), differences in solubilities and the P:N ratios indicate the presence of three phospholipids, lecithin, cephalin and a diamino-phospholipid.

Perlman (88) showed that the phospholipid content of the fatty extract of fresh cream shows a variable decrease with the increase in fat content of the cream, indicating a variable loss of the original milk phospholipids in the skim milk. It has been

Table II.

LECITHIN* CONTENT OF DAIRY PRODUCTS

Authors	Milk %	Skim %	Cream %	Butter %	Buttermilk %
Crane et al. (1943)	.013-.052 avg. .0351 (.47-.99) (avg. .705)				
Chapman (1928)	.0345-.0709 avg. .0447 (1.6)	.0082-.029 avg. .0165 (10.73)	.1824-.2155 avg. .1981 45% (.43)		.1036-.1480 avg. .1302 (20.25)
Gould et al. (1940)	.037-.052 avg. .046 (.65-1.25) (avg. .993)				
Heinmann (1939)	.035-.036 (.69-.72)	.016-.018 (19-22)	.156 40% (.387)	.216-.209 (.262)	.126 (16.93-19.55)
Holm et al. (1933)	.0337	.0169	.1816 41%	.1819	.1872
Horrall (1933)	(.857)	(13.91)	(.428)	(.232)	(19.66)
Mohr et al. (1936)	.037	.0155	.1685 23%	.206	.1142
Perlman (1935)	.029		.155 41%		
Thurston et al. (1935)	.0289				.0898

* Represents total phospholipid calculated from lipid P
Values in parenthesis represent lecithin content % of fatty extract

reported (88), (97, p.831), (32) that the lipid phosphorus content of cream, skim and whole milk decreases rapidly with age, even when stored at 40° F.

Thurston et al. (116) reported that agitation of milk causes reduction in the lecithin content of the cream and a corresponding increase in the lecithin content of the skim milk. This observation is in agreement with the work of Mohr and Brockmann (75, p.81), Palmer and Tarassuk (86), and Sharp, Myers and Guthrie (106). Although they do not specify lecithin, they definitely report the release of a surface-active agent during the churning of cream.

Total and lipid phosphorus decrease during the first two washings of cream; after the third or fourth washing it does not change materially (97, p.831), (32).

Methods of Determination of Lipolysis

The production of acidity resulting from the liberation of fatty acids during lipolysis has been employed as a criterion for the estimation of lipolytic activity. The following are some of the general principles which have been used:

a) Titratable acidity of the entire reaction mixture: Methods using direct titrations of raw whole milk (79), (81), cream and homogenized milk have been employed quite extensively. Simple synthetic triglycerides, such as tributyrin (59, p.806) or other esters, have been used as substrates in the reaction mixture.

The greatest disadvantage of the methods based on this principle

is the slow development of fat acidity which entails long incubation periods. Moreover, the constituents of milk such as proteins and mineral salts interfere during titration, causing fading of the end point.

Any synthetic substrate, such as tributyrin, when added to the reaction mixture, considerably shortens the incubation period. The enzyme activity, however, varies considerably for different synthetic substrates and therefore the enzymes involved might be different. For this reason, whenever a synthetic substrate (viz. tributyrin) is used, the results should be expressed in terms of tributyrinase rather than in terms of lipase.

b) Titratable acidity of steam distillate: Titratable acidity of steam distillate of milk and milk products, or of steam distillate of the reaction mixture containing synthetic substrates (99, p.833), has been used to a limited extent. Gould and Johnson (25, p.178) noted, however, that steam distillation of milk fats high in free fatty acids recovered only 5.7 per cent of the acids. On the other hand, steam distillation of the acidified combined alkaline and water washings of ether solutions of milk fats gave a 32.7 per cent recovery. Their study indicated that a steam distillation gave a higher percentage recovery in the case of a fat of low acid-degree than of high acidity.

c) Titratable acidity of churned and of solvent-extracted fat: Some investigators have employed titratable acidity (or acid degree) of churned (99, p.833) or solvent-extracted fat from milk and milk

products (42, pp.719-720), (51, pp.504-505). According to Gould and associates (23, p.172), (52, p.435) the churning method is not satisfactory because water-soluble acids are lost, first during separation of the cream and later during churning. They conclude that although the solvent extraction is not completely satisfactory, it is better than the churning method. Even the improved solvent extraction method, used by them, does not yield quantitative recovery of fatty acids (52).

d) Changes in surface tension: The progress of lipolysis can be followed by the changes in surface tension of the product or derivatives of the product; however, this is not a quantitative method (13, p.519), (36, p.882), (38, p.391), (17, p.521), (111). Although Tarassuk (110, p.156), (111) claims that surface tension measurements are more sensitive than the organoleptic method for detecting rancidity.

The surface tension of normal milk decreases about one or two dynes/cm. during the first two days of storage and remains fairly constant thereafter. On the other hand, as the rancidity develops, the surface tension of milk decreases from a value of 49-51 dynes/cm. to a value of 39-40 dynes/cm. or even lower (111, p.54). According to Tarassuk (110, p.156) a surface tension below 45 dynes/cm. at 20-21° C. is considered definitely rancid when the decrease in surface tension is caused by lipolysis. Dunkley (13, p.519) confirmed this and reported less overlapping of surface tension ranges for "not rancid" and "rancid" classes of milk than

of the acid degree ranges for the same classes of cream. His results are summarized in the following Table III.

Table III.

SURFACE TENSION AND ACID DEGREE OF NORMAL AND RANCID MILK

Class	No. of Samples	Surface tension at 20° C. Avg. dynes/cm.	Std. Deviation	Range dynes/cm.	No. of Samples	Acid Degree* Avg.	Range
Not Rancid	421	46.4	0.95	44.4-50.4	45	0.98	0.44-2.10
Slightly Rancid	193	44.2	1.02	41.8-47.1	15	2.59	1.47-4.88
Rancid	238	40.7	2.52	32.3-46.1	32	3.46	1.09-5.94

* ml. of 1N NaOH per 100 g. of fat

Dunkley states (13, p.518):

> The decrease in surface tension under conditions favorable for lipolysis increased with the fat content. These results indicate that variations in the fat content may account for a large part of the variability in surface tension of normal milk samples.... The percentage of fat has sufficient influence on both the surface tension of milk and the surface tension at which rancidity can be detected organoleptically that it should not be overlooked when surface tension is used as a measure of lipolysis.
>
> Other possible variables in the sampling procedure such as differences in rate of cooling of samples and exposure to light could cause variations in surface tension. (p.519)

Hlynka and Hood (38, p.391) studied the relation between lipase activity as indicated by surface tension measurements and "flavor as judged by odor". Their results with 144 samples of milk gave a correlation coefficient of 0.23, which they considered quite significant! (Since the standard error is not given, this value of correlation coefficient is practically meaningless.) Moreover, they assumed "that the surface tension of freshly drawn milks is fairly constant." (38, p.390)

Cardoso and Wancolle (5) reported surface tension values of 49.4 to 53.3 (average 51.5) dynes/cm. at 20° C. for one hundred samples of raw milk. Pasteurization at 63° C. for thirty minutes caused the average surface tension of twenty samples to change from 51.8 to 53.5 dynes/cm. Changes in the surface tension caused by preservatives were negligible. The surface tension of milk held at 5° C. for ten days decreased by 0.9 to 2.4 dynes/cm., although in the whole period the values fluctuated at random and were lower on the second day than on the eleventh. These authors finally concluded that surface tension measurements are of no value in indicating the quality of milk.

It is interesting to note that the addition of certain fatty acids to raw skim milk lowers the surface tension. This decrease is, however, not the same for different fatty acids and is very slight with the exception of oleic acid.

In connection with surface tension measurements, Sommer states (108, p.672):

> In working with pure liquids the various methods (of measuring s.t.) show good agreement; but on solutions, especially those containing colloidal substances, the results do not agree so closely. The results vary according to whether the tension is measured on a fresh surface (little or no adsorption) or on an old surface (adsorption). The surface tension values that have been reported for milk, therefore, vary somewhat according to the conditions under which the tests were made.

The above statement is very well examplified by the work of many early investigators and confirmed by Sharp and Krukovsky (105, p.745) who report that surface tension of milk at 25° C. is lower when the previously cooled milk is warmed to that temperature as compared to the one which is cooled from higher temperatures.

e) Colorimetric determination of end-products of lipolysis: Krukovsky and Knaysi (65, pp.659-661) described a colorimetric method of determining free fatty acids in milk fat, in which neutral red dye is used as an indicator. Measurements are made against standards containing added oleic acid.

Greenbank and Wright (27) reported a method using naphthyl esters of fatty acids (e.g. a-naphthyl acetate) as substrates. Lipase action hydrolyzes the ester into a-naphthol and the fatty acid (acetic acid in case of a-naphthyl acetate). When 2,6-dibromoquinonechloroimide (B.Q.C.) is added, a purple color due to the formation of benzenone 2,6-dibromoindonaphthol is developed and the color is determined around 5,400 Å. They regard one unit of lipase as liberating one mcg. of a-naphthol.

The assumption is that the enzyme lipase is responsible for

hydrolysis of this substrate. In this connection Nachlas et al. (78, p.354) reported that long-chain fatty acid esters of B-naphthol are preferentially attacked by "lipase", whereas, "unspecific esterase" preferentially attacks short-chain esters.

Recently Hofstee (41, pp.128-130) described a method of determining esterases based on the principle that salicylic acid strongly absorbs ultraviolet light of a wavelength of 290-300 mu, but the esters of salicylic acid, such as acetyl salicylic acid (or aspirin), do not absorb in this region.

Electron Microscope Study of Milk Constituents

So far only three studies have reported on the electron microscopy of milk. The first report published in Sweden, 1949, (30) stated that the fat globules are chiefly of an oval shape, and the membranes of the fat globules have a definite structure and may, therefore, be of histological origin.

A recent report from France (3) stated that the casein particles are approximately spherical and distributed at random with the majority having a diameter of 90 to 125 mu. The coagulation of milk by rennet leads to a three-dimensional network.

An electron microscope study by Hostettler and Imhof (46, pp.351-354, 400-402) revealed that casein constitutes a polydispersed system of roughly spherical particles 40 to 200 mu in diameter. At the isoelectric point, casein assumes a network of thread-like filaments.

EXPERIMENTAL

Products and Their Preparation

Milks: The individual milk samples, used throughout the experiments, were collected at the college barn immediately after milking and before cooling.

Large volumes of mixed milk, whenever necessary, were obtained from the college dairy products plant. Skim milk and cream were usually obtained from the plant. The separation, using a De Laval Air-tight separator, was normally carried out between 40° and 45° F. However, when separation at higher temperatures was desired, it was carried out by means of a Montgomery Ward (Bench model) hand separator. To obtain skim milk practically free from fat, regular skim was run through a Sharples super-centrifuge at 30,000 r.p.m. Ca-caseinate-Ca-phosphate was obtained by the method of Ramsdell and Whittier (92, p.413) by using the super-centrifuge at speeds greater than 45,000 r.p.m.

Pasteurization of the products was carried out in a laboratory water bath, usually at 75° C. for five minutes. Departures from this will be described in connection with the experiments themselves.

Emulsions: For the preparation of emulsions simulating natural milk, a small single-cylinder hand homogenizer was used. In most cases the mixture was passed through the homogenizer three times, but in some cases as many as five times were necessary. These emulsions were often compared microscopically with natural milk for approximate size distribution of the fat globules.

Homogenization at higher pressures was carried out in a small, two-stage Manton-Gaulin Model 25 homogenizer with a 25 gallon per hour capacity. The exact pressures used will be shown with the respective experiments.

Butteroil: In order to obtain butteroil practically free from phospholipid the following method, based on the procedure used by El-Rafey, et al. (14, p.810) was employed. Fresh, sweet cream, unsalted butter obtained from the college creamery was melted in large beakers in an oven maintained at 60° C., care being taken to avoid undue exposure to light. The top layer was skimmed off and the serum was separated from the melted fat using large separatory funnels. The melted fat was filtered through Whatman #12 fluted filter paper in a 60° C. oven, the entire process being accomplished in about five hours. The resulting crystal-clear butteroil was stored at -10° F. in one-pound lots in brown bottles.

Phospholipids: In the early experiments animal lecithin (practical grade) obtained from Eastman Kodak Co. was used. It was partially purified by dissolving in a minimum amount of cold ether, filtering, and adding two volumes of acetone to the filtrate to precipitate the lecithin. The precipitate was re-dissolved in ether and re-precipitated at about 0° C. This gave a cream-colored, sticky precipitate which eventually turned brown even when kept under acetone. This lecithin dissolved with great difficulty in both the fat and aquaeous phases. Moreover, it imparted a brownish color and a definite odor to the product in which it was dissolved. For this

reason, its use was abandoned and replaced by a purified soybean phospholipid product known as "Asolectin" obtained from Associated Concentrates.

"Asolectin" has several advantages. It is a slightly yellow, waxy, granular substance, readily soluble in fat, and easily dispersed in water. According to the manufacturer, it consists of 95 per cent phospholipids comprising about 30 per cent lecithin, 30 per cent cephalin and 35 per cent lipositol (an inositol phosphatide). The phosphorus content is 3.1 per cent. The disadvantage of this product is that it does not contain the same constituents or the same proportion of the phosphatides as those naturally occurring in milk. Moreover, it imparts a foreign flavor to the product.

An interesting observation recorded when using these phospholipids was that the depth of the cream layer in synthetic (or re-made) milks was greater with animal lecithin than with Asolectin, under similar conditions.

Washed creams: The technique of Storch (1897) was used to obtain washed cream (83, p.473). A hand separator was utilized for obtaining large quantities of washed cream, small quantities were recovered by centrifugal separation by using an I.E.C. centrifuge. The procedure was as follows:

Cream was diluted with twice its volume of distilled water at about 38° C. (100° F.), and separated. This process was repeated three times. The cream so obtained is referred to as 4-times washed cream.

Methods

Surface tension: Surface and interfacial tension measurements were made with a Du Noüy tensiometer, the liquid being placed in a petri dish for surface tension. In all measurements of interfacial tension 100 ml. beakers were used as containers and readings were made at about 38° C. (100° F.). Temperature at which surface tension measurements were made is shown with the results, although for later experiments a temperature of 38° C. (100° F.) was preferred because it minimized temperature manipulation and churning. The procedure for measurement of interfacial tension, say, between skim milk and milk fat, was as follows:

With skim milk only in the beaker, the platform was raised until the ring was immersed about 1/2 cm. in the skim milk. A quantity of milk fat was then carefully poured (or layered) on the surface of the skim milk to a depth of approximately 1.5 cm.; just deep enough to prevent the ring from entering the upper surface before the interfacial film broke. The rest of the procedure was the same as in the case of surface tension determination. After every determination the ring was rinsed with water, followed by a mixture of organic solvents, and then flamed. The following table gives surface tension values and standard deviations obtained for distilled water, glycerol and benzene. It also includes, for comparison, the pooled s value of milk for 15 random samples:

Substance	Surface Tension dynes/cm.	Standard Deviation
Benzene (21° C.)	28.9	0.09
Glycerol (21° C.)	62.9	0.16
Double-distilled water (23°-24° C.)	71.5	0.07
Whole milk	-	0.40*

* 0.4 is the pooled s for 15 random samples. By applying Box's method, F = 2.31 with 1 and 1,600 degrees of freedom. This is not significant and, therefore, the hypothesis that the population variances are equal, is verified.

Colorimetric determination of lipase: The method of Greenbank and Wright (27) was followed. To a desired volume of milk a 30 per cent solution of a-naphthyl acetate is added with agitation, to make a 0.15 per cent solution of a-naphthyl acetate. The sample is then incubated at 40° C. for one hour. Twenty-five ml. of the sample is transferred to an erlenmeyer flask and 3 ml. of a 12 per cent solution of $ZnSO_4$ is added with agitation. Six ml. of a 5 per cent solution of Na metaborate ($NaBO_2$) is added with agitation and the solution is filtered immediately. The clear filtrate should have a pH of 7.7 (±0.1). The density of purple color, developed by adding four drops of a 0.04 per cent solution of 2,6-dibromoquinonechloroimide (BQC) is determined using a colorimeter. The authors used a Klett-Summerson colorimeter with a filter transmitting a narrow band near 540 mu. A liberation of one mcg. of a-naphthol may be

considered as representing one unit of lipase.

The authors report that as little as 25 ml. of the sample may be used if it is to be activated by shaking. They, however, used 200 ml. in their work. In order to determine the size of the sample needed (for this study) the following experiment was designed.

Mixed milk was divided into four samples. Samples one and two were 200 ml. each, and samples three and four were 100 and 50 ml., respectively. Sample one was pasteurized at 90° C. for five minutes to destroy lipase, otherwise all the samples were treated alike, as mentioned in the above procedure.

Sample	Size of the Sample in ml.	% transmission at 540 mu
1	200 (control)	85.0
2	200	78.5
3	100	78.0
4	50	77.0

The results indicate that the size of the sample has no significant effect. It was decided, therefore, to use a 100 ml. sample in the colorimetric determinations of lipase in milk.

It was observed in this experiment that the pH of the filtrate was much lower than 7.7, but that a pH of about 7.7 can be obtained by adding ten instead of six ml. of $NaBO_2$ as suggested by the authors. This was true both for the milk of Jerseys and Holsteins. Hence ten ml. of $NaBO_2$ was used.

In order to select a suitable wavelength for measurement of

color formed after addition of BQC, different amounts of a-naphthol were added to a Clark and Lubs buffer of pH 7.6 (KH_2PO_4 and NaOH) to make up ten ml. in the spectrophotometer tubes. The color was measured at different wavelengths between 450 and 650 mu using a Coleman model 6A spectrophotometer. The results indicated a maximum absorption at approximately 600 mu. As this does not coincide with the wavelength suggested by the authors, another experiment was repeated in which the buffer solution was not used and instead the clear filtrate at pH 7.7 obtained after the addition of $ZnSO_4$ and $NaBO_2$ solutions, was taken. The results, illustrated in Figure 1, indicate maximum absorption between the wavelengths of 570 and 580 mu. A wavelength of 575 mu was chosen and used in all experiments. At this wavelength Beer's law is followed up to a concentration of 30 mcg. of a-naphthol.

To determine the accuracy of the method, different amounts of a-naphthol were added to 25 ml. of milk before the addition of $ZnSO_4$ and $NaBO_2$ solutions. The results shown in Table IV indicate that "recovery" of a-naphthol added to milk is not quantitative, as compared to its addition to the filtrate.

The authors of the method did not specify the type of control to be used. This was investigated. The results of four experiments are summarized in Table V.

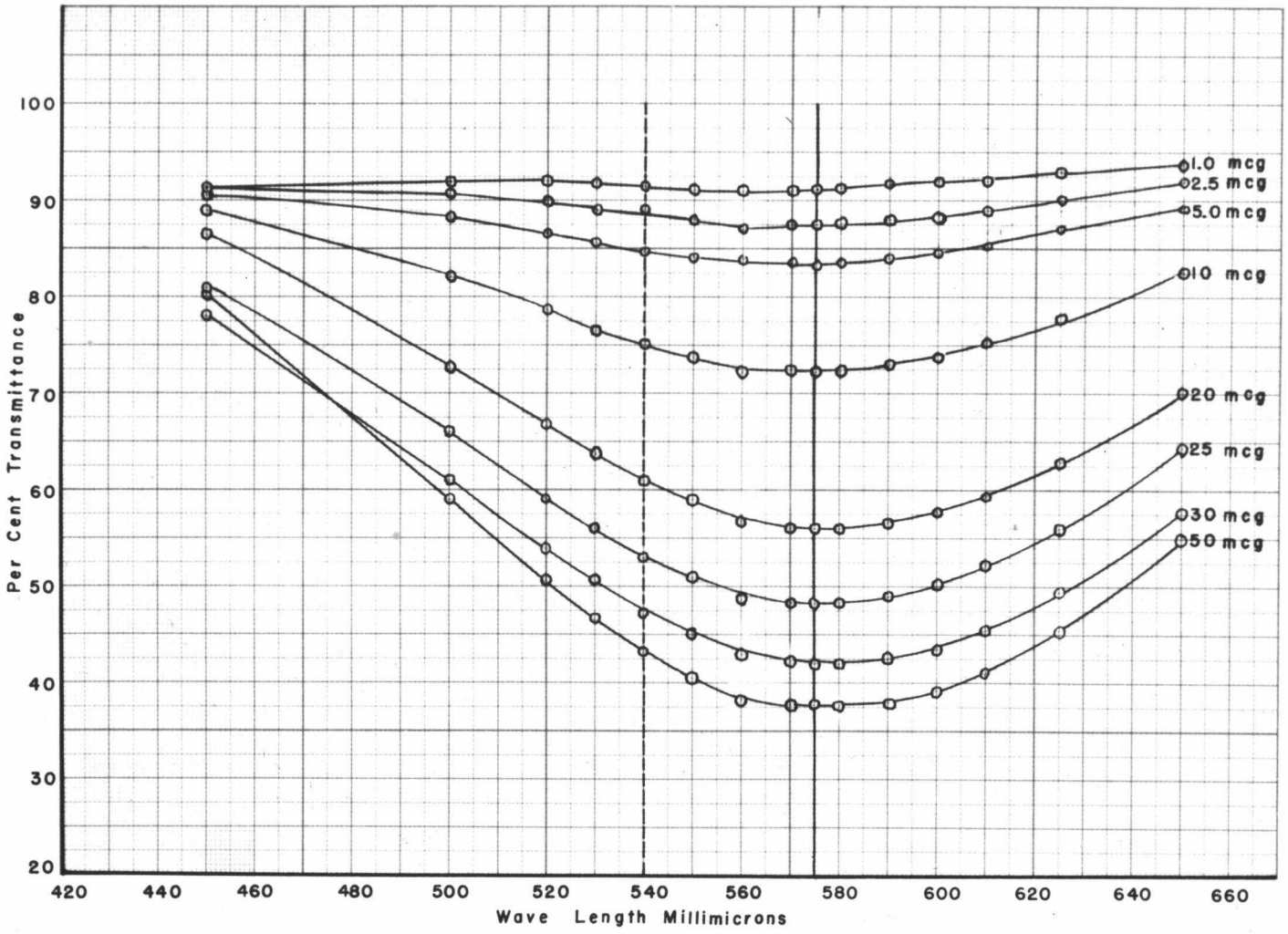

Figure 1. Absorption characteristics of benzenone 2-6 dibromoindonaphthol.

Table IV.

EFFECT OF ADDITION OF a-NAPHTHOL ON PER CENT TRANSMISSION

Sample	Addition of a-naphthol/ 10 ml. of milk	% Trans-mission at 575 mu	Addition of a-naphthol/10 ml. of the filtrate	% Trans-mission at 575 mu
	in mcg.*		in mcg.*	
1 (control)	0	100	0	100
2	10	99.7	1	91.1
3	20	97.9	2	89.2
4	30	95.3	2.5	87.5
5	40	95.3	5	83.4
6	60	92.2	10	72.3
7	80	83.8	20	56.0
8	120	76.9	25	48.2
9	200	69.0	30	42.0
10	-	-	50	37.7

* Average of duplicate readings

Table V.

THE EFFECT OF DIFFERENT TREATMENTS ON THE TRANSMITTANCY OF THE CONTROL

Treatment	% Transmission at 575 mu*			
Experiment	I	II	III	IV
1. Pasteurized**, no substrate	99.0	98.5	99.5	98.3
2. Pasteurized + substrate, not incubated	-	-	96.7	94.3
3. Pasteurized + substrate, incubated	84.1	70.4	96.0	81.7
4. Raw + substrate, not incubated	89.2	91.2	93.5	89.4
5. Raw + substrate, incubated	59.2	53.2	65.1	54.0
6. Same as #5, but double the amount of substrate	-	-	63.5	55.2

* Average of duplicate readings
** Pasteurized at 70° C. for five minutes
Substrate is 0.15 per cent solution of a-naphthyl acetate

If the hydrolysis of a-naphthyl acetate takes place by the action of the enzyme esterase only, then the treatments 2, 3 and 4 should be practically the same. However, treatment 3, in which the enzyme is "theoretically" destroyed, does result in some hydrolysis, which may indicate that chemical hydrolysis is taking place during incubation. This is important because similar chemical hydrolysis may also take place in the unpasteurized sample (treatment 4) in addition to enzymatic hydrolysis, thus giving a higher apparent esterase activity. For this reason, incubated pasteurized milk with substrate (treatment 3) was used as a control for all colorimetric determinations of the enzyme activity.

Determination of lipid phosphorus: The milk fat was extracted by the Mojonnier method (77, pp.39-43). It was found convenient to use porcelain ashing dishes instead of Mojonnier aluminum dishes for fat. The fat was then ashed according to the method of Halliday (29, pp.103-104) and phosphorus determined by the Fiske and Subbarow method (15, pp.375-400); the methods are as follows:

Five ml. of saturated alcoholic (95%) solution of $MgNO_3$ is added to the fat obtained by the Mojonnier method. This is heated on a steam bath for about 15 hours and then carefully charred on an electric hot plate. The charred sample is placed in a muffle furnace and heated to 600° C. for three hours, during which time complete oxidation takes place. The samples are then cooled. The ash is dissolved either in concentrated trichloroacetic acid solution or concentrated HCl acid. However, it was observed that the

final pH of the diluted solution played an important role in the color formation. It was found that when either concentrated CCl_3COOH or HCl was used, the final pH should be 1.0 or below. The most important factor is to have the same pH for standards as for the unknown.

The dissolved ash is then filtered through #44 Whatman filter paper and made up to a definite volume (e.g. 25 ml.) including the washings. Depending upon the concentration of phosphorus, an aliquot of the diluted solution is transferred to the spectrophotometer tube and diluted to nine ml. so that the final pH of the solution is 1.0. To this 0.5 ml. of 2.5 per cent solution of ammonium molybdate and 0.5 ml. of amino naphthol sulphonic acid reagent are added and mixed. The tubes are placed in the dark for ten minutes and then read at 660 mu (Coleman model 6A spectrophotometer), against the blank which is prepared exactly as above, substituting 5 per cent trichloroacetic acid for the filtrate. It was observed that the order in which the reagent is added influences the color formation and hence the sequence mentioned above was used throughout.

To obtain a standard curve, a solution of monopotassium phosphate (KH_2PO_4) (recrystallized twice from water and dried at 110° and 120° C.) in 5 per cent trichloroacetic acid, was used as a source of P, and treated exactly in the same manner as the filtrate. As a precautionary measure, the standard curve was obtained every time the determination of phosphorus was made.

The reducing agent, amino naphthol sulphonic acid (Eastman Kodak Co.) was purified according to the method described by Fiske

and Subbarow (15, p.389). Both the reagents (ammonium molybdate and amino naphthol sulphonic acid) were prepared every month and stored in brown bottles at 45° F.

Electron microscopy: To date, several attempts to study the milk fat membrane material with the help of microscopes have failed because of the minuteness of the material. Therefore, it was decided to study the interfacial membrane with the help of an electron microscope. According to Wyckoff (119, p.79) the present-day electron microscopes in good condition probably have a limiting resolution near 30 or 40 Å. In this study an RCA Model EMU-2D microscope was used.

Preparation of samples: Milk is a very complex biological fluid; hence, when it is examined after dilution the results are difficult to interpret. The major constituents of milk were, therefore, isolated and examined after suitable dilution. Their identities were established in comparison with known substances.

In order to obtain the membrane material in its natural state, many methods were tried. Several solvents (such as acetone, ethyl alcohol, ethyl ether, isopropyl alcohol, dioxane, ethylene chloride, carbon tetrachloride, chloroform, hexane, "heptane") were tried either alone or in different combinations. Only those methods which gave good results are described below.

The membrane lipo-protein was obtained from washed cream. The cream was washed four times, as indicated earlier and will be referred to as washed cream.

A method using four parts of hexane with one part of acetone has been used by Sell et al. (103, pp.1222-1223) for the extraction of lecitho-protein from egg yolk. According to them, this mixture did not denature the protein, whereas acetone alone denatured it completely. This mixture of solvents is used for extracting milk globule membrane lipo-proteins. Excess of this solvent mixture is repeatedly added to the washed cream, vigorously stirred, and allowed to stand for a few minutes or centrifuged. A pinkish-white precipitate settles down. The extraction is continued until all the fat is removed. This is centrifuged for five minutes and the solvent mixture is decanted. The precipitate is washed with distilled water to remove traces of solvent, and then suspended into double-distilled water. In later experiments hexane was replaced by "Heptane" without any deleterious effects.

The method used by Storch in 1897 (83, p.473) to isolate the membrane material was found to be quite suitable. Washed cream is treated with excess of a mixture of one volume of 95 per cent ethyl alcohol and two volumes of ether. The mixture is shaken and centrifuged. This treatment is repeated to remove all free fat, leaving a pinkish-white precipitate at the bottom of the centrifuge tube, which is suspended in distilled water. Later, slightly better results were obtained by using a mixture of one volume of EtOH and three volumes of EtOEt.

Dioxan (diethylene dioxide) was used by Rimpila and Palmer (97) for the isolation of the membrane protein from washed creams. They

added dioxan in the ratio of one cream to seven dioxan. When excess of dioxan is added to washed cream, agitated and centrifuged, a white colloidal layer floats on the top. Even after repeated similar treatments, all the free fat is not removed. Moreover, it is very difficult to remove dioxan from the prepared sample. Therefore this method is not as suitable as the first two mentioned above.

Methylene chloride and ethyl ether mixture has also been used to remove lipid material without denaturing the protein. Though it gave better results than dioxan, it was not as satisfactory for electron microscope work as the first two methods.

Preparation of film: Collodion and Formvar films are used quite extensively. A 2.2 per cent solution of collodion in amyl acetate is used. In order to prepare the membrane, a dish of about ten inches in diameter is filled with cold water; then about five drops of collodion solution are carefully put on the surface of water in the center of the dish. This spreads almost instantly to an extremely fine film that covers most of the surface of water. After a minute or two it begins to reflect light, and that area which looks gray possesses the desirable thickness. On this portion of the film, about ten 200-mesh stainless steel circular wire screens are placed with their rough edges pointing upward. The wire screens are spaced evenly so that they can be picked up on the surface of a glass microscope slide. This is achieved by placing a microscope slide on the screens, pushing it down into the water, turning it over, so that the slide carrying the collodion-covered screens may be brought to the

surface. The slide is allowed to dry and is then ready for use. Formvar film is also prepared in the same way except that a solution of 0.2 per cent Formvar in ethylene chloride is used.

A sample of the suspension in double-distilled water is drawn into a capillary pipette and a small drop is placed on the collodion film in the middle of a screen. This is allowed to dry at room temperature. Drying should not be hastened because such a practice leads to distortion of the sample.

The proper dilution of the sample which is placed on the screens is often difficult to determine. The proper dilution naturally depends upon the type of the sample. Satisfactory results are obtained in case of milk, for example, by diluting one ml. of milk to about 350 to 500 ml. (or per cent transmission at a wavelength of 550 mu should not be less than 95 per cent--Coleman model 6A spectrophotometer with a cell width of 19 mm.)

Shadow casting: Shadow casting improves the image considerably by highlighting the raised objects and thus giving a sort of three dimensional appearance. In addition, the height of the object casting a shadow can be calculated. This subject has been covered in detail by Wyckoff (119).

The samples in this investigation were placed two cm. below and six cm. from the vertical dropped from the filament. (Evidently the maximum length of shadow produced under these conditions is twice the height of the detail causing it.) In the beginning, gold was used for shadow casting, but it has a disadvantage of having a

tendency to crystallize and thus produce a granular appearance in the image. Palladium and chromium were also used and the latter was found quite satisfactory. In order to obtain sharp shadows, the vacuum must be less than 10^{-4} mm. of mercury.

The size of the particles or structures revealed by the electron microscope can be determined either by direct comparison with standard reference particles of known size, or with a diffraction grating. A polystyrene latex has been widely used. In this investigation a diffraction grating of 300 lines per mm. was used for calibration.

RESULTS

Section I: Surface Tension

I. Effect of Storing Milk at Low Temperatures on its Surface Tension

Milk from individual cows was divided into a number of samples which were stored at about 1° C. (34° F.) (no preservatives added). At different intervals, the surface tension of individual samples was determined, after they were tempered for one hour at 25° C. (77° F.). The results are shown in Table VI.

The results indicate that although the initial surface tension was fairly constant, it varied from 46.9 to 48.9 dynes/cm. for Jerseys and 46.7 to 47.5 dynes/cm. for Holsteins. The initial surface tension was taken by cooling the samples to 25° C. (77° F.), whereas the rest of the determinations were made by warming the milk from 1° C. to 25° C. (34° F. to 77° F.). Cooling and warming of milk always results in a decrease in surface tension and this is clearly indicated by the results. The average drop in surface tension was about two dynes (range 0.2 to 4.2). Some samples, even after storage at 1° C. (34° F.) for one month had practically the same surface tension values as initially. The results show that no sample exhibited lipolysis on mere cooling, as indicated by the decrease in surface tension and by its taste.

Table VI.

EFFECT OF STORING AT 1° C. (34° F.) ON SURFACE TENSION OF MILK

Cow	Surface tension in dynes/cm. at 25° C. (77° F.) Time after milking				
	2 hr.	3 days	7 days	14 days	1 month
219	47.9	-	46.2	-	-
219	47.8	44.1	-	-	-
210	46.9	-	42.7	-	42.6
225	48.9	44.7	-	-	-
230	47.8	47.0	-	46.7	-
251	47.0	-	-	47.1	47.6
263	47.3	-	41.8	-	43.2
271	47.6	45.6	-	-	-
494	47.5	47.1	46.7	-	47.7
523	46.9	-	45.3	-	33.4*
564	47.2	-	-	40.6	43.7
567	46.7	46.5	-	45.7	-

* Slightly churned

II. Effect of Incubation at 38° C. (100° F.) on Surface Tension of Milk

(A) Incubated after Cooling:

Six samples from individual cows were cooled to 10° C. (50° F.), divided into different groups and incubated at 38° C. (100° F.) (except those on which initial surface tension measurements were made). Initial surface tension was determined after tempering the non-incubated samples at 25° C. (77° F.) for one-half hour. The surface tension on the incubated samples was determined after incubating at 38° C. (100° F.) for two hours, ten hours and 18 hours. All the samples were tempered as mentioned above. The results are reported in Table VII-A.

As the initial determination was made by warming the samples from 10° C. to 25° C. (50° F. to 77° F.), the surface tension values are lower than they would have been if the samples were cooled from 38° C. to 25° C. (100° F. to 77° F.). However, after two hours of incubation the surface tension values increased slightly more than one dyne/cm. Perhaps this is due to the establishment of an equilibrium in a complex system which has undergone temperature changes. As the interval of incubation was prolonged, the surface tension values decreased progressively. This is expected because the samples had been subjected to a process similar to temperature activation (pre-cooled to 10°C. (50° F.), re-warmed to 38° C. (100° F.) and re-cooled to 25° C. (77° F.)).

Table VII.

EFFECT OF INCUBATION ON SURFACE TENSION OF MILK

A. Incubated after Cooling

Sample	Surface tension in dynes/cm. at 25° C. (77° F.) Time of incubation at 38° C. (100° F.)			
	0 hr.	2 hr.	10 hr.	18 hr.
1	44.6	46.1	42.5	40.0
2	45.3	47.0	42.7	40.7
3	46.9	48.2	44.4	38.3
4	46.8	47.2	43.6	39.0
5	44.3	45.8	45.7	38.3
6	43.6	45.9	43.5	40.9

B. Incubated Without Cooling

Cow No.	Surface tension in dynes/cm. at 38° C. (100° F.) Time of incubation at 38° C. (100° F.)				
	0 hr.	3 hr.	18 hr.	3 days	6 days
TG-2	43.7	45.4	45.8	45.7	45.6
273	44.8	42.4	43.7	43.7	44.6
533	45.4	44.8	45.6	46.4	46.1
564	44.9	42.4	45.1	44.8	44.6
566	45.6	44.5	45.4	45.2	45.9

(0.1 per cent HCHO was added as a preservative)

(B) Incubated Without Cooling:

Five samples from individual cows were incubated at 38° C. (100° F.) immediately after milking and without cooling. One-tenth per cent HCHO was added to all samples as preservative against bacterial growth. The surface tension determinations in this experiment were made at 0-hr.; 3-hr.; 18-hr.; 3-day and 6-day intervals at 38° C. (100° F.). This temperature of 38° C. (100° F.) was preferred to 25° C. (77° F.) because lowering of temperature may introduce changes in surface tension which may not be realized or understood. In addition, milk is very susceptible to churning at 25° C. (77° F.) even when stirred rather carefully. The results are reported in Table VII-B.

These results are the opposite of those in Table VII-A. In this experiment the surface tension decreased slightly after three hours of incubation and then increased slightly upon continued incubation. But the surface tension values on the sixth day were (except in case of sample 1) the same as the initial values, thus indicating that no lipolysis had occurred.

III. Effect of Temperature Activation, With and Without Stirring, on Surface Tension of Milk

Five samples from individual cows were divided into three groups after the initial surface tension measurements were made. The first group was cooled to 1° C. (34° F.). The second and third groups were temperature activated by keeping them in the 1° C. room for three

hours after which they were incubated at 30° C. (86° F.) for one and one-half hours and finally returned to 1° C. room. Samples belonging to the third group were mixed by occasional careful rotation; those of the second group were left undisturbed.

The samples of the first group were removed from the 1° C. room after three days and tempered in the 25° C. (77° F.) waterbath for one hour before surface tension determinations were made at 25° C. (77° F.) The surface tension measurements on the remaining samples were made on the seventh day, after similar treatment as mentioned above. The results are summarized in Table VIII.

Surface tension of samples which were stirred occasionally was slightly lower than the surface tension of those which were not stirred. This may be due to the fact that stirring produces change at the interface between fat and serum. The surface tension was lowered considerably by temperature activation (pre-cooling, re-warming and re-cooling) of samples. The surface tension values of the samples which were merely cooled, were considerably lower than the initial surface tension values, determined immediately after milking; this is due to the cooling and warming of the samples.

IV. Surface Tension of Different Components of Milk

Mixed whole milk (samples A and C), cooled to about 5° C. (41° F.) and stored overnight, was separated at ca. 7° C. (45° F.). The skim milk so obtained is indicated as S.M.-I (fat less than 0.1 per cent). This S.M.-I was then put through a Sharples

Table VIII.

EFFECT OF TEMPERATURE ACTIVATION, WITH AND WITHOUT STIRRING, ON SURFACE TENSION OF MILK

Surface tension in dynes/cm. at 25° C. (77° F.)

Cow	Initial	After 3-day incubation			After 7-day incubation		
		Group 1	Group 2	Group 3	Group 1*	Group 2**	Group 3**
TG-2	46.4	42.0	38.9	37.4	40.0	38.4	35.9
273	46.3	41.7	39.3	38.2	41.1	36.8	36.0
533	47.3	42.4	37.6	37.2	43.7	37.2	36.7
564	48.5	41.8	36.4	37.4	41.2	35.6	36.4
566	47.4	42.1	38.2	36.0	43.3	35.6	34.8

* Samples tasted old, slightly cowy but definitely not rancid
** Samples were very rancid and bitter

Group 1: Cooled to 1° C. (34° F.)

Group 2: Temperature activated--undisturbed

Group 3: Temperature activated--mixed by occasional careful rotation

supercentrifuge at 30,000 r.p.m.; the super skim milk obtained is termed S.M.-II (no fat by Babcock). S.M.-II was once again put through the supercentrifuge at ca. 42,000 r.p.m. to obtain S.M.-III. The cream was washed four times (4 x W.C.) at 38° C. (100° F.).

Another sample of cold 5° C. (41° F.) mixed whole milk (sample B) was separated at 38° C. (100° F.) and then treated in the same way as mentioned above. The total solids were determined by gravimetric (Mojonnier) method on the components of samples A and B. The results are tabulated in Table IX.

The removal of cream increased the surface tension of skim milk from that of whole milk. The increase was greater in case of 38° C. (100° F.) skim milk.

Considerable of the Ca-caseinate-Ca-phosphate complex is removed by supercentrifuging skim milk; this increased the surface tension of the skim milk slightly.

The apparent lowering of surface tension during washing is probably due to slight churning during the process of washing.

It is difficult to determine the surface tension of cream at 25° C. (77° F.) because it is very susceptible to churning. The lower surface tension of cream and four-times washed cream of sample B may be due to slight lipolysis due to temperature activation (original sample cooled to 1° C. (41° F.), separated at 38° C. (100° F.) and cooled to 25° C. (77° F.)).

Table IX.

SURFACE TENSION OF DIFFERENT COMPONENTS OF MILK

	Surface tension in dynes/cm. at 25° C. (77° F.)				
	Samples				
	A		B		C
	Surface Tension	% Total Solids	Surface Tension	% Total Solids	Surface Tension
Whole milk	47.3	12.9	44.7*	13.14	45.15
S.M.-I	49.4 (45° F.**)	9.21	55.0 (100° F.**)	9.33	50.7 (45° F.**)
S.M.-II (30,000 r.p.m.)	49.8	7.98	55.4	-	51.35
S.M.-III (42,000 r.p.m.)	50.2	-	55.25	7.48	-
Cream	43.4	43.8	41.8	48.4	43.4
4 x W.C.	41.9	43.92	38.85	42.6	43.3

* Slightly churned during mixing
** Temperature of separation

V. Interfacial Tensions Between Distilled Water and Milk Fat, With and Without Phosphatides

Asolectin, a mixed phosphatide, was used at concentrations of 0.5 per cent and 1.0 per cent in the aqueous phase and 1.0 per cent in the fat phase. Interfacial tensions were determined immediately after the two surfaces were brought into contact as described in the experimental section (p.29) at 38° C. (100° F). Interfacial tension changes until the equilibrium is established. The results, averages of at least three readings, are shown in Table X.

Spreading Coefficient (s) is calculated as follows (31, pp.41-43):

$$s = \gamma_{m.f.} - (\gamma_{H_2O} \neq \gamma_{m.f./H_2O})$$

$\gamma_{m.f.}$ = surface tension of fat phase

γ_{H_2O} = surface tension of aqueous phase

$\gamma_{m.f./H_2O}$ = Interfacial tension between the two phases

s was calculated only from the interfacial tension obtained by the upward (↑) pull of the ring.

VI. Interfacial Tensions Between Super Skim Milk and Milk Fat With and Without Phosphatides

Asolectin was used at concentrations of 0.5 and 1.0 per cent in the aqueous phase and 1.0 per cent in the fat phase. The average results of at least three readings are shown in Table XI. Results for one sample of acid whey obtained from skim milk when adjusted to

Table X.

INTERFACIAL TENSIONS AND SPREADING COEFFICIENTS BETWEEN DISTILLED WATER AND MILK-FAT WITH AND WITHOUT ASOLECTIN

Surface and interfacial tensions in dynes/cm. at 38° C. (100° F.)

	Surface Tension					
	I			II		
Double distilled H_2O	69.8			69.8		
d.d. H_2O + 0.5% Asolectin	37.55			38.9		
Milk fat	34.6			34.6		
Milk fat + 1% Asolectin	34.1			34.1		
	Up σ_f/a	Down	s	Up σ_f/a	Down	s
m.f./d.d. H_2O	24.3	19.7	-59.5	24.6	20.8	-59.8
m.f. + 1% Asolectin/d.d.H_2O	9.6	10.5	-45.3	9.6*	8.5*	-45.3
m.f./d.d.H_2O + 0.5% Asolectin	4.8	9.3	-07.75	7.8	9.7	-10.75
m.f./d.d.H_2O + 1% Asolectin	-	-	-	3.9	4.1	- 7.6
m.f. + 1% Asolectin/d.d.H_2O + 0.5% Asolectin	5.75	6.4	- 9.2	3.8	4.0	- 8.6

* Both readings changed to 10.5 dynes/cm. two hours after the milk fat was layered on distilled water

The amount of Asolectin added is the percentage of the phase in which it is incorporated, e.g. when it is added to fat, it is the percentage of the fat phase and when added to aqueous phase it is the percentage of the aqueous phase.

Table XI.

INTERFACIAL TENSIONS AND SPREADING COEFFICIENTS BETWEEN SKIM MILK AND MILK FAT, WITH AND WITHOUT ASOLECTIN

Surface and interfacial tensions in dynes/cm. at 38° C. (100° F.)

	I	II
	Surface tension	
Skim milk (s.m.)	46.5	45.8
Super skim milk (s.s.)	47.2	45.8
s.s. + 0.5% Asolectin	34.3	31.3
s.s. + 1% Asolectin	32.55	-
milk fat (m.f.)	34.6	34.6
m.f. + 1% Asolectin	34.1	34.1
Acid whey (pH adjusted to 6.5)	45.7	-

	I			II		
	Up	Down	s	Up	Down	s
	Interfacial Tension			Interfacial Tension		
m.f./s.m.	14.6	12.8	-26.5	-	-	-
m.f./s.s.	14.3	11.7	-26.9	12.2	9.25	-24.8
m.f./s.s. + 0.5% Asolectin	5.8	2.8	- 5.5	7.1*	3.6*	- 3.8
m.f./s.s. + 1% Asolectin	5.1	2.7	- 3.05	-	-	-
m.f. + 1% Asolectin/s.m.	7.4	6.0	-19.8	-	-	-
m.f. + 1% Asolectin/s.s.	5.2	5.1	-18.3	-	-	-
m.f. + 1% /s.s. + 0.5% Asolectin/ Asolectin	4.1	3.5	- 4.3	5.2	3.4	- 2.4
m.f. + 1% Asolectin/whey	6.1	4.4	-17.7	-	-	-

* Both readings were 3.6 dynes/cm. 15 minutes after the milk fat was layered on the super skim milk

(Refer to footnotes of Table X.)

pH of about 6.5 with NaOH, are also reported.

VII. Effect of Incorporation of Cholesterol in Milk Fat on the Interfacial Tensions Between Aqueous and Lipid Phase With and Without Phosphatides

One per cent Asolectin was incorporated in milk fat to which 0.3 per cent cholesterol had been added. The surface and interfacial tensions and spreading coefficients are reported in Table XII. The interfacial tension readings for the first four samples were taken 15 minutes after the milk fat was layered on the aqueous phase. However this interval was not sufficient to equalize the up and down readings and therefore the interfacial tension determinations at the first contact were again resumed.

VIII. Effect of Added Asolectin on the Surface Tension of Distilled Water, Skim Milk and Whey

Varying amounts (from 0.01 to 1.0 per cent) of Asolectin were added to distilled water, skim milk and rennet whey. The solutions were kept over night at 50° F. and then warmed to 38° C. (100° F.) for the surface tension determinations. Results are reported in Table XIII and Figure 2.

Table XII.

EFFECT OF INCORPORATION OF CHOLESTEROL IN MILK FAT ON THE INTERFACIAL TENSION BETWEEN AQUEOUS AND LIPID PHASES

Surface and interfacial tensions in dynes/cm. at 38° C. (100° F.)

	Surface tension
Milk fat	34.55
m.f. + 0.3% Cholesterol	34.7
Super skim milk (s.s.)	52.8

Samples		Up $\gamma_{f/a}$	Down	s
(1)	*m.f./d.d. H_2O	21.4	19.6	-56.6
(2)	*m.f. + 0.3% Cholesterol/d.d. H_2O	20.75	17.0	-56.0
(3)	*m.f./s.s.	12.9	9.25	-31.15
(4)	*m.f. + 0.3% Cholesterol/s.s.	12.4	8.8	-30.5
(5)	m.f. + 0.3% Cholesterol + 1% Asolectin / s.s.	6.5	4.1	-25.1
(6)	m.f. + 0.3% Cholesterol + 1% Asolectin / d.d. H_2O	9.85	7.9	-45.45

* Readings taken 15 minutes after the milk fat was layered over the aqueous phase

(Refer to footnotes of Table X.)

Table XIII.

EFFECT ON SURFACE TENSION OF DISTILLED WATER, SKIM MILK AND WHEY BY ADDITION OF ASOLECTIN

% Asolectin	Surface tension in dynes/cm. at 38° C. (100° F.) Asolectin in:		
	Dist. H_2O	Skim milk	Rennet whey
0.0	70.1	49.4	48.4
0.01	69.0	-	-
0.02	64.1	-	-
0.025	-	48.9	47.4
0.03	55.8	-	-
0.04	47.1	-	-
0.05	45.3	47.4	46.9
0.075	43.9	47.3	46.9
0.1	40.1	44.3	44.9
0.25	-	41.6	38.8
0.5	33.1	41.1	35.2
0.75	-	41.5	36.6
1.0	32.9	39.75	30.9

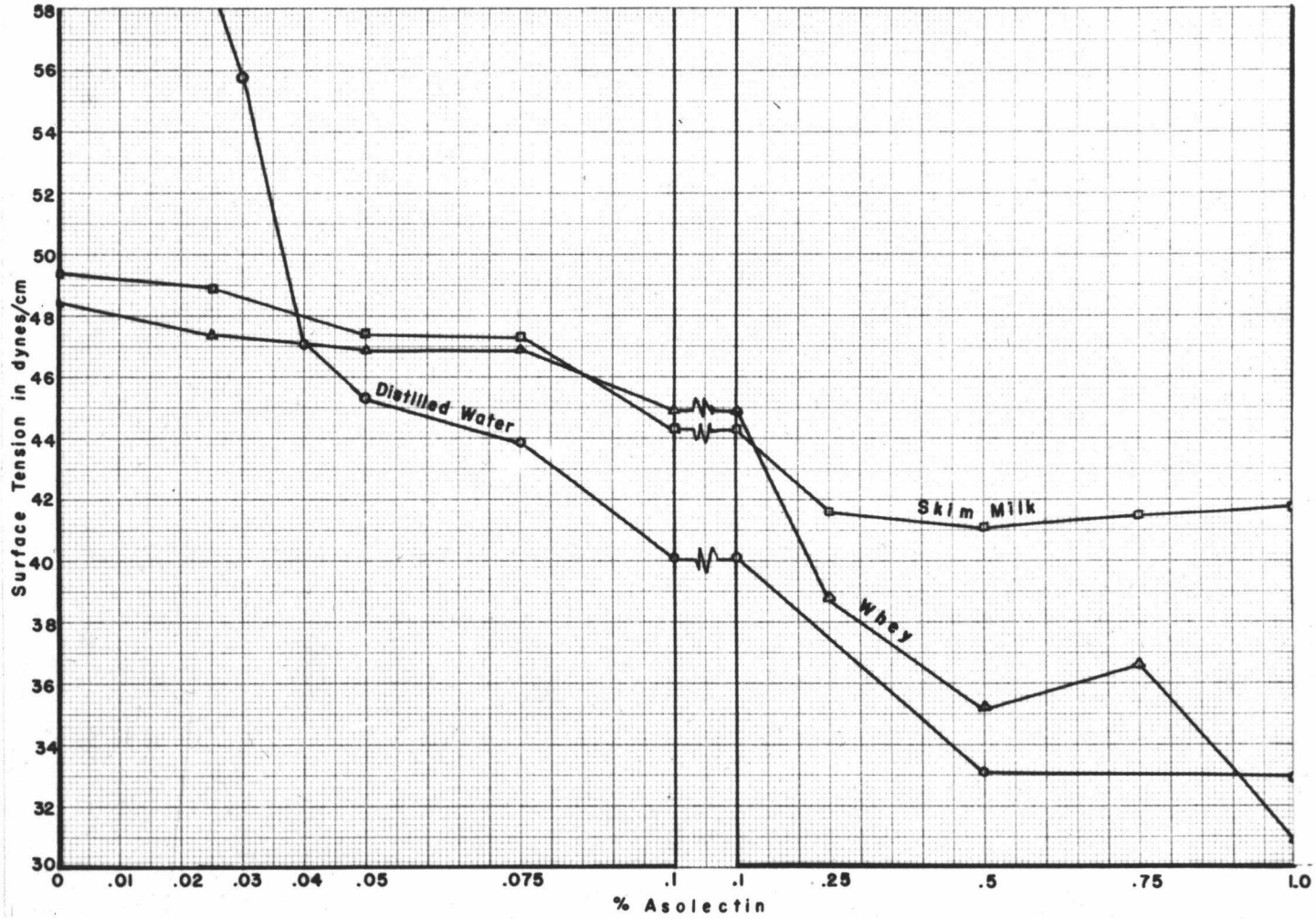

Figure 2. Effect of concentration of Asolectin on the surface tension of distilled water, skim milk and whey.

IX. Effect of Increasing Amounts of Asolectin in the Aqueous Phase on the Interfacial Tensions Between Milk Fat and Distilled Water, Milk Fat and Skim Milk, and Milk Fat and Whey

The solutions of the above experiment were used for determinations of interfacial tensions. The results are reported in Tables XIV and XV and Figures 3, 4 and 5.

The surface tension of distilled water is about 20 dynes/cm. higher than skim milk or whey; this is due to the presence of surface-active substances, such as proteins. The surface tension of milk fat is lower than that of milk plasma, and the addition of phosphatides to the fat phase has little effect on the surface tension. Phosphatides added to the distilled water reduce the surface tension considerably, but similar amounts added to skim milk or whey cause negligible reductions (Figure 2). In the case of distilled water, the critical concentration of asolectin (as indicated by a sharp break in the curve) appears to be 0.04 per cent; with skim milk and rennet whey there is a very gradual decrease in surface tension with increasing concentration of asolectin. However, between the concentrations of 0.5 and 0.75 per cent there is a slight increase in surface tension of whey; this increase is reflected in the corresponding increase in interfacial tension values.

The values for interfacial tensions reported are averages of at least three readings. The reproductibility of interfacial tensions was low and sometimes the values for the same sample varied as much as three dynes/cm. or more. In spite of this limitation, the values

Table XIV.

INTERFACIAL TENSIONS BETWEEN MILK FAT AND DISTILLED WATER WITH ASOLECTIN IN THE AQUEOUS PHASE

Surface and interfacial tensions in dynes/cm. at 38° C. (100° F.)

Milk fat	I γ 36.7		II γ 32.7		
Aqueous phase	Up $\gamma_{f/a}$	Down	Up $\gamma_{f/a}$	Down	s
d.d. H_2O	25.7	19.45	23.5	21.75	-60.9
" + 0.01% Asolectin	-	-	16.75	16.4	-53.05
" + 0.02% "	-	-	13.9	17.9	-45.3
" + 0.03% "	-	-	16.0	16.5	-39.1
" + 0.04% "	13.7	10.7	13.2	13.7	-27.6
" + 0.05% "	15.1	15.2	14.6	15.2	-27.2
" + 0.075% "	-	-	10.1	12.6	-21.3
" + 0.10% "	17.95	14.8	10.6	11.4	-18.0
" + 0.25% "	12.5	9.9	-	-	-
" + 0.50% "	10.8	4.7	2.65	5.7	- 3.05
" + 1.0% "	-	-	2.8	3.15	- 3.15

(See Table XIII for surface tension of water and water and asolectin)

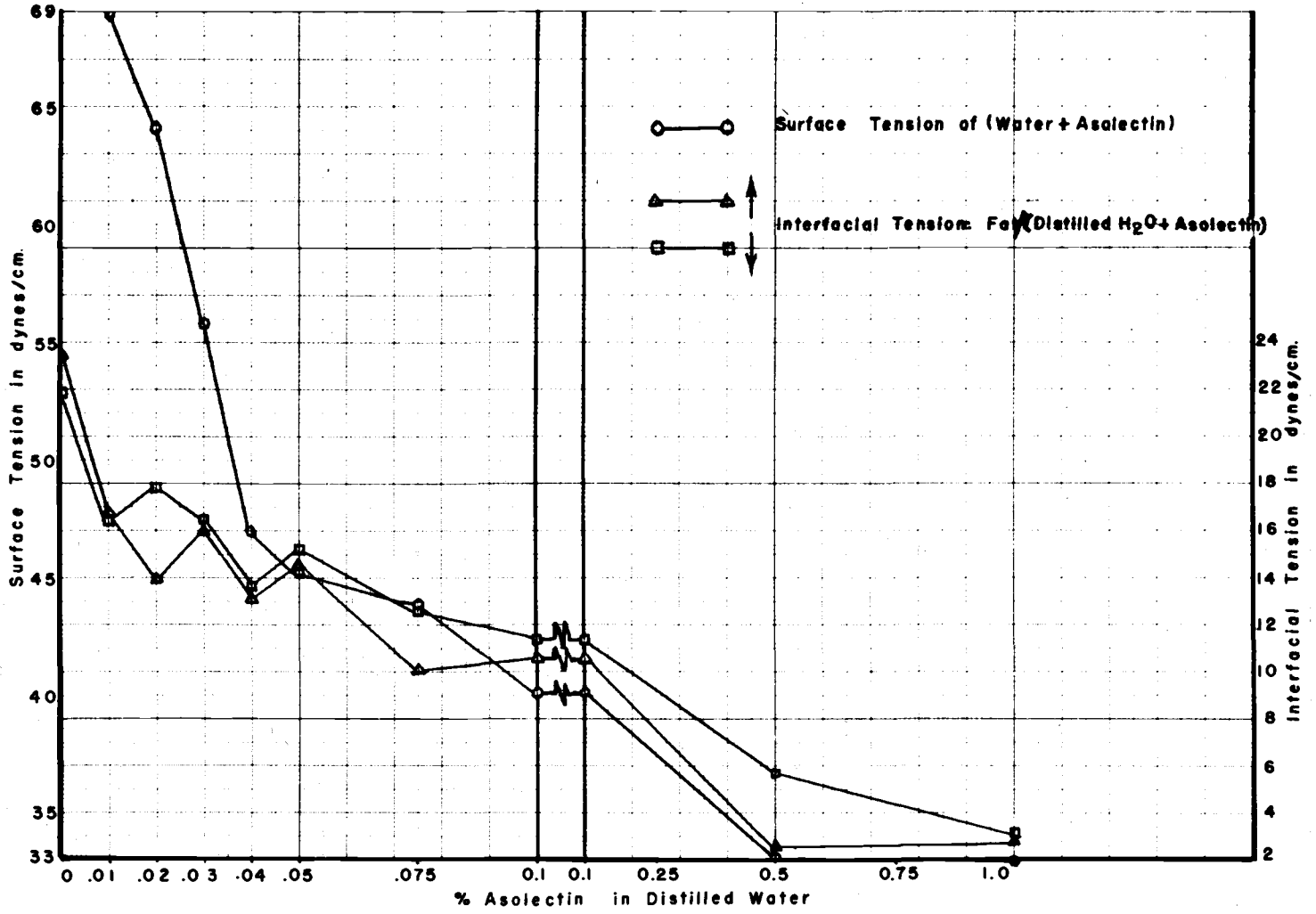

Figure 3. Effect of concentration of Asolectin on the surface properties of distilled water.

Table XV.

INTERFACIAL TENSIONS BETWEEN MILK FAT AND SKIM MILK OR WHEY WITH ASOLECTIN IN THE AQUEOUS PHASE

Surface and interfacial tension in dynes/cm. at 38° C. (100° F.)

Fat	γ 33.35			γ 33.35		
% Asolectin in aqueous phase	Skim milk			Whey		
	Up $\gamma_{f/a}$	Down	s	Up $\gamma_{f/a}$	Down	s
0	13.2	12.2	-29.25	13.2	11.5	-28.25
0.025	13.4	12.55	-28.95	13.2	11.4	-27.25
0.05	13.2	12.1	-27.25	13.45	12.3	-27.0
0.075	13.35	11.6	-24.3	13.35	11.75	-26.9
0.1	12.5	11.8	-23.45	11.2	10.1	-22.75
0.25	12.8	11.65	-21.05	4.6	3.65	-10.05
0.5	12.3	10.2	-20.05	1.5	1.2	- 4.75
0.75	11.7	10.4	-19.85	8.5	5.8	-11.75
1.0	6.6	3.9	-13.0	1.3	1.0	≠ 1.15

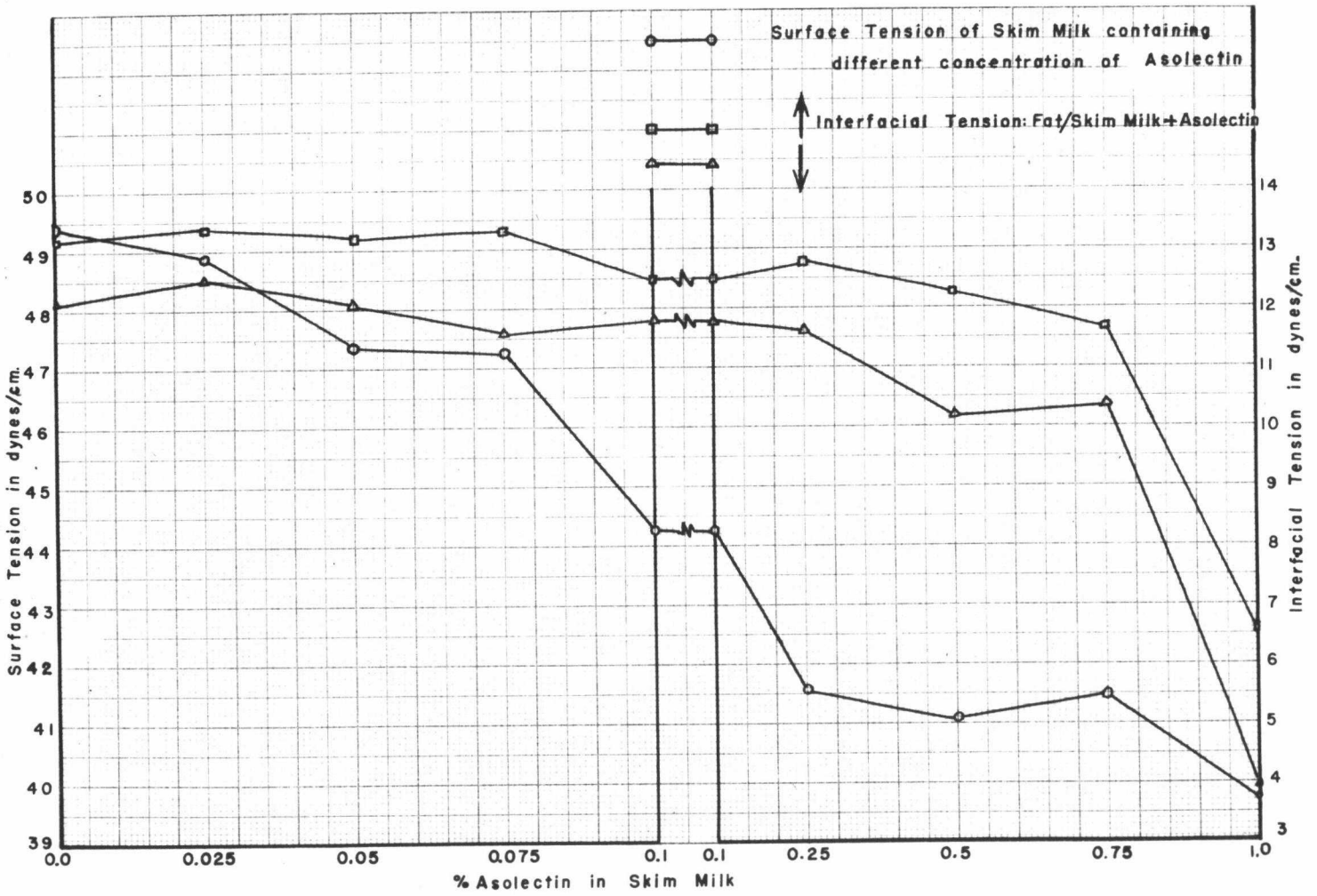

Figure 4. Effect of concentration of Asolectin in the surface properties of skim milk.

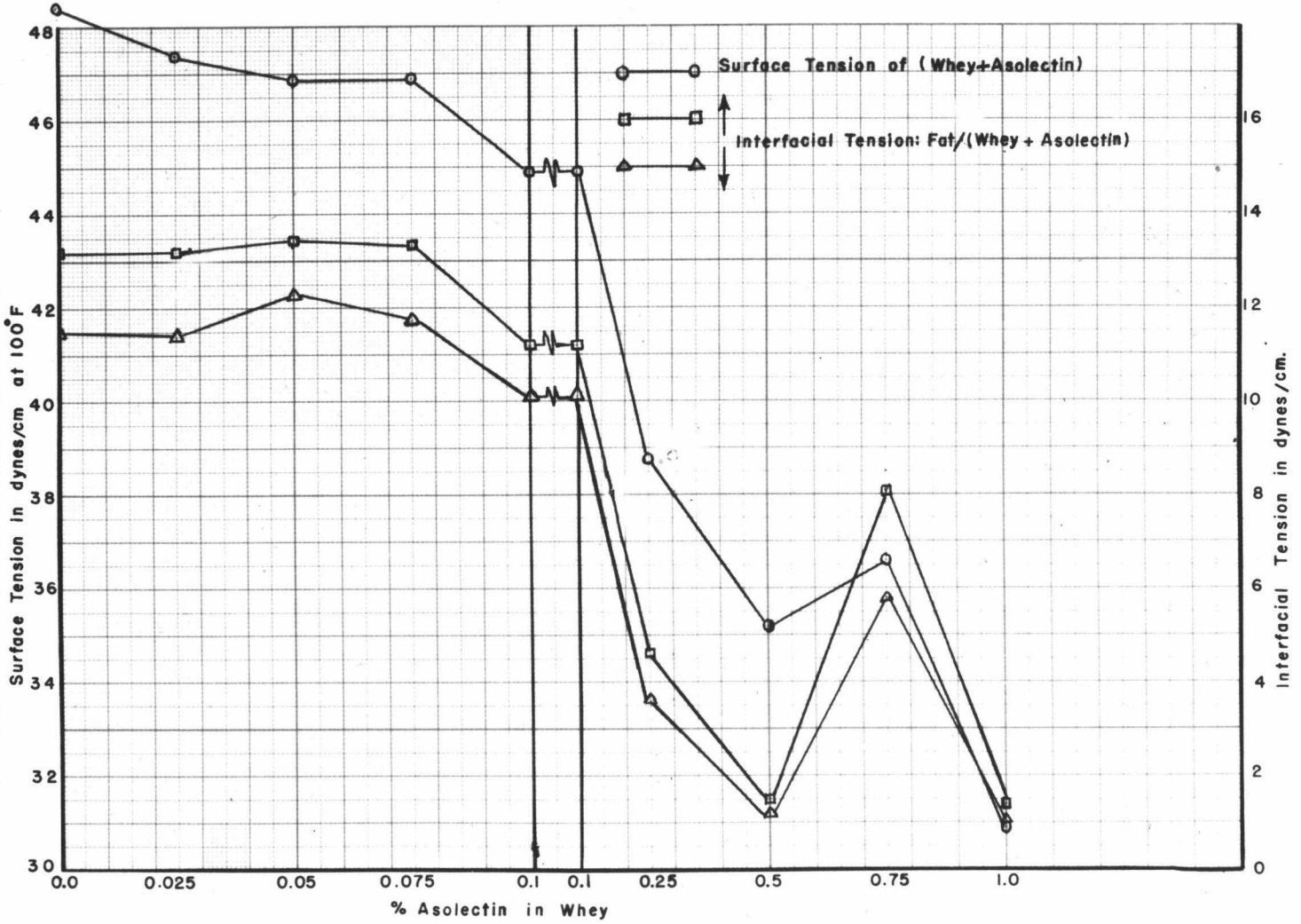

Figure 5. Effect of concentration of Asolectin on the surface properties of rennet whey.

obtained by the ring method are comparable to those reported by Palmer (83, pp.475-478) for butteroil and water containing different milk proteins, using the drop weight method, and by Sommer (108, p.672) for butteroil and milk serum, using the capillary rise method.

It should be emphasized that the interfacial tension readings were made immediately after the fat was layered on the aqueous phase and, therefore, they do not represent conditions at equilibrium. This is indicated by the fact that the interfacial tensions taken two hours after pouring milk fat containing Asolectin on distilled water are from one to two dynes higher than those made immediately after pouring (refer to Table X). Usually the interfacial tension obtained by the upward pull (↑) of the ring is higher than that by downward push (↓) of the ring. This is especially apparent when the distilled water is the continuous phase and when phospholipid has been added to it. However, when two hours were allowed for equilibrium to be established, the same values for up and down pulls were obtained. It was noticed that an opaque layer, probably of hydrated phospholipid, was visible at the interface at equilibrium. In another case (refer to Table XI) equilibrium was established in 15 minutes. For this reason some readings, as indicated in Table XII, were taken 15 minutes after the fat was poured, but the up and down values still did not coincide. It is apparent that a 15 minute period is insufficient for equilibrium to be established. However, since it is not practically feasible to delay the readings for two

hours, the interfacial tension determinations at the first contact were again resumed.

Although the addition of phospholipid to the fat phase has practically no effect on its surface tension, it produces a drop of 15 dynes/cm. in interfacial tension for milk fat and distilled water, and of about seven dynes/cm. for milk fat and skim milk or whey (refer to Table XI).

The inclusion of phospholipids to the aqueous phase, however, leads to a considerable decrease in its surface tension. This is reflected in the lowered interfacial tensions (refer to Figures 3, 4 and 5). When a five per cent emulsion of milk fat is prepared in skim milk containing different amounts of Asolectin in the aqueous phase, the surface tensions of the emulsions are practically the same as those of the skim milks from which they are prepared (refer to Tables XVII and XIII). This probably is to be expected since, as shown in Figure 2, only gradual decreases in the surface tension of skim milk are produced by increasing concentration of asolectin in skim milk.

The addition of asolectin in either phase fails to give positive spreading coefficients of the water on fat, but makes the s values less negative than the system without any asolectin, thus indicating that the water has greater tendency to spread over milk fat in the presence of a phosphatide. This is particularly true when it is added in the aqueous phase (see Tables X and XI). A comparison of spreading coefficients for distilled water and the

skim milk or whey indicates that for the former the values change from -60 to -3, whereas in the latter they change from -29 to -4 (see Tables XIV and XV). The drop is quite rapid in the case of distilled water. This is largely due to the fact that the addition of Asolectin decreases the surface tension of water considerably more than that of skim milk or whey (see Tables XII and XIII).

X. Surface Tension of "Remade" Creams

(A) Two groups of remade creams were prepared. One group was made in super skim milk obtained by separating skim milk at 45,000 r.p.m. and the other group in rennet whey obtained from this super skim milk. Each group consisted of three samples which received treatments as follows:

Milk A: 10% milk fat emulsified in super skim milk or whey,

Milk B: 10% (milk fat + 1% Asolectin) emulsified in super skim milk or whey,

Milk C: 10% (milk fat + 1% Asolectin + 0.3% Cholesterol) emulsified in super skim milk or whey.

The ten per cent emulsions were prepared at 38° C. (100° F.) by homogenizing at 300 and 200 pounds in two stages. The emulsions were cooled, kept at 1° C. (34° F.) for 24 hours, tempered to 38° C. (100° F.) for one-half hour, at the end of which surface tension readings were taken. The results are reported in Table XVI-A.

The surface tensions were practically the same for emulsions prepared in super skim milk and whey. A slight decrease in surface tension occurred when the samples were cooled and then warmed before

Table XVI-A.

SURFACE TENSION OF "REMADE" CREAMS

	Dynes/cm. at 38° C. (100° F.)		
	(i)	(ii)	(ii*)
milk fat (m.f.)	34.7	-	-
Super skim milk (s.s.)	52.8	50.3	-
Whey	52.5	-	-
10% m.f. in s.s.	44.7	46.3	43.05
10% (m.f. + 1% Asolectin) in s.s.	30.9	35.1	34.35
10% (m.f. + 1% Asolectin + 0.3% Cholesterol) in s.s.	30.0	32.6	31.1
10% m.f. in whey	44.6	-	-
10% (m.f. + 1% Asolectin) in whey	30.3	-	-
10% (m.f. + 1% Asolectin + 0.3% Cholesterol) in whey	30.9	-	-

(1) The surface tension measurements were made after the samples were stored at 34° F. for 24 hours, and tempered to 100° F. for one-half hour.

(ii) Measurements were made immediately after emulsification.

(ii*) Samples (ii) were stored at 34° F. for three days and tempered at 100° F. for one-half hour for surface tension measurements.

surface tension measurements were made. A similar phenomenon occurs in natural milk.

(B) Twenty per cent creams were prepared as follows:

(1) Super skim milk / milk fat

(2) Super skim milk / milk fat containing 1% Asolectin

(3) Pasteurized s.s. / m.f. containing 1% Asolectin

(4) Rennet whey / milk fat

(5) Rennet whey / milk fat containing 1% Asolectin

(6) Pasteurized whey / m.f. containing 1% Asolectin

(7) Approximately 3% Ca-caseinate-Ca-phosphate suspension / milk fat

(8) Approximately 3% Ca-caseinate-Ca-phosphate suspension / m.f. containing 1% Asolectin

All the samples were homogenized in a hand homogenizer at 38° C. (100° F.). Surface tension measurements were made immediately after homogenization. Results are tabulated in Table XVI-B.

All the samples with the Asolectin have considerably lower surface tensions than those not containing the phosphatides and therefore the decrease must be due to the phosphatides. As seen in earlier experiments, the phosphatide, Asolectin, when added to the fat, has little effect on the surface tension of fat. The same amount when incorporated in the aqueous phase, however, lowers the surface tension considerably. This experiment indicates that when phosphatides are supplied in the fat phase, they move to the interface and interact in some way (e.g. by solvation, hydration or combination) with the constituents of the aqueous phase to lower the interfacial

Table XVI-B.

SURFACE TENSION OF "REMADE" CREAMS

Sample	Dynes/cm. at 38° C. (100° F.)
1. Super skim milk ≠ milk fat	45.7
2. s.s. ≠ (m.f. containing 1% Asolectin)	33.8
3. Pasteurized s.s. ≠ (m.f. containing 1% Asolectin)	34.5
4. Rennet whey ≠ milk fat	47.3
5. Whey ≠ (m.f. containing 1% Asolectin)	33.6
6. Pasteurized whey ≠ (m.f. containing 1% Asolectin)	32.0
7. 3% Ca-caseinate-Ca-phosphate ≠ m.f.	46.8
8. 3% Ca-caseinate-Ca-phosphate ≠ (m.f. containing 1% Asolectin)	32.55

All emulsions contained 20 per cent fat and were pasteurized at 75° C. (167° F.) for five minutes.
Amount of Asolectin on the fat basis.

Table XVI-C.

SURFACE TENSION OF "REMADE" CREAMS

Sample	Dynes/cm. at 38° C. (100° F.)
Super skim milk containing 0.2% Asolectin ≠ 20% m.f.	34.2
Pasteurized s.s. " " " ≠ " "	coagulated
Rennet whey " " " ≠ " "	31.8
Pasteurized whey " " " ≠ " "	32.1

Pasteurized at 75° C. (167° F.) for five minutes.

tension, which is reflected by the decrease in surface tension.

(C) If the lowering of surface tension was mainly due to the phosphatide Asolectin which migrates to the interface and then becomes solvated (or hydrated), the surface tension of the emulsion should approach the same value when an equal amount of phosphatide is added to the aqueous phase, assuming, of course, that all the phosphatide migrates to the interface.

In the previous experiments (Table XVI-A and B), one per cent Asolectin was incorporated in the fat phase--the concentration being calculated on the fat basis. This is equivalent to 0.2 gram in 100 gram of 20 per cent cream. Creams (20%) were prepared in a hand homogenizer with super skim milk or whey, in which 0.2 gm. of Asolectin had been dispersed. Surface tension determinations were made at 38° C. (100° F.) immediately following homogenization and pasteurization (at 75° C. (167° F.) for five minutes). The results, tabulated in Table XVI-C indicate essentially the same surface tension as the samples containing an equivalent amount of phosphatide supplied in the fat phase (refer to Table XVI-B, samples 2, 5 and 6). This supports the migration and solvation idea suggested above.

XI. Effect of Increasing Temperatures on Surface Tension of Remade Milks

Five per cent emulsions were prepared in reconstituted skim milk (10%) prepared from spray dried non-fat dry milk solids (milk preheated to 75° C. (168° F.) for 15 minutes before spray drying).

Asolectin was added at different concentrations (from 0.1 to 1.0 per cent) to either the fat or the aqueous phase. The percentage of Asolectin added was based on the phase in which it was incorporated. The emulsification was done at 100° F. under 2500 pounds pressure (2000 lbs. - first stage, and 500 lbs. - second stage) for the samples of group A; and at 1000 pounds (500 lbs. in the first and second stages) for those of group B.

The samples were cooled to 1° C. (34° F.) for 15 to 20 hours and then tempered for one-half hour at different temperatures, 10°, 20°, 25°, 30° and 38° C. (50°, 68°, 77°, 86° and 100° F.) for the surface tension determinations. The results which are averages of four to six readings are tabulated in Table XVII and illustrated in Figure 6.

Surface tension decreases with increasing amounts of Asolectin and increasing temperatures. Figures indicate that emulsion 6 (milk fat containing 1 per cent Asolectin), emulsion 7 (skim milk containing 0.1 per cent Asolectin) and emulsion 8 (natural milk) behave in a similar manner. The phosphatide content of sample 6 was one per cent on the fat basis or 0.05 gm. of Asolectin per 100 gms. of the five per cent emulsion and that of sample 7 is 0.1 per cent of the skim milk or 0.095 gm. of Asolectin per 100 gm. of the five per cent emulsion.

Table XVII.

EFFECT OF INCREASING TEMPERATURE ON THE SURFACE TENSION OF REMADE MILKS

Type of Emulsion	Surface tension in dynes/cm. at temperatures									
	A					B				
	10° C.	20° C.	25° C.	30° C.	38° C.	15° C.	20° C.	25° C.	30° C.	38° C.
1. Skim milk	54.6	50.8	49.5	46.6	46.3	55.0	52.4	52.5	49.55	50.4
2. s.m. + milk fat	53.6	49.8	48.0	47.2	46.5	53.0	49.2	47.9	47.2	46.9
3. m.f. containing 0.1% Asolectin + s.m.	51.7	49.4	47.3	47.0	46.25	51.8	48.6	47.4	46.8	46.45
4. m.f. containing 0.5% Asolectin + s.m.	51.5	48.4	46.65	46.3	45.9	50.2	47.7	46.1	45.9	45.95
5. m.f. containing 0.75% Asolectin + s.m.	52.0	47.8	46.8	45.4	45.6	48.8	46.7	45.05	44.3	43.8
6. m.f. containing 1.0% Asolectin + s.m.	50.4	47.2	45.6	44.4	44.8	47.2	44.8	40.9	39.1	41.7
7. m.f. + s.m. containing 0.1% Asolectin	50.1	46.95	45.5	43.4	44.2	46.9	42.4	40.8	39.15	38.9
8. m.f. + s.m. containing 0.5% Asolectin	41.8	36.6	32.6	31.7	31.7	42.1	36.9	33.2	33.3	32.65
9. m.f. + s.m. containing 1.0% Asolectin	41.2	33.7	33.4	30.65	29.6	36.2	31.8	31.2	31.3	30.2
10. Natural milk (Cow TG-1)	-	-	-	-	-	46.4	45.5	42.2	39.35	39.6

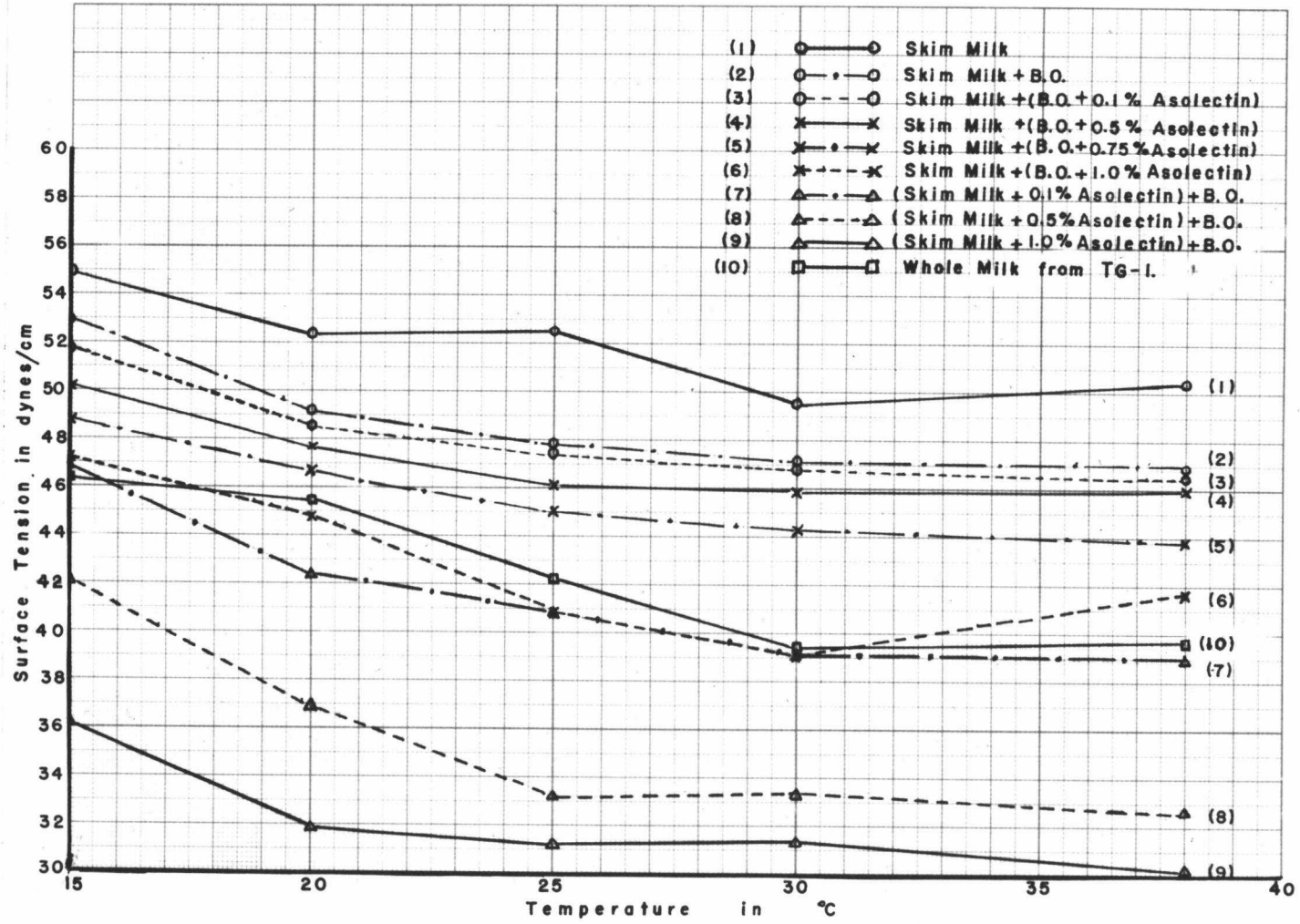

Figure 6. Effect of temperature on surface tension of natural and remade milks.

Section II: Lipolysis as Determined by Titration of Acidity

I. Effect of Homogenization Immediately After Milking

Individual samples from different cows were homogenized immediately after milking and before cooling. Homogenization was achieved by passing the milk three times through a hand homogenizer. Each homogenized sample was divided into two groups of 50 ml. each and was pasteurized at 80° C. (176° F.) for three minutes. Two drops of one per cent phenolphthalein were added to all and the pH adjusted to 8.3 with NaOH before incubation at 38° C. (100° F.). Formaldehyde, 0.02 per cent, was added as a preservative against bacterial growth. After 40 hours of incubation the samples were titrated with 0.1 N alcoholic KOH to the phenolphthalein endpoint. The results are reported as ml. of 0.1 N alcoholic KOH required per 100 ml. of milk (Table XVIII). They indicate a greater acid development in raw milk than in either non-homogenized raw or homogenized pasteurized milk from the same source.

II. The Use of an Antiobiotic as a Preservative Against Bacterial Growth

It has been reported (19, p.782) that formaldehyde partially inhibits lipase action in non-homogenized milk, but has no effect on homogenized milk. It was decided, therefore, to try antibiotics to prevent the growth of bacteria.

Table XVIII.

EFFECT OF HOMOGENIZATION IMMEDIATELY AFTER MILKING

Cow	ml. of N/10 alc. KOH required to re-neutralize to the phenolphthalein endpoint 100 ml. of milk after incubation at 37°-38° C.* for 40 hours		
	Raw	Raw Homogenized	Pasteurized Homogenized
226	2.94	5.64	3.44
246	2.42	10.96	2.68
263	5.00	10.74	3.40
270	5.02	11.86	1.04
G-1	4.96	9.68	1.32
547	3.00	6.70	1.68
531	3.74	4.64	0.72
561	2.44	2.12	2.48
568	3.08	3.08	1.36

* Results are averages of duplicates

Penicillin acts well against Gram positive organisms, but is not so effective against Gram negative organisms. On the other hand, streptomycin is effective against both types. Pseudomonas aeruginosa, which is more resistant to streptomycin, requires 2000 mcg./ml. to inhibit its growth, (91, p.255). Milk often contains a high initial count of a variety of microorganisms, and therefore it was necessary to try different concentrations of streptomycin.

Individual samples of milk were obtained from five cows. One ml. of sample from each cow was added to five sterilized test tubes and treated as follows:

i) 1 ml. of milk (control)

ii) 1 ml. of milk + streptomycin equivalent to 2000 mcg./ml.

iii) " " " " + " " " 1000 mcg./ml.

iv) " " " " + " " " 500 mcg./ml.

v) " " " " + formaldehyde, final concentration = 0.1%

These samples were incubated at 38° C. (100° F.) for 43 hours—by which time the controls had coagulated. The remainder were plated on tryptone glucose beef extract agar for the standard plate count (no inhibitor for streptomycin was added). The plates were incubated at 38° C. (100° F.) and examined after 24, 48 and 72 hours. No growth was shown by any plate.

This indicates that a streptomycin concentration as low as 500 mcg./ml. can be used as a preservative against bacteria.

III. Determination of the Susceptibility of Milk to Lipolysis at 38° C. (100° F.)

(A) Each of ten individual milk samples from the college herd was divided into four 100 ml. parts and treated as follows:

i) pasteurized at 62° C. (143° F.) for 30 minutes,

ii) raw,

iii) raw, neutralized to phenolphthalein endpoint by the addition of NaOH,

To the samples of groups i, ii, and iii streptomycin equivalent to 1000 mcg./ml. was added.

iv) raw, 0.1% formaldehyde added as a preservative.

The samples were then incubated at 38° C. (100° F.) as soon as possible after milking and without being allowed to cool. After incubating for 44 hours one ml. of one per cent phenolphthalein was added and they were titrated against 0.1 N alcoholic KOH to the phenolphthalein endpoint. The average results of duplicates are compiled in Table XIX.

The results indicate that maximum acidity developed in the samples which were neutralized to pH 8.3 before incubation. The samples containing formaldehyde as a preservative showed slightly more acidity than the samples containing antibiotic. An occasional sample of pasteurized control developed more acidity than raw samples. This did not occur in the preceding experiment and is difficult to explain.

Table XIX.

SUSCEPTIBILITY OF MILK TO LIPOLYSIS AT 38° C. (100° F.)

Sample	Cow	ml. of 0.1 N alc. KOH required to neutralize 100 ml. of milk to phenophthalein endpoint after incubation at 38° C. (100° F.) for 44 hours			
		i Control	ii Raw	iii Raw Neutralized to pH 8.3	iv Raw + HCHO 0.1%
1	M-1 (45.7*)	23.4 (48.0)	21.4 (47.1)	1.5 (42.3)	23.3 (45.1)
2	199	26.5	25.3	0.9	27.1
3	222	26.9	26.1	2.4	27.4
4	225	32.6	30.8	2.5	32.6
5	230	31.5	27.9	3.5	30.9
6	G-1	24.2	23.7	3.3	25.7
7	H-21	20.8	20.4	1.2	22.1
8	556	24.4	24.1	1.2	25.6
9	561 (45.9*)	22.3 (47.1)	21.5 (47.0)	1.3 (43.9)	24.2 (45.5)
10	572	27.0	26.7	1.2	27.9

The values in parenthesis are surface tension values in dynes/cm. at 38° C. (100° F.). Those with (*) were determined one hour after milking.

Groups i, ii and iii contained streptomycin equivalent to 1000 mcg./ml.

The surface tension values of two samples, 1 and 9, give no indication of rancidity development. The pasteurized samples had the highest surface tension. It is recognized, however, that pasteurization causes an increase in the surface tension of milk.

(B) As no definite conclusions could be drawn from the foregoing results, another experiment was designed to show the susceptibility to lipolysis of milk incubated at 38° C. (100° F.).

Ten individual samples were collected from the college herd. Each sample of 50 ml. was treated as follows:

(I) Raw milk:

i) titrated before incubation,

ii) titrated after incubation for 48 hours at 1° C. (34° F.) and 38° C. (100° F.);

(II) Milk pasteurized at 62° C. (143° F.) for 30 minutes:

i) titrated immediately after pasteurization,

ii) titrated after incubation for 48 hours at 1° C. (34° F.) and 38° C. (100° F.).

To all samples, except raw milk which was not incubated (I-i), 0.5 mg. of streptomycin per ml. was added. Before titration, 50 ml. of distilled water and one ml. of one per cent phenolphthalein were added and then titrated to the phenolphthalein endpoint with 0.1 N alcoholic KOH. Average results of duplicates expressed as ml. of 0.1 N alcoholic KOH required to neutralize 100 ml. of milk are reported in Table XX.

Table XX.

LIPOLYSIS OF MILK INCUBATED AT 1° C. (34° F.) and 38° C. (100° F.)

Sample		ml. of 0.1 N alc. KOH required to neutralize 100 ml. of milk to phenolphthalein endpoint					
		Before incubation		After incubation for 48 hours at			
		Pasteurized	Raw	Raw		Pasteurized	
				34° F.	100° F.	34° F.	100° F.
1	M-1	12.6	12.3	12.6	14.9	12.4	12.4
2	199	17.6	17.6	19.5	19.4	16.6	16.2
3	222	15.8	16.5	16.7	19.3	16.8	16.6
4	225	17.5	17.8	18.1	20.2	17.0	17.8
5	230	18.7	19.0	18.8	19.8	17.6	18.4
6	G-1	14.8	15.7	15.6	18.6	15.4	16.0
7	H-21	14.8	15.7	15.8	15.9	15.4	15.2
8	543	14.0	15.2	15.2	15.2	15.2	14.4
9	561	17.1	19.0	18.7	18.8	18.2	19.4
10	572	16.7	16.9	16.3	17.1	17.8	17.2

IV. The Effect of Different Amounts of Asolectin in the Fat and Aqueous Phase on Lipolysis

Five per cent emulsions were prepared by emulsifying milk fat in reconstituted skim milk (11%) obtained from spray dried non-fat milk solids (milk preheated to 75° C. (168° F.) for 15 minutes before spray drying). Asolectin was added at different concentrations (from 0.1% to 1.0%) in the fat or the aqueous phase. The percentage is based on the phase in which it was incorporated, for example, one per cent Asolectin in fat phase is equal to 0.05 gm. in 100 ml. of five per cent milk and one per cent Asolectin in 100 ml. of five per cent milk. The emulsification was done at 38° C. (100° F.) at 1000 pounds (500 pounds in the first stage and 500 pounds in the second stage).

All the samples were autoclaved for five minutes at 15 pounds steam pressure and cooled. The samples were divided into three parts of 45 ml. each and treated as follows:

a) 45 ml. of emulsion + 5 ml. of pasteurized milk (control),

b) " " " " + 5 ml. of raw milk,

c) " " " " + 5 ml. of raw milk + 0.1% HCHO.

To the samples of groups a and b, 1000 mcg. equivalent of streptomycin per ml. was added.

The samples were inoculated with five ml. of whole milk (fifth milking after calving). The samples were then incubated at 38° C. (100° F.) for 48 hours, at the end of which period 50 ml. of distilled water and one ml. of one per cent phenolphthalein were added

and the samples titrated against 0.1 N alcoholic KOH to the phenolphthalein endpoint. The average results of duplicates are reported in Table XXI.

Table XXI.

EFFECT OF DIFFERENT AMOUNTS OF ASOLECTIN IN THE FAT AND AQUEOUS PHASE ON LIPOLYSIS

Treatment (The following are all 5% emulsions)	ml. of 0.1 N alcoholic KOH required to neutralize 100 ml. of remade milk to phenolphthalein endpoint, after incubation at 100° F. for 48 hours.*		
	Control	Raw	
	Antibiotic (a)	Antibiotic (b)	HCHO (c)
Milk fat / skim milk	12.8 (48.9)	18.1 (44.85)	23.7 (44.9)
(m.f. / 0.1% Asolectin) in s.m.	14.0	20.2	25.1
(m.f. / 0.5% Asolectin) in s.m.	14.1 (46.35)	20.2 (41.8)	25.1 (42.55)
(m.f. / 0.75% ") in s.m.	14.4	25.1	26.5
(m.f. / 1.0% ") in s.m.	13.14 (46.65)	25.0 (38.9)	27.4 (40.7)
m.f. / (s.m. / 0.1% Asolectin)	16.1	22.3	24.9
m.f. / (s.m. / 0.5% Asolectin)	15.7	22.8	26.0
m.f. / (s.m. / 1.0% Asolectin)	15.3 (31.3)	22.9 (30.55)	25.8 (30.65)

* Average of three readings

(Refer to footnote of Table X)

The values in parenthesis are surface tension values in dynes/cm. at 38° C. (100° F.) determined after incubation

Section III: Colorimetric Determination of Lipase

I. Colorimetric Determination of Esterase in Individual Milk Samples

Samples from four individual cows were divided into two groups. One group was pasteurized at 80° C. (176° F.) for five minutes to destroy the enzyme. The results are reported in Table XXII.

Table XXII.

COLORIMETRIC DETERMINATION OF ESTERASE IN INDIVIDUAL MILKS

Sample	Per cent transmission at 575 mu		
	Pasteurized Control	Raw	Difference
251	96.0	54.9	41.1
521	66.2	47.1	19.1
Floss	91.1	56.2	34.9
Mike	92.8	71.8	21.0

The controls, except sample 521, showed very little hydrolysis of a-naphthyl acetate. More hydrolysis had occured in the raw samples. The difference indicates the amount of hydrolysis brought about by a heat-labile substance.

II. Distribution of the "Heat-labile" Esterase in Milk

Milk was obtained from one cow, and was immediately separated at a temperature of about 35° C. (95° F.). The skim milk so obtained after cooling to 21° C. (70° F.), was super-centrifuged at 30,000 r.p.m. and again at 40,000 r.p.m. These samples were divided into two groups, one of which was pasteurized at 75° C. (167° F.) for five minutes and served as a control.

The above procedure was repeated with the milk from another cow. The results (averages of the duplicates) are tabulated in Table XXIII.

The results definitely indicate the presence of a heat-labile esterase in raw whole milk. However, the enzyme is practically absent in skim milk or super skim milk and therefore should be expected to be associated with the cream. The following experiment was performed to determine the esterase activity of the cream and washed cream:

Mixed whole milk (cooled overnight) was separated at about 7° C. (45° F.) in a commercial De Laval separator. The skim milk (total solids equal 9.74 per cent) was super-centrifuged at 31,000 r.p.m. The cream was washed four times with distilled water at 38° C. (100° F.). All the samples, except the super skim milk, were adjusted to the same total solids as that of skim milk by the addition of distilled water. This was done in order to have uniform total solids in different products. The samples were divided

Table XXIII.

DISTRIBUTION OF HEAT-LABILE ESTERASE IN MILK

Product	Per cent transmission at 575 mu					
	Sample I			Sample II		
	Control #	Raw	Difference	Control #	Raw	Difference
Whole milk	84.5	44.0	+40.5	68.8	46.0*	+22.8
Skim milk (hand separated)	51.0	42.9	+ 8.1	47.0*	46.5*	+ 0.5
Super skim milk (30,000 r.p.m.)	42.8	44.5	- 1.7	47.5*	47.3*	+ 0.2
Super skim milk (40,000 r.p.m.)	47.0	47.4	- 0.4	47.0	46.5*	+ 0.5

* Very cloudy filtrate, and most of them took more than two hours to filter

Controls: pasteurized at 75° C. (167° F.) for five minutes

into two groups; one group was pasteurized at 77° C. (170° F.) for five minutes. Two samples (whole milk and super skim milk) were boiled momentarily to determine if they differed from the control pasteurized samples. The results are reported in Table XXIV.

This experiment confirms that the heat-labile esterase is definitely associated with cream. In this experiment, however, the cream was separated at about 7° C. (45° F.) when the milk fat was in solid state. There is an indication that when the cream is washed at 38° C. (100° F.) the heat-labile esterase is not retained with the washed cream. The skim milk possessed slight heat-labile esterase activity, whereas the super skim milk was devoid of it.

In a similar experiment the total solids were adjusted in all cases, except the super skim milk, to that of skim milk (9.33%) by the addition of distilled water. In addition, the total solids of four-times washed cream were adjusted to 9.33 per cent with super skim milk, skim milk and the first wash water. The results are reported in Table XXV.

Results once again confirm that the a-naphthyl esterase activity accompanies cream separated at 40° F. However, in this experiment four-times washed cream retained some activity. When skim milk, super skim milk or the first washing obtained during the washing of cream, was combined with this washed cream, the esterase activity showed a large increase. The pasteurized control samples of skim milk or super skim milk either alone or in combination

Table XXIV.

DISTRIBUTION OF HEAT-LABILE ESTERASE IN DIFFERENT COMPONENTS OF MILK ADJUSTED FOR TOTAL SOLIDS

Product	Per cent transmission at 575 mu		
	Control	Raw	Difference
Whole milk	64.3 (61.0)*	50.2	14.1
45° F.-cream	93.2	82.6	10.6
Cream washed 4 times at 100° F.	93.0	93.4	- 0.4
45° F.-skim milk	54.8	49.5	5.3
Super skim milk (31,000 r.p.m.)	52.6 (53.6)*	53.4	- 0.8

* The values in brackets are those of samples which were momentarily boiled. The controls were pasteurized at 77° C. (170° F.) for five minutes.

All samples, except super skim milk (total solids equal 9.48 per cent) were adjusted to 9.74 per cent total solids with distilled water.

Table XXV.

DISTRIBUTION OF HEAT-LABILE ESTERASE IN DIFFERENT COMPONENTS OF MILK ADJUSTED FOR TOTAL SOLIDS

Product	Per cent transmission at 575 mu		
	Control	Raw	Difference
Whole milk	53.8	48.5	5.3
Skim milk	50.2	53.8	- 3.6
Super skim milk	56.0 (57.9)*	54.8	1.2
45° F.-cream	82.5	63.5	19.0
Four-times washed cream	91.2	81.5	9.7
4 x W.C. + super skim milk	79.7	50.0	29.7
4 x W.C. + first washing	92.2	63.8	28.4
4 x W. C. + skim milk	66.8	47.0	19.8

* Momentarily boiled; all the other control samples were pasteurized at 77° C. (170° F.) for five minutes.

All samples, except super skim milk, were adjusted to 9.33 per cent total solids with distilled water, and in addition with skim milk, super skim and first washing in case of four-times washed cream.

with the washed cream gave lower per cent transmission than the others. Perhaps this might indicate the possibility of a heat-stable agent which is responsible for hydrolysis of a-naphthyl acetate. This activity is not eliminated even by momentary boiling of the sample. It is very unlikely that the esterase enzyme is responsible for this activity, however it might be due to chemical hydrolysis of a-naphthyl acetate brought about, particularly, by adsorption on some constituents of skim milk.

Another experiment was performed in order to establish whether the heat-labile esterase activity is associated with cream separated cold at 7° C. (45° F.) or with cream obtained at a higher temperature, 38° C. (100° F.). The samples were treated as follows:

1) Mixed whole milk (12.86 per cent total solids) diluted with distilled water to total solids equal to 9.25 per cent.

2) H.T.S.M. (9.21 per cent total solids): skim milk obtained at about 40° C. (105° F.) with a hand separator.

3) L.T.S.M. (9.25 per cent total solids) skim milk obtained at about 7° C. (45° F.) with a commercial separator.

4) H.T.-cream (43.83 per cent total solids) obtained as in #2. Diluted with distilled water to 9.25 per cent total solids.

5) L.T.-cream (44.24 per cent total solids) obtained as in #3. Diluted with distilled water to 9.25 per cent total solids.

6) Super skim milk (s.s.) (7.98 per cent total solids); #3, L.T.S.M. was super-centrifuged at 30,000 r.p.m.

7) 4 x W.C. (53.92 per cent total solids); #5 L.T.-cream was washed four times at 100° F. Diluted with distilled water to 9.25 per cent total solids.

8) 4 x W.C. diluted with super skim milk to make 9.25 per cent total solids.

9) 4 x W.C. diluted with "agglutinin-rich 'cream-serum'"* to 9.25 per cent total solid. (*L.T.-cream was washed twice with cold distilled water--ca. 15° C. (59° F.). The cream was then warmed to 43° C. (110° F.) for 15 to 20 minutes and separated in a hand separator. The "skim" so obtained is called the "cream-serum" which contains the so-called agglutinin.) (108, pp.417-418).

10) 4 x W.C. diluted with a suspension of Ca-caseinate-Ca-phosphate complex to total solids of 9.25 per cent. (Ca-caseinate-Ca-phosphate was obtained as a centrifugate in the bowl of the super centrifuge during preparation of sample 6.)

All these samples were divided into two groups. Members of one group were pasteurized at 76° C. (169° F.) for five minutes and used as controls. The results are reported in Table XXVI.

The results indicate that 43° C. (110° F.)-cream as well as 7° C. (45° F.)-cream showed high esterase activity. The "cream-serum" rich in agglutinin exhibits high esterase activity. The activity to hydrolyze a-naphthyl acetate is almost completely lost during the process of washing. The esterase activity also appears to accompany the centrifugate, Ca-caseinate-Ca-phosphate complex.

Freezing the samples immediately after incubation did not inhibit the esterase activity in samples 4, 5, 6 and 7, but the activity was almost the same in other samples.

Table XXVI.

DISTRIBUTION OF a-NAPHTHYL ESTERASE IN MILK

	Sample	Per cent transmission at 575 mu I Control	Raw	Difference	II Control	Raw	Difference
1	Whole milk	59.0	50.3	∕ 8.7	52.2	45.0	∕ 7.2
2	H.T.S.M.	53.2	48.5	∕ 4.7	50.0	45.0	∕ 5.0
3	L.T.S.M.	49.7	50.7	- 1.0	39.2	45.0	- 5.8
4	H.T.-cream	93.0	79.0	∕14.0	85.9	54.4	∕31.5
5	L.T.-cream	93.2	84.8	∕ 8.4	88.7	62.1	∕26.6
6	Super skim milk	52.7	54.6	- 1.9	55.0	42.9	∕12.1
7	4 x W. C.	93.0	91.0	∕ 2.0	91.2	82.1	∕ 9.1
8	4 x W.C. ∕ s.s.	53.8	54.5	- 0.7	46.8	48.9	- 2.1
9	4 x W.C. ∕ "serum"	80.0	56.0	∕24.0	76.0	53.2	∕22.8
10	4 x W.C. ∕ Ca-caseinate-Ca-Phosphate	78.0	58.5	∕19.5	61.2	49.0	∕12.2

(II: All samples were frozen immediately after incubation and thawed after three days for examination.)

Section IV: Phosphorus Determination

I. Determination of Phosphorus in Butteroil and Asolectin

Butteroil, low in phosphorus, was prepared by melting and filtering fresh sweet cream butter at 60° C. To this butteroil, one per cent Asolectin--a mixed phosphatide--was added. Asolectin contains about 3.1 per cent phosphorus. A modification of the Fiske and Subbarow method, as described earlier (pp.35-36) was employed to determine phosphorus.

	mg. P/100 gm. of fat
Butteroil	0.231 mg. (average of 8 readings)
Butteroil + 1% Asolectin	30.05 mg. (average of 7 readings)

If Asolectin contains 3.1 per cent P, one gram of Asolectin will have 0.0031 gm. or 31 mg. of P. Therefore, the value of (30.05 - 0.231) = 29.819 mg., obtained by actual determination, is sufficiently close to the value given by the manufacturer to confirm the accuracy of the method.

II. Determination of Lipid Phosphorus in Different Types of Cream

Mixed whole milk kept cold (ca. 4° C. (40° F.) overnight was separated in a commercial separator at about 7° C. (45° F.). The cream was divided into two lots; one was washed four times with distilled water at 38° C. (100° F.), the other five times at temperatures less than 15° C. (60° F.). The phosphorus contents of these samples are shown in Table XXVII.

Table XXVII.

LIPID PHOSPHORUS AND PHOSPHOLIPID IN DIFFERENT TYPES OF CREAM

Sample	Lipid-P/ 100 gm. fat	Fat	Lecithin*/ 100 gm. fat	Lecithin* in sample
	mg.	%	mg.	mg. %
Mixed whole milk	24.1	4.37	625.15	27.32
45° F.-cream	14.95	43.33	387.8	168.0
45° F.-cream, 4 x washed at 38° C. (100° F.)	7.97	52.8	206.7	109.16
45° F.-cream, 4 x washed at less than 15° C. (60° F.)	9.3	51.8	241.24	124.96

* The general term lecithin is used for convenience to designate the fat-like substances containing phosphorus. Lecithin = mg. per cent lipid phosphorus x 25.94. The factor assumes that the lecithin is of the stearyl-oleyl type.

The results show that about 62 per cent of the lipid phosphorus of milk goes with the 45° F.-cream and that approximately 47.0 per cent of lipid phosphorus of the cream is removed by washing the cream at 38° C. (100° F.). On the other hand, only 37.8 per cent is lost when the same cream is washed at temperatures less than 15° C. (60° F.) when the milk fat is largely in the solid state. This indicates some relationship between retention of phospholipids and the physical state of the fat.

III. Determination of the Distribution of Lipid Phosphorus in Milk

Mixed whole milk was obtained from the college creamery. A part of it was separated in a commercial separator at about 4° C. (40° F.), the remaining portion in a hand separator at about 43° C. (110° F.). The cream obtained at 4° C. (40° F.) was washed four times at 43° C. (110° F.). The first wash water, which is rich in euglobulin (108, p.417) was kept for the determination of lipid-P. The results are reported in Table XXVIII.

This confirms the conclusion of the last experiment that the amount of lipid-P in the product depends on the temperature of separation. Cream separated when the milk fat is in solid state contains more lipid-P (64 per cent of the total in milk) than the cream separated at higher temperatures (ca. 50 per cent of the total in milk). The first wash water of 4° C. (40° F.) cream washed at 43° C. (110° F.) contains a large amount of lipid-P. The amount which leaves the cream must be either in a free state

Table XXVIII.

AMOUNT OF LIPID PHOSPHORUS AND LECITHIN IN DIFFERENT FRACTIONS OF MILK

Product	% Fat	Lipid-P/ 100 g. fat	Lecithin/ 100 g. fat	Lecithin in product
		mg.	mg.	mg. %
Mixed whole milk	4.32	27.65	717.2	30.98
7° C. (40° F.)-cream	39.72	17.68	458.6	182.2
43° C. (110° F.)-cream	40.68	13.08	339.3	138.0
7° C. (40° F.)-cream washed four times	42.57	10.77	279.4	118.9
7° C. (40° F.)-skim milk	0.07	700.0	18,158.0	12.71
43° C. (110° F.)-skim milk	0.118	582.7	15,115.0	17.83
First wash water of 7° C. (40° F.)-cream washed at 43° C. (110° F.)	0.056	541.0	14,033.0	7.86

(Refer to the footnote of Table XXVII)

or in loose combination and hence can pass from the fat to the aqueous phase.

IV. Effect of Washing on the Lipid Phosphorus Content of Cream

Mixed whole milk cooled overnight to about 4° C. (40° F.) was separated at 7° C. (45° F.). The cream so obtained was washed six times at 38° to 43° C. (100° to 110° F.), and a sample each of two-, four- and six-times washed cream was kept for lipid phosphorus determination. The six-times washed cream was then churned in a test tube and the lipid phosphorus determined on the buttermilk obtained from this churning. The results are tabulated in Table XXIX.

The results once again verify that the first two washings of the cream remove most of the phospholipids; it is reduced from 13.22 mg. to 8.27 mg. of lipid-P per 100 gm. of lipid material. This is further reduced to 7.07 mg. per cent after the fourth washing. It then remains practically constant as indicated by the amount of 7.25 mg. per cent after the sixth washing. The last figure actually shows a slight increase, which is within the possibilities of experimental error or may be due to slight churning which generally leads to oiling off in washed cream. About half of the phospholipids are more or less free and, therefore, can migrate from the fat to the aqueous phase. The other half, being in some sort of combination with the membrane material, can be considered "fixed" in the cream phase. The latter can be

Table XXIX.

EFFECT OF CREAM WASHING ON ITS LIPID PHOSPHORUS AND LECITHIN CONTENT

Product	% Fat	Lipid-P/ 100 g. fat	Lecithin/ 100 g. fat	Lecithin in product
		mg.	mg.	mg. %
Milk	4.31	29.86	774.57	33.38
7° C. (45° F.)-skim milk	0.092	631.5	16,381.0	15.07
7° C. (45° F.)-cream	50.0	13.22	342.9	171.5
2 x washed cream	62.10	8.27	214.5	112.4
4 x washed cream	61.9	7.07	183.4	113.5
6 x washed cream	59.78	7.25	188.06	133.2
Buttermilk of 6 x washed cream	2.6	27.63	716.7	18.63
First washing at 43° C. (110° F.)	0.083	561.6	14,567.9	12.09

(Refer to the footnote of Table XXVII)

partially removed by mechanical agitation or churning which leads to the displacement of the membrane. This is indicated by the higher content of lipid phosphorus in buttermilk. A considerable amount remains in butter largely in the form of "entrapped" churned membrane material. This is indicated by the fact that when the butter is melted the butteroil is low in lipid phosphorus.

V. Total Phosphorus Content of Lipo-protein Membranes Obtained from Washed Cream by Different Methods

Cream, obtained by separating at 10° C. (50° F.) mixed whole milk which had been cooled to, and kept at 4° C. (40° F.) overnight, was washed five times with distilled water at about 43° C. (110° F.). The washed cream was shaken repeatedly with ether at room temperature until all the fat was removed. The pinkish-white membrane material was allowed to settle. This membrane was dried below 50° C. (122° F.) under reduced pressure to constant weight.

The membrane material was isolated also using the following solvents: a mixture of two volumes of ethyl ether and one volume of ethyl alcohol; a mixture of one volume of acetone and four volumes of "Heptane", and a mixture of one part of ethylene chloride and one part of ethyl ether. The phosphorus of the membrane material was determined directly and therefore represents total rather than lipid phosphorus. Another sample of cream was treated in a similar manner, except that it was washed six times at 38° C. (100° F.). The results are reported in Table XXX.

Table XXX.

AMOUNT OF PHOSPHORUS IN THE INTERFACIAL MEMBRANE MATERIAL OBTAINED BY DIFFERENT METHODS

Product	Lipid-P/100 gm. of fat	
	I	II
	mg.	mg.
10° C. (50° F.)-cream	15.7 (37.5% fat)	13.22 (50% fat)
Washed cream	8.4 (5-times washed, 38.26% fat)	7.25 (6-times washed, 59.78% fat)
Interfacial membrane obtained by	**Total P/100 gm. of dry membrane material**	
	mg.	mg.
Ether extraction	478.1	680.8
(2 vol. EtOEt + 1 vol. EtOH) extraction	144.8	235.9
(1 vol. Acetone + 4 vol. Heptane) extraction	447.4	556.4
(1 vol. Ethylene Chloride + 1 vol. EtOEt) extraction	207.0	-

The membrane obtained by ether extraction had the highest phosphorus content, due perhaps to the fact that ether does not break the lipo-protein complex. On the other hand, alcohol is known to extract some of the phospholipid from the complex and thus the residual phosphorus in the alcohol extracted membrane was low. It has been reported (103, pp.1222-1223) that the lipo-protein complex is not broken when a mixture of one volume of acetone with four volumes of Hexane is used. This appears to be true when "Heptane" is used instead of Hexane. Therefore, when the mixture of one volume of acetone plus four volumes of "Heptane" was used for extracting the membrane, the values of total phosphorus were comparable with, although not as high, as those obtained by ether alone. As mentioned earlier, a mixture of equal proportions of ethylene chloride and ethyl ether has been used for removal of lipid material without denaturing protein. The result shows, however, that a considerable amount of total phosphorus is retained in the membrane after extraction with this mixture.

Section V: Electron Microscope Studies of Milk Constituents

Skim milk constituents: Milk is a complex biological fluid, consisting of substances in true solution (such as lactose, mineral salts), in colloidal suspension (such as proteins, certain calcium salts) and in the emulsified state (e.g. milk fat). The whey proteins are of extremely small dimensions, whereas the Ca-caseinate-Ca-phosphate entities are larger. The fat globules which form the discontinuous phase of the emulsion range from one to ten microns in diameter, the bulk of them ranging from two to five microns.

Little information is available regarding the preparation of milk samples for examination with the electron microscope. Milk must be diluted considerably (about 400 to 500 times) in order to obtain a clear image in which the constituents do not overlap and obscure their identity. It is difficult to identify the various images obtained even when a material as complex as milk is diluted. The identity of various components was established by examining the individual constituents of milk when purified (or isolated) by the available methods.

The electron microscope used in this investigation has a minimum magnification of about 1,100 diameters. For this reason it is not possible to examine specimens that can be seen with the naked eye. Therefore, it is necessary to remove all but the smaller fat globules. Skim milk was centrifuged at 2,000 r.p.m. for five minutes, the lower portion removed, diluted with distilled

water and re-centrifuged. This procedure was twice repeated. Finally, the diluted skim milk so obtained was examined after air-drying and shadowcasting. The image shows two general types of structures as illustrated in Figures 7 and 8.

Figure 7 shows many spherical aggregates of Ca-caseinate-Ca-phosphate complex of different dimensions (ranging from 100 mu to 220 mu) surrounding a bacterium. Figure 9 represents a very dilute suspension of low-heat skim milk powder; here also, the globular aggregates of Ca-caseinate-Ca-phosphate predominate. The sizes of the particles are comparable.

Figure 10 shows Ca-caseinate-Ca-phosphate complex obtained by the method of Ramsdell and Whittier (92, p.413). The spheres are symmetrical and are of assorted dimensions. This Figure substantiates the identity of spherical objects of Figures 7 and 9.

Figure 8 shows a typical fat globule with its membrane. It has not retained its spherical shape, but has flattened during drying or shadowcasting. The surface forces which maintain the fat globule in a spherical form in milk undoubtedly are responsible for maintaining a circular contour of the globules as seen in most of the electron micrographs. The ridges represent flattened or shrivelled membrane. Figure 11 shows similar structures obtained from diluted four-times washed cream. Unfortunately, none of the images, even under maximum magnification, showed any details of the periphery of the fat globule and its "membrane".

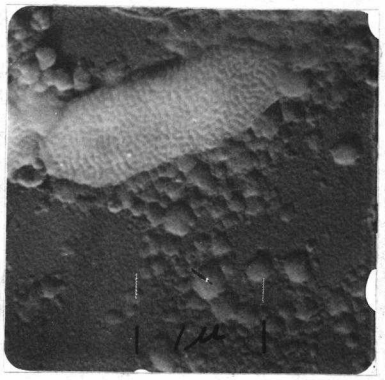

Figure 7.
Ca-caseinate-Ca-phosphate.

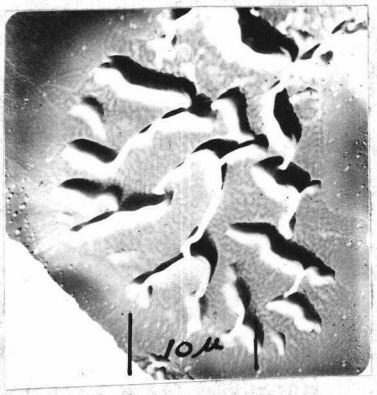

Figure 8.
Fat globule with its membrane.

Repeatedly centrifuged and diluted skim milk.

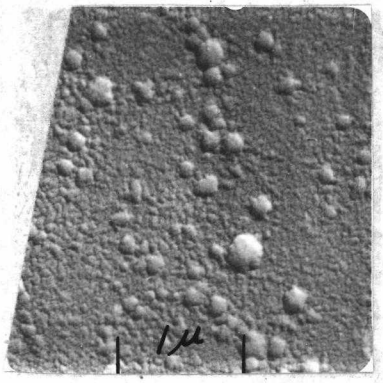

Figure 9.
Low-heat skim milk powder.

Figure 10.
Ca-caseinate-Ca-phosphate obtained by super centrifuging.

Figures 12 and 13 show whey proteins obtained from rennet whey by heat precipitation at pH 5.6 and acid precipitation at pH 4.8, respectively. The former indicates a definite change of physical state perhaps due to denaturation, whereas, in the latter the proteins show little change from the typically sphere-like entities. Figure 14 represents lactalbumin precipitated at its isoelectric point by 50 per cent ethyl alcohol at 1° C. (34° F.). The change in physical state does not appear to be as drastic as in Figure 12.

The Fat Globule "Membrane": In order to obtain the interfacial membrane in its "native" state, different treatments were used on a five-times washed cream. The membrane obtained by ether, two volumes of ether plus one volume of ethyl alcohol, one volume of acetone plus four volumes of "Heptane", and absolute ethyl alcohol on the samples of washed cream are shown in Figures 15, 16; 17,18; 19,20; and 21 respectively.

Figure 21 shows structures which are very likely due to the "denaturation" resulting from dehydration of protein by absolute alcohol. It was found that whenever alcohol alone was used or in proportions higher than one volume of ethyl alcohol and one volume of ethyl ether, the electron microscope image contained a considerable number of objects resembling long, hollow, slender cylindrical crystals. These structures were sometimes observed in buttermilk extracted with ether to remove the free fat (see Figure 45). These cylindrical entities apparently are due to the loss of

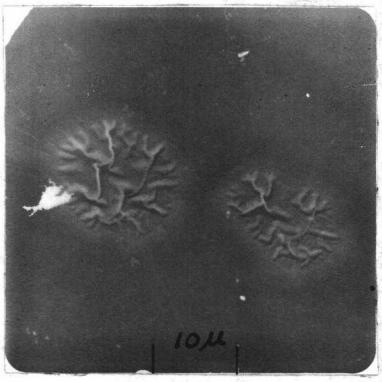

Figure 11.
Fat globules with membranes from washed cream.

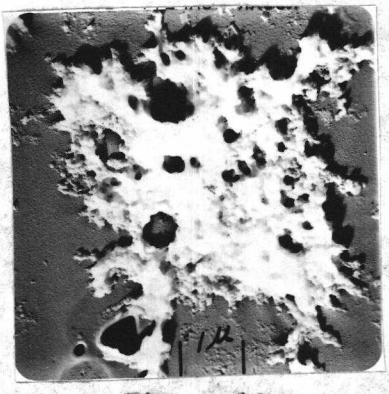

Figure 12.
Whey proteins--heat precipitated at pH 5.6.

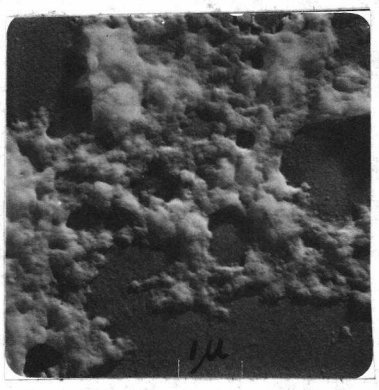

Figure 13.
Whey proteins--acid precipitated at pH 4.8.

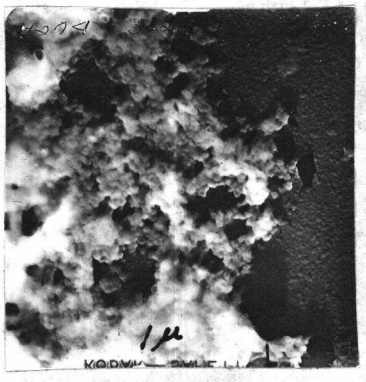

Figure 14.
Lactalbumin--precipitated by 50% alcohol at 35° F.

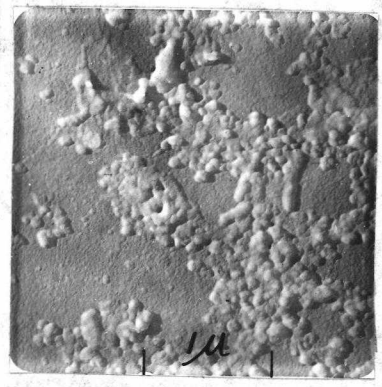

Figure 15.

Figure 16.

Lipo-proteins obtained from washed cream
by extracting with ether.

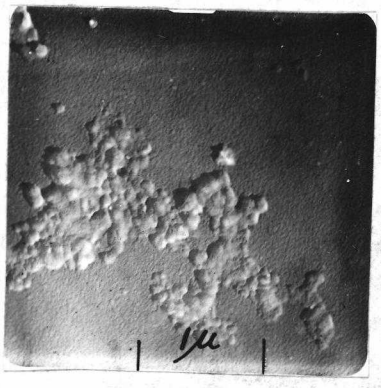

Figure 17.

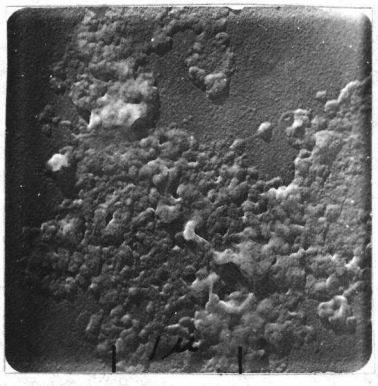

Figure 18.

Lipo-protein obtained from washed cream
by treatment with 2 vol. EtOEt / 1 vol. EtOH.

identity of globular proteins caused by dehydration or by the removal of lipids from the lipoprotein complex, resulting in a partially denatured protein. It is known from studies of lipoproteins that whenever the lipid-protein linkages are broken the protein is slightly denatured (Chick, Biochem.Jour. p.418, 1914).

In this connection it is interesting to refer to a statement by Frey-Wyssling (18, p.136):

> Rigorous dehydration removes not only the water between the macromolecular spheres, but also the hydration water inside the globular molecules, so that their structure is destroyed and the solubility of the protein is abolished. This physico-chemical transformation of soluble proteins is called denaturation. There are some indications that the denaturation of globular proteins consists of an unfolding of the wrapped-up polypeptide chains. The inverse reaction, the transformation of the "denatured" protein into globular molecules, is usually impossible in vitro, but it must occur readily in vivo.

Membranes obtained with ether alone do not generally show as much granular appearance as those obtained by 2 EtOEt / 1 EtOH or 1 acetone / 1 "Heptane". Figure 22 shows the globular membrane obtained from the buttermilk treated with ether to remove the free fat. The buttermilk was obtained by churning four-times washed cream. It appears from the clarity and the granular appearance of the images that more phospholipid is available to ether extraction after churning than from the unchurned washed cream.

Figures 23 and 24 illustrate the membranes obtained by formaldehyde plus ether and by formaldehyde, ether and alcoholic KOH, respectively. Both the figures indicate the adverse effect of

Figure 19.

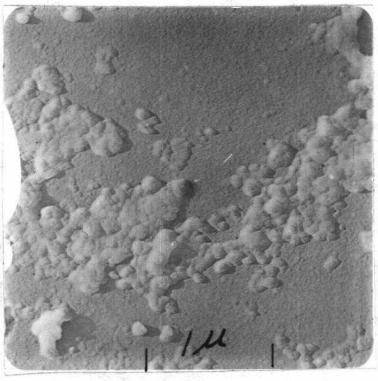

Figure 20.

Lipo-protein obtained from washed cream by treatment with a 4:1 mixture of "Heptane" and acetone.

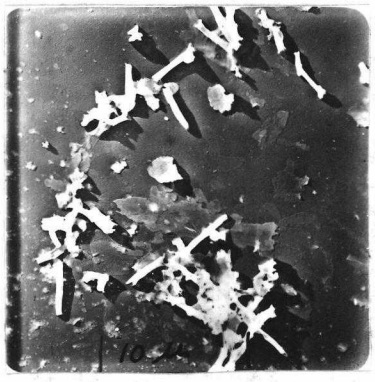

Figure 21.
Washed cream extracted with absolute EtOH at room temperature.

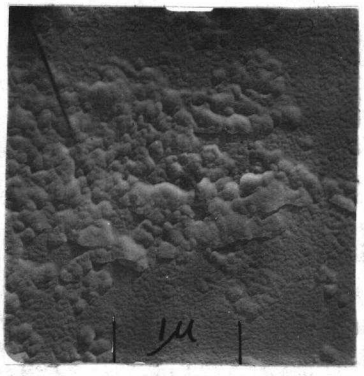

Figure 22.
Buttermilk from washed cream treated with ether to remove free fat.

formaldehyde which was used as a "fixing" agent. The latter figure shows a sort of "disintegrated" membrane, this is due to the effect of alcoholic KOH, and reflects the use of such drastic treatments used to break the emulsion in milk.

Figures from 25 to 29 show interfacial membranes extracted from washed cream obtained from milk of different cows. The four-times washed creams were treated in an identical manner with two volumes of ethyl ether plus one volume of ethyl alcohol. These Figures indicate that the membranes are not exactly identical although they are all made up of small spherical entities which are more or less symmetrically arranged and closely packed and connected.

Examination of egg yolk lipoprotein: In order to substantiate the identity of milk lipoproteins, lipovitellin from egg yolk was examined in the electron microscope. Figure 30 illustrates lipoprotein from egg yolk extracted by 1 EtOH / 2 EtOEt. For comparison, Figure 28 shows the lipoprotein of the interfacial membrane from milk. The structure of material in the two pictures is similar; however, the spherical globules of lipovitellin are much larger than those of milk lipoproteins.

In this connection it is interesting to note that the electron micrographs of the preparations of thromboplastic lipoproteins of blood, obtained by Chargaff (8, p.19), revealed the presence, together with some aggregated material, of a large percentage of almost perfect spheres with a diameter of 80 to 120 mu. He

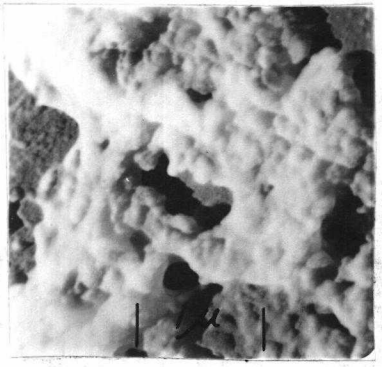

Figure 23.
Lipo-protein obtained by treatment with formaldehyde / ether.

Figure 24.
Washed cream treated with alcoholic KOH / ether / formaldehyde.

(Formaldehyde was used as a fixing agent.)

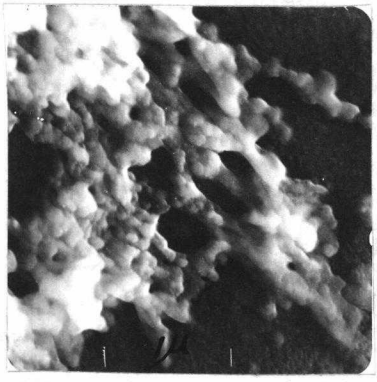

Figure 25.
(Jersey)

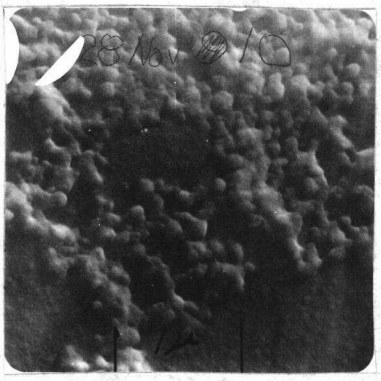

Figure 26.
(Jersey)

Lipo-proteins obtained from washed creams of different cows by treatment with 2 vol. EtOEt / 1 vol. EtOH.

Lipo-proteins obtained from washed creams of different cows by treatment with 2 vol. EtOEt + 1 vol. EtOH.

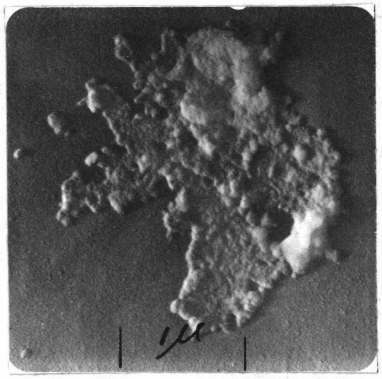

Figure 27.
(Holstein)

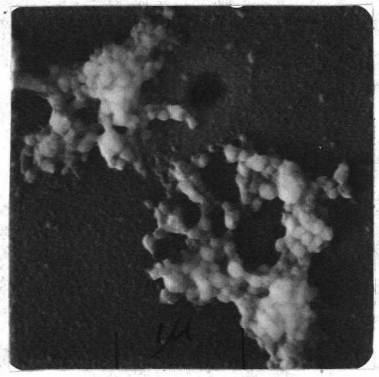

Figure 28.
(Holstein)

Figure 29.
(Holstein)

further reports that the particles are probably in a highly hydrated state.

Figures 32 A, B and C represent very interesting and unusual patterns, possibly artifacts resulting when the egg yolk lipoproteins were prepared and examined. Caution should be exercised in interpreting electron micrographs.

The images of milk fat or its emulsion in water could not be seen clearly in the electron microscope as they always appeared to be out of focus. However, after repeated trials, one picture (Figure 33) was obtained. For this sample a very small amount of milk fat was emulsified with Tween 20 in water. In this sample the fat globules have not retained a circular appearance as they are not protected by an interfacial membrane as in the case of the fat globules in milk.

Similar difficulties were also encountered with the aqueous solutions of lecithin and Asolectin; although spherical globules could be seen, they could not be photographed due to their out-of-focus appearance (see Figure 36). This property of lipids to appear out of focus may be due to the fact that lipids are probably not as opaque to the electrons as are proteins.

One sample of cold, 1° C. (35° F.), gravity cream, washed twice with warm 43° C. (110° F.) distilled water, extracted with ether and examined in the electron microscope revealed in addition to a few membranes and cylindrical crystals a large number of phospholipid-like spheres which were out of focus. These spheres are

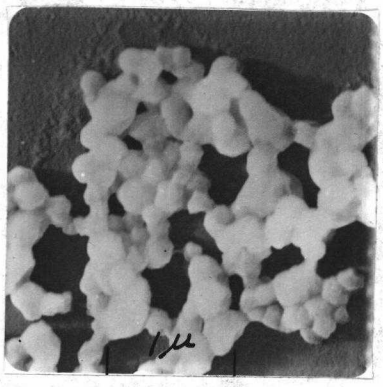

Figure 30.
Lipo-proteins from egg yolk.

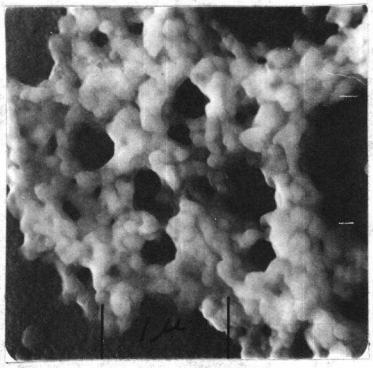

Figure 31.
Lipo-proteins from milk.
(Jersey)

Magnification ca. 16,800 X.

(Lipovitellin has larger entities than the lipo-proteins from milk.)

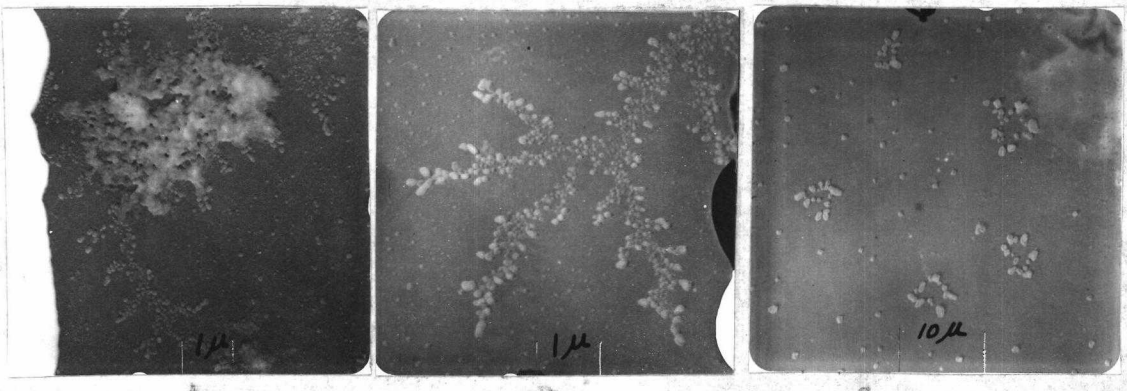

Figure 32.

Probable artifacts resulting when egg yolk lipo-proteins are prepared and examined.

illustrated in Figure 34.

In a few cases the preparation of animal lecithin in distilled water revealed biconvex lens-shaped as well as spherical entities. Most of these lens-shaped objects were arranged at the periphery of circular patterns, as shown in Figure 35.

Interfacial membranes of "remade" creams: Figures from 38 to 43 represent interfacial membranes obtained from "remade" creams. The emulsions, prepared at 200 pounds pressure in a two-stage homogenizer, were aged overnight at 1° C. (34° F.). The creams were obtained by prolonged centrifuging of the emulsions at 2,000 r.p.m. These creams were washed only twice because of their tendency to churn and oil off. The washed creams were treated repeatedly with ether to remove free fat. The residual membranes were examined after diluting and shadowcasting.

The emulsions were prepared as follows:

Figure 37: 10% milk fat in super skim milk (obtained at 41,000 r.p.m.).

Figure 39: 10% milk fat including 1% Asolectin in super skim milk (41,000 r.p.m.).

Figure 40: 10% (milk fat including 1% Asolectin and 0.3% cholesterol) in super skim milk (41,000 r.p.m.).

Figure 41: 10% milk fat in rennet whey.

Figure 42: 10% milk fat including 1% Asolectin and 0.3% cholesterol in cheddar cheese whey.

Figure 43: 10% milk fat in 3% egg albumin solution.

Figures 44 and 45: 10% milk fat (including 1% Asolectin) in 3% egg albumin solution.

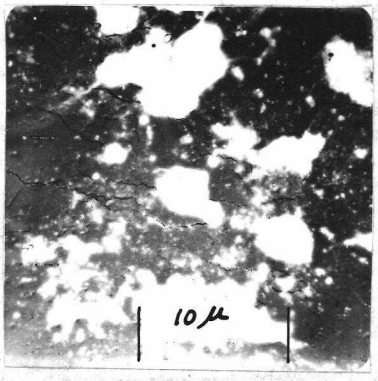

Figure 33.
Milk fat emulsified with Tween 20 in water.

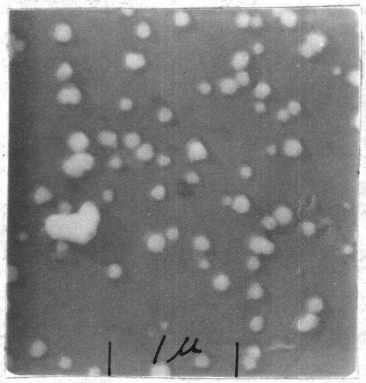

Figure 34.
Washed gravity cream extracted with ether.

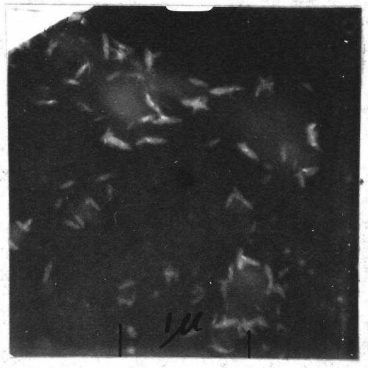

Figure 35.
Animal lecithin in distilled water.

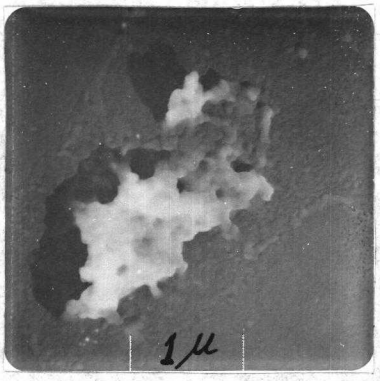

Figure 36.
Hydrated Asolectin at the interface between fat and distilled water after the equilibrium is established at the interface (sample treated with ether to remove free fat).

Membranes from artificial emulsions
(obtained by extraction with ether).

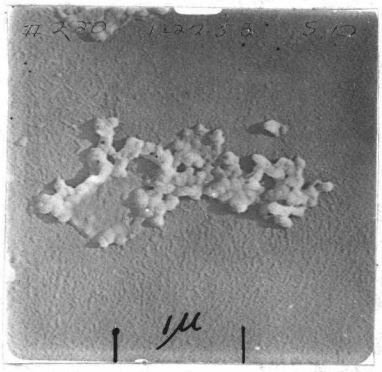

Figure 37.
10% milk fat in super skim milk.

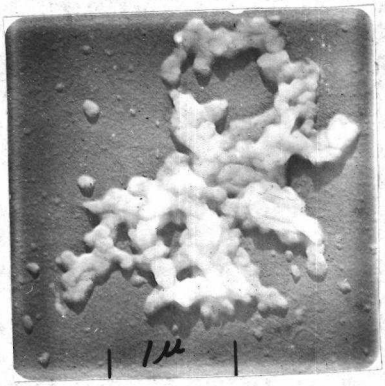

Figure 38.
10% milk fat in skim milk (membrane obtained by 3 EtOEt / 1 EtOH).

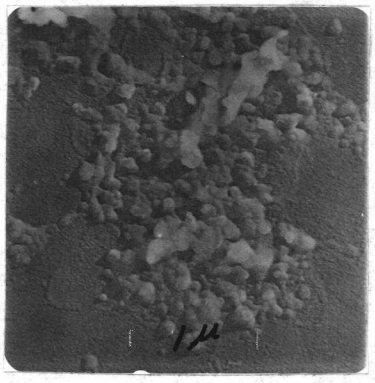

Figure 39.
10% milk fat (with 1% Asolectin) in super skim milk.

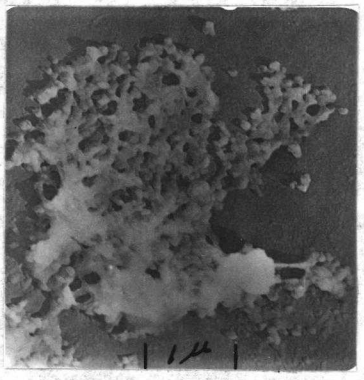

Figure 40.
10% milk fat (including 1% Asolectin and 0.3% cholesterol) in super skim milk.

Membranes from artificial emulsions
(obtained by extraction with ether).

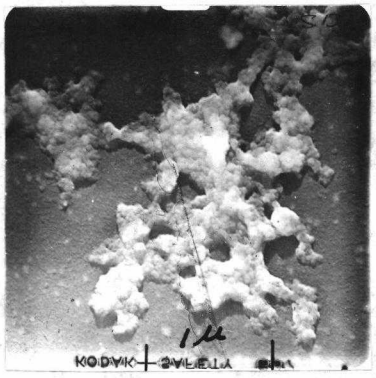

Figure 41.
10% milk fat in rennet whey.

Figure 42.
10% milk fat (including 1% Asolectin and 0.3% cholesterol) in cheddar cheese whey.

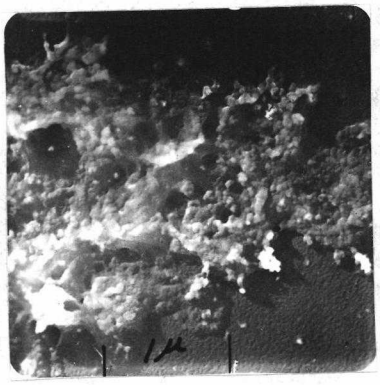

Figure 43.
10% milk fat in 3% egg albumin.

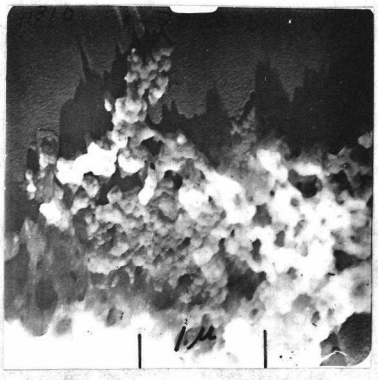

Figure 44.
10% milk fat (with 1% Asolectin) in 3% egg albumin.

Figure 46: Milk fat in Ca-caseinate-Ca-phosphate suspension. Membrane extracted with 3 EtOEt / EtOH.

It is rather difficult to interpret these electron micrographs; however, it can be said that there is definite aggregation of protein entities in all these cases. Of course, these samples cannot be expected to duplicate exactly the natural membrane because the conditions and the constituents present during the formation of the "remade" membrane are not the same as those when the natural membrane is formed.

The Figures 41 and 42 indicate that the membrane obtained with whey does not resemble the natural membrane as closely as the one obtained from super skim milk (Figures 37 to 40). On the other hand, the large Ca-caseinate-Ca-phosphate entities (Figure 46) do not appear to play an important role in the formation of the membrane. The inclusion of phospholipid in the fat phase appears to increase the size of the globule entities of the membrane (Figures 39, 40 and 44). The membrane obtained from buttermilk emulsion shows the membrane which appears to be broken up or disintegrated (Figure 47). Figure 45 shows the long cylindrical crystals obtained from the repeatedly ether-extracted artificial emulsion of fat (containing Asolectin) in egg albumin.

Membranes from artificial emulsions.

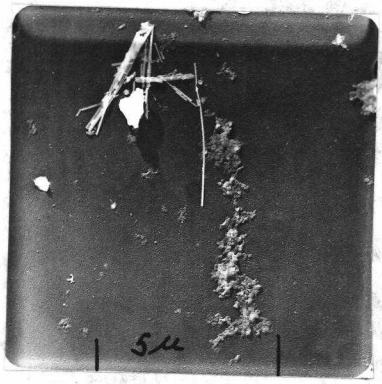

Figure 45.
10% milk fat (including 1% Asolectin) in 3% egg albumin.

(Long crystal-like structures are physico-chemically changed or denatured protein.)

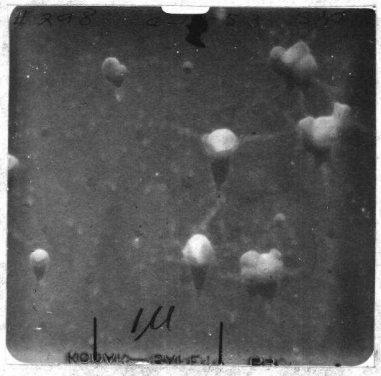

Figure 46.
Milk fat in Ca-caseinate-Ca-phosphate solution.

(Membrane obtained by treatment with 3 EtOEt / 1 EtOH.)

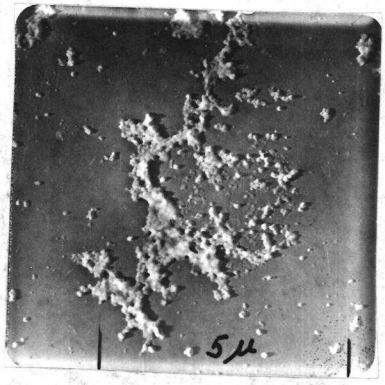

Figure 47.
Milk fat (containing 1% Asolectin) in buttermilk.

(Membrane extracted with 2 EtOEt / 1 EtOH.)

DISCUSSION

Spontaneous rancidity which requires only cooling was not encountered in the samples examined. Therefore this study was restricted to induced lipolysis.

The results in Table XVIII show that homogenization at 38° C. (100° F.) immediately after milking produced lipolysis as indicated by an increase in acidity. The results of Table XXI give evidence that rancidity was invariably produced by inoculation of raw milk into pasteurized synthetic emulsions of milk fat in skim milk with and without added phospholipid. Induced lipolysis of milk by temperature activation involves pre-cooling, re-warming and re-cooling. The results of Table VII-A indicate that the first two steps of pre-cooling and re-warming produced considerable lowering of surface tension, but complete temperature activation involving all three steps with and without occasional stirring produced intense rancidity in all samples (see Table VIII).

All these results definitely demonstrate that although a lipase (or esterase) is always present in active form in all milks it is unable to act upon either the phospholipid or the fat itself in milk, as long as the natural membrane around the fat globule in the freshly secreted milk remains unaltered. The natural membrane, therefore, appears to act as a barrier between the enzyme and the substrate. The efficiency of this lipoprotein membrane as a barrier is reduced by treatments which induce lipolysis in milk or cream.

Phospholipids appear to play an important role in the reactions taking place at the interface. As shown in Tables X and XI, the inclusion of Asolectin in either the fat or the aqueous phase reduces interfacial tension, thereby making the spreading coefficient less negative. This facilitates spreading of the aqueous phase over milk fat. This is particularly true when the phosphatide is added to the aqueous phase. As illustrated in Figure 6, a five per cent milk fat-skim milk emulsion containing one per cent phosphatide in the fat phase or 0.1 per cent in the aqueous phase exhibits changes in surface tension with increase in temperature from 10° C. to 38° C. (50° F. to 100° F.), similar to a sample of natural milk. This indirectly indicates that in natural milk the phospholipids may occur partly in the fat phase and partly in the aqueous phase.

As shown in Table XXI, the mixed phosphatide, Asolectin, when added to either phase increases lipolysis of pasteurized milk fat-skim milk emulsions inoculated with a small amount of raw milk. Phosphatides when included in the fat phase show greater increase in lipolysis than when added to the aqueous phase. Whether this enhancing of lipolytic activity is due to the surface-activity of the phosphatide or its action as an enzyme activator (or coenzyme) is not established.

Evidence as shown in Tables XXVII and XXVIII proves that a greater amount of phospholipid is retained in cream obtained when milk is separated at a temperature at which the milk fat is in

solid state than when the fat is in the liquid state. The mere warming of a 45° F.-cream results in a migration of a part of phospholipid from the fat to the aqueous phase. If the same phenomenon occurs in whole milk pre-cooled to 7° C. (45° F.) and subsequently warmed to 38° C. (100° F.), the action of the first two steps of temperature activation is explained.

In this connection, Frey-Wyssling (18, p.146) makes the interesting statement that the homopolar cohesive bonds between lipids and polypeptides are loosened by small amounts of energy and are therefore sensitive to temperature changes, which makes them more mobile. The evidence of the presence of such bonds in serum lipoproteins is reported by Macheboeuf (72, p.372).

The decrease in surface tension, as shown in Table VII, which results by cooling of natural milk to 10° C. (50° F.) or below and warming above the melting point of the milk fat may be explained by the release of surface-active phospholipids from the fat to the aqueous phase. This view of phospholipid migration is supported by the results of Table XVI-B and C. They show that the lowering of surface tension in synthetic emulsions of milk fat containing Asolectin in skim milk is about the same as in the case when an equivalent amount of phosphatide is added to the aqueous phase. This indicates that the phospholipid in the aqueous phase is the cause of lowered surface tension and agrees with observations of others that churning of milk causes the release of lecithin from the cream to the skim milk and ultimately into the buttermilk.

The latter has the lowest surface tension of any milk product. From these observations it may be postulated that in the early stages of rancidity the lowering of surface tension may be due to the release of phospholipids to the aqueous phase.

The hypothesis of the migration of phospholipid is further supported by Anderson (2, p.519) who reports that the nuclear envelope is composed of two components: (i) a structural layer very likely consisting of a meshwork of fibrils, and (ii) a mobile component, rich in phospholipid believed to be associated with the structural component in such a manner as to leave open the pores or interstices. He further states that movement of the mobile component away from the nuclear envelope may result in the formation of blebs or free floating spheres. This phenomenon may account for so many phospholipid spheres being observed in the electron micrograph of twice-washed gravity cream extracted with ether to remove free fat (Figure 34).

The colorimetric determination of milk "lipase" indicates that a heat-labile esterase is concentrated in cream, which is in contradiction to the observations of other investigators. The pasteurized samples of skim milk or super skim milk either alone or in combination with washed cream, gave higher color density than similar controls of whole milk or cream. Hydrolysis of a-naphthyl acetate has occurred in the pasteurized samples. Moreover, the results shown in Tables XXIV and XXV indicate that this "heat-stable" esterase activity is not destroyed even by

momentary boiling of the samples. It is very unlikely that any enzyme could withstand boiling temperatures and therefore it appears doubtful if the esterase enzyme is responsible for the hydrolysis of a-naphthyl acetate. The action of the cream in inhibiting hydrolysis in the pasteurized samples is unexplained. It might, however, be due to the chemical hydrolysis of a-naphthyl acetate which somehow is hindered in pasteurized samples containing cream. Because of such discrepancies no attempt has been made to report the results in "lipase" units, a unit representing the liberation of one mcg. of a-naphthol.

In connection with the "heat-stable" esterase, Shipstead and Tarassuk (107, p.616) report:

> If a dehydrated product is made from naturally lipolytically active milk, hydrolytic rancidity will develop in powder upon aging. The lipolytic activity in this case is not stopped by pre-heating the milk before drying as high as 93° C. (200° F.) for 15 seconds. How high it is necessary to heat milk before the lipase of naturally active milk is completely inactivated has not been established.

Caution should be exercised in interpreting electron micrographs. The air-drying of samples often produces artifacts due to surface tension forces (see Figure 32 A, B and C). Figures 8 and 11 are excellent illustrations of fat globules of milk which have not retained their spherical shape but have flattened and assumed a wrinkled appearance. They have a definite circular form unlike the fat globules emulsified in water with Tween 20, which show irregularly shaped-masses of fat as indicated in Figure 33. The

fat globule membrane, therefore, appears to be responsible for the form and the wrinkled appearance. The basic structure in lipoproteins obtained from washed cream and from egg yolk is similar and appears as fibrils made up of spherical entities. The large holes in the membrane are perhaps artifacts. The membranes obtained from the milk of different cows are basically similar in structure.

For the electron microscope work, best results for isolating the lipoprotein membrane from washed cream were obtained by a mixture of two or preferably three volumes of ether with one volume of alcohol. The results of Table XXX indicate that the phosphorus content of the fat globule membrane isolated from the same sample of washed cream depends on the type of solvent used for the isolation of the membrane. The total phosphorus content is minimum when a mixture of two volumes of ether and one volume of ethyl alcohol is used. It may be that the removal of a considerable part of the phospholipid by this mixture permits clearer images. When absolute alcohol is used for isolating the membrane from the washed cream, the protein undergoes drastic changes as shown in Figure 21. This apparently is due to the loss of identity of globular proteins by dehydration or by the removal of lipids from the lipoprotein complex resulting in a partially denatured protein. Denaturation of whey proteins is illustrated in Figure 12.

Electron micrographs (Figures 37 to 44) of membranes obtained

from synthetic emulsions of milk fat (with and without phosphatides) in skim milk, whey or egg albumin indicate that protein entities are aggregated in the form of membranes. The tremendous surface energy at the interfacial boundry apparently causes distortion of protein entities, and leads to some flattening and uncoiling of globular proteins to form protein chains. This spreading of the protein molecules might cause distortion and re-arrangement of the molecules so that all hydrophilic groups become oriented toward the aqueous phase and the lipophilic groups toward the lipid phase. This may make certain reactive groups available for conjugation with lipids to form lipoproteins. Such "strain" results in a permanent damage or denaturation so that when the emulsion is broken the protein entities do not revert to their original state but appear as aggregates in the form of "membrane".

It is not easy to visualize the role phosphatides may play in the formation of these interfacial membranes. However, phosphatides possessing both hydrophylic and lipophilic groups are known to orient at the interface of fat and water. Lecithin, with both acidic (phosphoric acid) and basic (amine) groups should easily form compounds with the groups in the polypeptide chains uncoiled during the orientation of the proteins at the fat globule interfaces and thereby could act as a "cementing" agent to stabilize the emulsion. The property of the lipoprotein complex so formed will depend largely upon the type of bonds (e.g. homo-or hetero-polar cohesive, or homo- or hetero-polar valency bonds--

see Frey-Wyssling, p.146) between lipids and proteins. In turn this will depend on the reactive groups available in the protein.

This membrane would be expected to act as a "barrier" for lipase action either on the phospholipids or on the fat itself. As mentioned earlier, however, a part of the phospholipid can be made to leave the interfacial membrane either by the shaking of warm milk or by warming pre-cooled milk; thus, the membrane becomes more permeable to the enzyme.

The above discussion suggests the following as some of the interrelated factors playing an important role in making the substrate more susceptible to the action of a lipase (or esterase) which is native to milk:

(a) The area of contact between the enzyme and the substrate, i.e. the interfacial area between milk fat and the plasma phase.

(b) The physical and chemical nature of the lipoprotein membrane around the fat globule which acts as a "barrier" between the enzyme and the substrate. The membrane is dynamic and its efficiency as a barrier is altered by temperature changes, agitation, homogenization, etc.

(c) The physical state of the fat, influenced by the temperature history of milk or cream, and its relation to the migration of phospholipids between two phases.

(d) The presence of phospholipids at the interface.

SUMMARY AND CONCLUSION

This investigation involved an approach to a better understanding of the development of hydrolytic rancidity in milk and cream through a study of the changes in physical and chemical properties of the interfacial membranes of natural and synthetic milk emulsions. Extensive use was made of the electron microscope to study the structure of the membrane under a wide variety of conditions and treatments.

The progress of lipolysis was followed by: (a) changes in surface tension determined by du Nuoy ring interfacial tensiometer. Surface tension measurements were made at 38° C. (100° F.) at which little temperature manipulation was necessary and churning was minimized; (b) increase in titratable acidity of the entire reaction mixture. To inhibit bacterial activity, formaldehyde (0.1%) and streptomycin (1000 mcg./ml.) were found effective. The latter, however, caused clumping of fat globules which rose to the surface and interfered with surface tension measurements. Moreover, samples containing the antibiotic consistently produced less acidity than those containing formaldehyde, thus indicating that it may partially inhibit lipolysis.

Cooling and warming of fresh milk produced a slight lowering in surface tension. Temperature activation of milk by cooling to 2° C. (36° F.), re-warming to 30° C. (86° F.) and re-cooling to 2° C. (36° F.), with and without stirring produced rancidity;

the occasionally stirred sample produced the lowest surface tension.

No sample of milk developed spontaneous rancidity on cooling. When milk samples were incubated at 38° C. (100° F.), immediately after milking and without cooling, only in the occasional sample was more acidity produced than in the pasteurized controls. The same samples, when homogenized immediately after milking, however, showed lipolysis in all cases. The addition of a small amount of raw milk to pasteurized emulsions of milk fat in skim milk invariably developed rancidity. This indicates that raw milk contains an active lipase (or esterase) which is unable to act on the globules whose membrane is intact; the latter acts as a barrier between the enzyme and the substrate. Induced lipolysis results from the exposure of the substrate to enzyme by different treatments such as agitation, homogenization, temperature manipulation.

It was found that inclusion of the mixed phosphatide, Asolectin, in either the fat or the aqueous phase reduces the interfacial tension and results in less negative values for spreading coefficients of the aqueous phase on the fat. Therefore, the inclusion of phosphatide in either phase increases the spreading ability of the aqueous phase on fat. This is more pronounced when Asolectin is added to the aqueous phase.

Incorporation of the phosphatide in the fat phase does not lower the surface tension of the latter. When this fat is emulsified in skim milk or whey, the surface tension of the

emulsion is lowered as if the phosphatide was added to the aqueous phase. This indicates the migration of the phosphatide from the lipid phase to the interface where it either becomes hydrated or associated with protein or both.

Surface tension of emulsions of milk fat in skim milk decreased with increasing amount of Asolectin in either phase. The emulsion containing one per cent Asolectin in the fat phase or 0.1 per cent in the aqueous phase behaved like normal milk, as regards the changes in surface tension with increasing temperatures from 10° C. (50° F.) to 38° C. (100° F.). Inclusion of phosphatide in synthetic emulsions of milk fat in skim milk increases lipolysis when the emulsions are inoculated with raw milk. Maximum rancidity is obtained when it is included in the fat phase. This indicates that besides its surface activity some other property plays an important part in lipolysis.

The colorimetric method of Greenbank and Wright (27) was used for determining the amount of lipase in milk. It was found to be inaccurate and hence gave only approximately quantitative results. There was a definite indication that a heat-labile esterase, associated with cream, was responsible for hydrolysis of a-naphthyl acetate. This esterase activity, however, can be more or less completely removed by washing the cream and hence the enzyme may be adsorbed on the fat globules. Skim milk and super skim milk which exhibited practically no esterase activity when examined alone, showed considerable activity when combined with four-times

washed cream.

Cream separated at about 38° C. (100° F.) has a lower lipid phosphorus content than that separated at about 7° C. (45° F.). The low temperature cream retains more lipid phosphorus when washed at temperatures at which the fat is in solid state than at temperatures when it is in liquid state. Considerable phospholipid originally associated with fat is lost to the aqueous phase when the 45° F.-cream is warmed to 38° C. (100° F.), indicating a relationship between the physical state of the fat and the retention of phospholipid. About 50 per cent of the lipid phosphorus of cold cream occurs either free or in loose combination and can be removed by washing. The other 50 per cent is tenaciously held to the fat (or very likely to the fat globule membrane) but can be largely removed by churning and appears in the buttermilk.

The total phosphorus content of the isolated residual fat globule membrane depends on the type of solvent used for extracting the membrane from the washed cream and decreases in the following order of the solvents used for extraction: ether; one volume of acetone with four volumes of "Heptane"; one volume of ethylene chloride with one volume of ether and two volumes of ether with one volume of ethyl alcohol.

The electron microscope investigation revealed that: (a) Ca-caseinate-Ca-phosphate entities occur as regular spheres of diameters ranging from 100 mu to 240 mu--the largest number being around 120 mu. (b) The lipoprotein membrane from washed cream

and from egg yolk showed a globular "sieve-like" structure. (c) Membranes obtained from milk of different cows although basically of the same type of structure are not entirely identical. (d) Lipids alone are difficult to photograph as they always appear to be out of focus. (e) The physico-chemical changes in proteins due to heat or treatment with absolute alcohol indicate a loss of identity of globular proteins. (f) The fat globules of milk showed ridges representing shrivelled membrane. The circumference of the globule retained a circular outline due to the presence of membrane. (g) The fat globules emulsified in water showed irregular fat masses. (h) The membranes of synthetic emulsions, when phosphatide was added to the fat, resembled the natural membrane more closely than those not having any added phosphatides. (i) The membrane obtained from the milk fat-skim milk emulsion had closer resemblance to the natural membrane than the one obtained from the milk fat-rennet whey emulsion.

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