Membranous Material of Bovine Milk Fat Globules. I. Comparison of Membranous Fractions Released by Deoxycholate and by Churning*

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ABSTRACT: The fat globules of milk are coated with a membrane composed of lipids and proteins. The nature of this membrane has been the subject of much investigation, but still is controversial. In the present work, water-soluble lipoprotein particles accounting for 45% of the total weight of the membrane were released from the intact fat globules by sodium deoxycholate (DOC), and were isolated by centrifugation. Chemical analyses of these lipoproteins showed that lipids and proteins were present in approximately equal amounts, and that 76% of the lipids were phospholipids. Ultracentrifugal analyses indicated that the particles were physically heterogeneous, with the predominant species showing a sedimentation coefficient, $s_{20,w}$, of *ca.* 13 S. Similar lipoproteins were isolated, but in smaller amounts, from untreated buttermilk, a source of membranous material used widely by previous investigators. However, DOC released water-soluble lipoproteins from buttermilk in amounts comparable to those released from intact fat globules. These findings led to the view that the membrane surrounding the milk fat globule consists of two types of lipid–protein complexes, approximately equal in amount, and distinguishable on the basis of their solubility in water. The water-soluble lipoproteins are considered to be adsorbed on a water-insoluble matrix, composed of lipids and proteins, bordering the triglyceride core of the fat globule.

Milk fat is present in bovine milk in the form of small globules, most of which are approximately 2–5 μ in diameter. Although these globules consist mainly of triglycerides (*ca.* 99% by weight), they are stable in an aqueous medium because of an interfacial material, referred to as membranous material. Among the major constituents of the membranous material are proteins, phospholipids, and triglycerides; the minor constituents include cholesterol, cholesteryl esters, and metals such as iron, copper, molybdenum, and zinc (King, 1955; Whitney, 1958; Jenness and Patton, 1959; Brunner, 1962; Richardson and Guss, 1965). Whereas extensive information is available on the chemical composition of the membranous material, comparatively little is known regarding its physical structure. King (1955), using chemical information, proposed a model in which the fat globule consists of a triglyceride core around which the other lipid constituents are organized in discrete concentric layers. He postulated that the innermost layer is composed of lipids with the least polar properties, and that the outermost layer is composed of phospholipids which possess hydrophilic groups. According to this model, the phospholipid layer in turn is surrounded by proteins attached to these hydrophilic groups.

Another concept of the structure of the milk fat globule was advanced by Morton (1954), who observed, with the aid of an electron microscope, small particles adhering to both intact and collapsed globules. Morton suggested that "the fat globule in milk is surrounded by a continuous protein membrane onto which microsomal particles may be adsorbed." He termed these particles "milk microsomes."

According to Morton's concept of the membrane, it seems possible that the adhering particles could be released without disrupting the remaining portion of the membrane. This paper describes a preparative procedure effective in releasing lipoprotein particles from intact fat globules and reports some chemical and physical properties of the released lipoproteins. The results obtained by this new approach are compared with data obtained from fractions prepared by the churning technique, widely employed in past studies of the membrane of the milk fat globule.

Preliminary Considerations

The apparent composition of the membranous material of the milk fat globule varies with the method of preparation. Much of the present knowledge of the membrane has been derived from studies made on buttermilk prepared as follows. The milk is centrifuged first in a cream separator to concentrate the fat globules in the cream. The cream is washed to remove milk plasma constituents that are not an integral part of the

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membrane. This is done by repeated dilution with water and reseparation to obtain washed cream. The washed cream then is churned to obtain buttermilk (continuous aqueous medium in which the membranous material is, for the most part, in suspension) and butter (continuous fat phase interspersed with buttermilk).

The key observation on the variety of membranous fragments found in buttermilk is Morton's electron-microscopic photograph showing an intact fat globule surrounded by smaller particles. From this it has been inferred that there are two types of particles, and that the smaller particles are detachable from the surface of the larger particle (the globule). If such particles could be released *in vitro*, their study should lead to a clearer understanding of the structure of the membrane as well as of the biochemical relationships among its various constituents.

Alexander and Lusena (1961) used sodium deoxycholate (DOC)[1] treatment and centrifugation to fractionate the membranous material released by freezing washed cream. However, it is difficult to assess the extent of change in the structure of the membrane components caused by freezing and thawing (King, 1955). We found, under the conditions described below, that DOC released a substantial amount of water-soluble lipoprotein particles from intact fat globules. These lipoproteins accounted for *ca.* 50% of the total membrane protein and >50% of the total phospholipids. However, an important question arises: did the DOC simply release the smaller particles or did it also alter significantly their original structure?

We attempted to answer this question by comparing the chemical composition and the ultracentrifugal behavior of the DOC-released lipoproteins with those of the water-soluble lipoproteins isolated from buttermilk which had not been in contact with DOC. Finally, the buttermilk was treated with DOC to release additional lipoproteins. The three lipoprotein preparations obtained by the three preparative methods were compared.

Experimental Procedures

Lipid Extraction. Lipids were extracted from samples by solvent extraction according to the method described by Freeman *et al.* (1957). The extracted lipids were dried at *ca.* 30° in a vacuum oven overnight prior to weighing.

Protein Nitrogen. The residue left after the lipid extraction was brought into solution by adding sodium hydroxide. The solution was transferred quantitatively to a micro-Kjeldahl digestion flask and brought to neutral pH with sulfuric acid. Kjeldahl nitrogen was determined according to the method described by Ma and Zuazaga (1942) and was considered to be protein nitrogen.

Lipid Analyses. Samples of the extracted lipid were analyzed for Kjeldahl nitrogen (Ma and Zuazaga) and for phosphorus (Smith *et al.*, 1959).

[1] Abbreviation used: DOC, sodium deoxycholate.

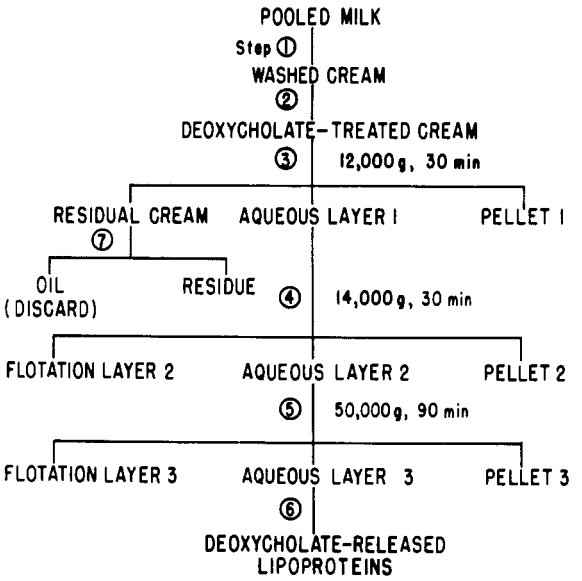

FIGURE 1: Outline of the procedure used to isolate water-soluble lipoproteins (deoxycholate-released lipoproteins) from the membrane of milk fat globules. This procedure is based on the action of sodium deoxycholate on intact washed cream.

Ultracentrifugal Analyses. The lipoprotein particles obtained by each of the preparative procedures described below were analyzed ultracentrifugally. Prior to the ultracentrifugation, samples of each water-soluble fraction were dialyzed at pH 7.0 against 0.02 M sodium phosphate buffer containing 0.1 M sodium chloride. The buffer was changed four times during a period of 24 hr. The sedimentation patterns were obtained with a Spinco Model E ultracentrifuge, in double-sector cells, at 20 ± 1°, 16 min after the rotor attained an operational speed of 42,040 rpm.

Procedure I. ISOLATION OF LIPOPROTEINS RELEASED BY DEOXYCHOLATE FROM WASHED CREAM. Figure 1 outlines the principal steps in the isolation of membranous material of bovine milk fat globules. Fresh, uncooled milk from several Holstein and Jersey cows was combined and held at 37° during the short time between milking and step 1. Step 1 included the separation of the cream from the milk at 37° and the washing of the cream in a DeLaval cream separator, Model 518. The cream was washed four times with an aqueous solution of 0.25 M sucrose and 0.15 M sodium chloride buffered at pH 7 with 0.15 M sodium phosphate. In each washing the cream was diluted with four times its volume of washing solution and reseparated at 37°. Concentrated solutions of the chemicals used to prepare the wash solution were passed through a column of Dowex A-1 chelating resin to remove contaminating copper. The concentrated wash solution was diluted with deionized water. Stainless steel utensils were used throughout the isolation procedure. The fat content of the washed cream was about 50% (w/w).

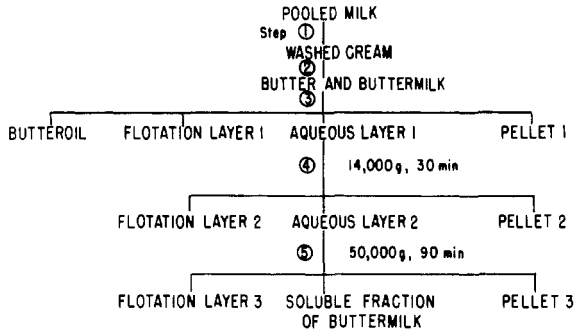

FIGURE 2: Outline of the procedure used to isolate the water-soluble membranous fraction from buttermilk (soluble fraction of buttermilk) obtained by churning washed cream.

In step 2, the washed cream was treated with DOC. Approximately 30 g of washed cream was weighed into each of seven 50-ml plastic centrifuge tubes for a Servall SS-34 rotor. Sufficient DOC solution was added to each tube to give a DOC concentration of 1% in the aqueous phase of the washed cream. The stock DOC solution contained 10% (w/v) sodium deoxycholate in 0.25 M sucrose buffered at pH 8.5 with 0.25 M Tris chloride. The tubes were capped, inverted several times to mix their contents, and then transferred to a water bath maintained at 37°. The washed cream–DOC mixture was allowed to react for 60 min during gentle shaking.[2] Under these conditions, DOC released some of the membranous material from the fat globules; the material included lipoproteins[3] which were centrifugally fractionated in subsequent steps.

Step 3 was the first stage in the differential centrifugation of the membranous material released by DOC. The tubes containing the reaction mixture were centrifuged at 12,000*g* for 30 min at 37°. Three discrete layers were formed in each tube: (1) a concentrated layer of fat globules, at the top, designated residual cream; (2) an aqueous, yellowish, middle layer designated aqueous layer 1 which contained the released lipoproteins; (3) at the bottom a reddish brown pellet designated pellet 1. The tubes were chilled in ice water to solidify the residual cream layer, and aqueous layer 1 then was removed from each tube through a pinhole puncture near the bottom of the tube. The tubes were cut open and the solidified cream layers and pellets were collected separately. The residual cream was stored at 4° until step 7 could be completed.

Aqueous layer 1 collected in step 3 was turbid due to the presence of large lipid-containing aggregates. Two additional preparative centrifugations were done to remove these aggregates. In the first of these (step 4), aqueous layer 1 was centrifuged in a Spinco Model L centrifuge, Type 30.2 rotor, at 14,000*g* for 30 min at 4°. Flotation layer 2, a turbid layer at the top of the preparative tube, was removed with a capillary pipet, as was aqueous layer 2. Pellet 2 and flotation layer 2 were stored for chemical analysis.

In step 5, aqueous layer 2 was centrifuged at 50,000*g* for 90 min at 4°. Flotation layer 3 was removed from the top of each tube with a capillary pipet. Aqueous layer 3, a clear yellow solution which contained the DOC-released lipoproteins, was removed in the same manner. Pellet 3 was removed with a spatula.

In step 6, the DOC was removed from aqueous layer 3 by gel filtration to minimize possible modification of the lipoproteins. A column of Sephadex G50, medium grade, 4 cm in diameter and 40 cm in height, was equilibrated with the eluent (0.025 M Tris chloride buffer, pH 8.5). About 50 ml of aqueous layer 3 was pipetted slowly onto the column. When the sample had entered the column, eluent was added, and the flow rate was adjusted to approximately 0.9 ml/min. The eluate was collected in 10-ml fractions, and the relative lipoprotein concentration in each was determined from the absorbance at 280 mμ. After the lipoproteins had been eluted, subsequent fractions were acidified to determine the presence of DOC by turbidity or Tyndall effect. The final fraction containing lipoproteins was well separated from the DOC elution front. This was considered presumptive evidence that the DOC had been removed from the lipoprotein solution. The eluates containing lipoproteins were pooled, and are designated as deoxycholate-released lipoproteins in the studies described below.

In step 7, the residual cream recovered in step 3 was portioned into plastic centrifuge tubes and warmed at 40° for 30 min. The tubes then were transferred to a prewarmed rotor and centrifuged at 10,000*g* for 30 min at 40°. After the run, the clear yellow oil (melted fat) was removed with a pipet. Below the oil was the membranous material released from the residual cream: (1) a layer resembling milky white paste, (2) a turbid aqueous layer, and (3) a pellet at the bottom of the tube. Collectively, the membranous material was designated as the residue.

Procedure II. ISOLATION OF WATER-SOLUBLE FRACTION OF BUTTERMILK. Figure 2 shows the principal steps in the isolation of a water-soluble membranous fraction from buttermilk. A sample of the washed cream prepared in procedure I was used. Step 1 was the same as that in Figure 1. In step 2, 30 g of washed cream was weighed into each of seven 50-ml centrifuge tubes, cooled overnight at 5°, and churned at 12° on a mechanical shaker.

In step 3, the mixture of butter and buttermilk in the tubes was centrifuged at 12,000*g* for 30 min at 37°. The fat separated as butteroil, indicating that the globules were destabilized. This was in contrast to the results in procedure 1, where the fat globules did not coalesce as an oil. Steps 4 and 5 were essentially similar to the same

[2] Experiments showed that increasing the reaction time from 60–180 min resulted in only a minor increase in the amount of membranous material released.

[3] In this paper the term lipoproteins refers to the water-soluble particles composed of lipids and proteins, whereas the term lipid–protein complex is used to denote either water-soluble or water-insoluble material composed of lipids and proteins.

TABLE I: Composition of Centrifugal Fractions of Membranous Material Obtained by Deoxycholate Treatment of Washed Cream.

Fraction[a]	Protein Nitrogen mg	Protein Nitrogen % of Total Protein N	Total Lipid (mg)	Lipid Nitrogen (mg)	Lipid Phosphorus mg	Lipid Phosphorus % of Total Lipid P
Washed cream	140.4	100	107.9×10^3	...	22.9	100
Residue (step 7)	45.0	32.2	924	4.4	8.0	34.8
Pellet 1	11.1	7.9	21.7	0.0	0.2	0.9
Flotation layer 2	2.7	1.9	37.4	0.2	0.1	0.4
Pellet 2	Combined with pellet 3					
Flotation layer 3	2.3	1.6	26.8	0.3	0.4	1.7
Pellet 3 and 2 combined	9.2	6.5	18.1	0.1	0.1	0.4
Deoxycholate-released lipoproteins	63.2	45.0	504	5.9	15.3	67.0
Total recovered	133.5	95.1	1532	10.9	24.1	105.2

[a] Refer to Figure 1 and text for description.

steps in Figure 1. In step 5, the aqueous yellowish solution above the pellet was removed and designated as the soluble fraction of buttermilk.

Procedure III. ISOLATION OF MEMBRANOUS MATERIAL, RELEASED BY DEOXYCHOLATE, FROM A MIXTURE OF BUTTERMILK AND BUTTER. Figure 3 presents the principal steps followed in the isolation of DOC-soluble membranous material both from buttermilk and from the membranous material occluded in butter. A sample of the washed cream prepared in procedure I was used. Steps 1, 2, and 3 were the same as those in Figure 2. The butter granules melted under these conditions, and the resultant butteroil was removed with a capillary pipet. In step 4, the mixture of buttermilk and membranous material from butter was treated with DOC and then centrifuged as in step 3 of Figure 1. Steps 5 and 6 were essentially similar to the corresponding steps in Figure 1. The yellowish eluate from the Sephadex column, and which contained lipoproteins, was designated as deoxycholate-soluble membrane material from buttermilk.

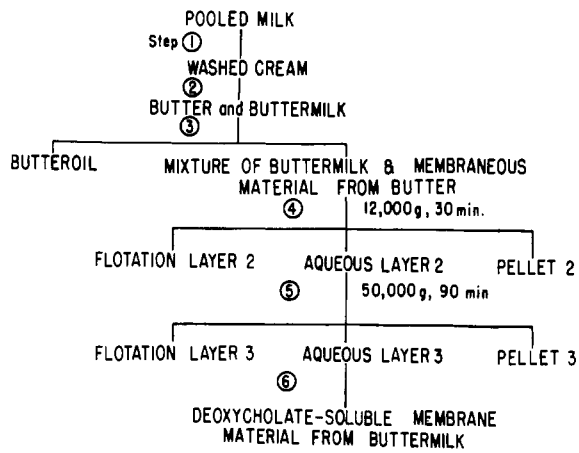

FIGURE 3: Outline of the procedure used to isolate water-soluble membranous material by treatment of buttermilk with deoxycholate (deoxycholate-soluble membrane material from buttermilk).

Results

Comparison of Chemical Composition of Various Fractions of Membranous Material. Table I presents the typical gross chemical composition of membranous fractions obtained by differential centrifugation of cream treated with DOC according to the preparative procedure in Figure 1. This procedure produced fractions that were either soluble or insoluble in water with respect to centrifugation at 50,000*g* for 90 min at 4°. The recovery of protein nitrogen and lipid phosphorus in the fractions was within ±5% of the total amounts of these constituents in the washed cream.

There were two distinct classes of membranous material among the various fractions obtained by procedure I (Table I). The first of these was the water-soluble lipoproteins released by DOC. The second was that membranous material which remained associated with the fat globules during the DOC treatment, but which was released from the globules in subsequent steps.

Since one of the aims of this investigation was to bring the membranous material into solution, the data for the fraction designated as DOC-released lipoproteins are of particular interest. This fraction accounted for 45% of the protein and 67% of the lipid phosphorus initially present in the washed cream. Using a conversion factor of 6.25 mg of protein/mg of protein nitrogen, the estimated mass of the protein was 395 mg. The weight of the lipids, determined gravimetrically, was 504 mg. Although this fraction contained a greater

TABLE II: Composition of Centrifugal Fractions of Membranous Material Obtained by Churning Washed Cream.

Fraction[a]	Protein Nitrogen mg	Protein Nitrogen % of Total Protein N	Total Lipid (mg)	Lipid Nitrogen (mg)	Lipid Phosphorus mg	Lipid Phosphorus % of Total Lipid P
Washed cream	140.4	100	107.9×10^3	...	22.9	100
Flotation layer 1	24.0	17.1	2763	6.2	11.0	48.0
Pellet 1	31.6	22.5	91.3	0.6	1.4	6.1
Flotation layer 2	5.1	3.6	126.3	0.6	1.0	4.4
Pellet 2	9.7	6.9	54.9	0.3	0.6	2.6
Flotation layer 3	4.2	3.0	156.0	0.6	1.0	4.4
Pellet 3	30.5	22.4	245.0	2.3	3.9	17.0
Soluble fraction of buttermilk	16.2	11.5	117.0	0.8	1.6	7.0
Total recovered	121.3	87.0	3534	11.4	20.5	89.5

[a] Refer to Figure 2 and text for description.

TABLE III: Composition of Centrifugal Fractions of Membranous Material Obtained by Deoxycholate Treatment of Buttermilk.

Fraction[a]	Protein Nitrogen mg	Protein Nitrogen % of Total Protein N	Total Lipid (mg)	Lipid Nitrogen (mg)	Lipid Phosphorus mg	Lipid Phosphorus % of Total Lipid P
Washed cream	140.4	100	107.9×10^3	...	22.9	100
Flotation layer 2	21.1	15.0	1933	2.3	3.8	16.6
Pellet 2	17.3	12.3	34.6	0.2	0.2	0.9
Flotation layer 3	3.4	2.4	170.4	0.6	1.0	4.4
Pellet 3	18.6	13.2	99.0	0.3	0.6	2.6
DOC-soluble membranous material from buttermilk	56.3	40.1	536.6	6.8	17.9	78.1
Total recovered	116.7	83.0	2774	10.2	23.5	102.6

[a] Refer to Figure 3 and text for description.

mass of lipids than it did protein, it was optically clear and behaved as a solution under ordinary laboratory conditions. Using a conversion factor of 25 mg of phospholipids/mg of lipid phosphorus, the estimated amount of phospholipids in the lipids was 382 mg or approximately 75% of the total lipids.

The water-insoluble membranous material was present mainly in the residue (step 7, Figure 1); this constituted about 32% of the protein and 35% of the lipid phosphorus initially present in the washed cream.

That buttermilk is a complicated mixture, rather than a solution, is indicated by its marked turbidity. The nature of this mixture is evident from Table II. The greatest partitioning of the buttermilk occurred in the first centrifugation (step 3), which yielded flotation layer 1 and pellet 1. These two insoluble fractions accounted for approximately 80% of the lipid and 46% of the protein nitrogen in the buttermilk. The cumulative amount of insoluble material obtained with successive differential centrifugation exceeded by far the amount of soluble fraction finally obtained. The soluble fraction of buttermilk in this particular experiment contained only 12% of the protein nitrogen present initially in the washed cream. This was considerably less than the 45% in the water-soluble DOC-released lipoproteins of procedure I.

While the yield of the soluble fraction of buttermilk (Table II) was relatively low, its lipid to protein mass ratio was similar to that of the DOC-released lipoproteins (Table I). Both fractions contained approximately 50% lipids but differed in composition of the lipids; for example, phospholipids constituted about 34% of the lipids present in the former, compared to 76% in the latter.

The results in Table III show that, although untreated buttermilk consists mainly of water-insoluble material,

buttermilk treated with DOC yielded an amount of water-soluble material, designated as the deoxycholate-soluble membranous material from buttermilk, which was comparable to that released directly from the fat globules by DOC (Table I, deoxycholate-released lipoproteins). Also, the gross chemical composition of these two water-soluble fractions is comparable; each contained approximately 50 to 60% lipids which in turn contained 75 to 85% phospholipids. These observations indicate that the deoxycholate-released lipoproteins and the deoxycholate-soluble membranous material from buttermilk may represent the same structural particles of the membrane.

Comparison of Ultracentrifugal Analyses. Figure 4A is the sedimentation pattern for the DOC-released lipoproteins obtained as outlined in Figure 1. The distribution in S is wide and, at the peak of the distribution, the rate of sedimentation was 13 S. Thus, the DOC-released lipoproteins were regarded as a population of physically heterogeneous particles. Since no turbidity boundary could be observed on the viewing screen of the optical system to migrate during the acceleration period of the rotor, this population was considered to be essentially free of contamination by larger aggregated material.

In Figure 4B, the sedimentation pattern for the water-soluble fraction from buttermilk isolated according to Figure 2, the distribution in S is wide. At the peak, the rate of sedimentation was approximately 11 S. Aside from the relatively small area of this pattern, which reflects the relatively low yield of this fraction, the distribution in S is similar to that for the DOC-released lipoproteins.

Figure 4C, the sedimentation pattern for the DOC-soluble membranous material obtained from buttermilk, is quite similar to the patterns for the DOC-released lipoproteins and the soluble fraction of buttermilk. As can be seen from Tables II and III, DOC increased the yield of water-soluble fraction from buttermilk approximately threefold.

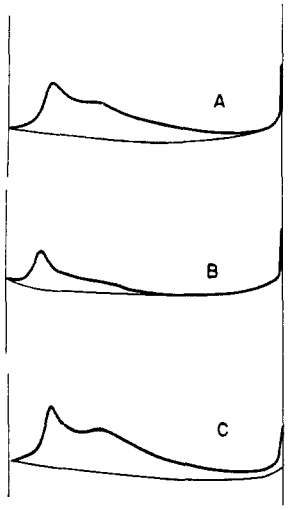

FIGURE 4: Comparison of the ultracentrifugal sedimentation patterns of the water-soluble fraction of the membranous material obtained from milk fat globules by three different preparative procedures (as outlined in Figures 1–3). Patterns A, B, and C are, respectively, for the deoxycholate-released lipoproteins, the soluble fraction of buttermilk, and the deoxycholate-soluble membrane material from buttermilk. The solvent system was 0.02 M phosphate buffer at pH 7.0, with ionic strength of 0.1 with respect to sodium chloride. The runs were made in a Spinco Model E analytical ultracentrifuge at 20 ± 1°, in double-sector cells. The patterns shown were obtained 16 min after the rotor attained the desired speed of 42,040 rpm. Sedimentation is toward the right.

Discussion

Water-soluble lipoproteins were released from the membrane of milk fat globules by all three procedures used. In procedure I (Figure 1), intact fat globules were stirred gently in a solution of dilute sodium deoxycholate. This procedure is considered the mildest of the three, and *ca.* 50% of the membranous protein appeared in the lipoproteins released.

In contrast to this procedure is procedure II, which entails the churning of the fat globules. Even though no deoxycholate was used here, some of the membranous fragments were water soluble and their lipid: protein mass ratio and sedimentation behavior were similar to those of the DOC-released lipoproteins. However, the yield of water-soluble lipoproteins was small.

In procedure III, where buttermilk was treated with sodium deoxycholate, the yield of water-soluble lipoproteins was increased markedly and was comparable to that of the DOC-released lipoproteins of procedure I. Again these lipoproteins were similar in gross chemical composition and sedimentation behavior to those of the DOC-released lipoproteins.

Since comparable lipoproteins were obtained with chemical (DOC) or physical (churning) procedures, and by a combination of both, it is apparent that these lipoproteins were capable of retaining their identity under the conditions of these different preparative procedures. Therefore, we regarded them as being representative of structural components of the membrane in the native state. Because procedure I is a stepwise way to fragment the membrane, and yields comparatively large amounts of water-soluble lipoproteins, we considered it as the method of choice for further studies of the membrane.

Table I shows that, among the membranous fractions obtained by using DOC, phospholipids were distributed almost entirely in the DOC-released lipoproteins and the residue. These two fractions contained the major portion of the membranous proteins. The pellet fractions, released concomittantly with the DOC-released lipoproteins, contained approximately 15% of the total protein but a negligible amount of phospholipids and, furthermore, their total lipid contents were relatively

low. Thus, while all phospholipids are associated with proteins, the data suggest there may be some proteins which are not associated with phospholipids.

The results obtained with the three preparative procedures may be explained, in most part, by the following tentative model of the structure of the milk fat globule. We consider the globule to be a triglyceride core surrounded by a membrane consisting of two types of lipid–protein complexes, distinguishable on the basis of their solubility in water. The insoluble lipid–protein complexes border the triglyceride core and provide a structural matrix on which the water-soluble lipoprotein particles are adsorbed. According to this model, the water-soluble lipoproteins, accounting for *ca.* 45% of the total protein N (Table I), are released from the structural matrix by DOC through a mechanism yet to be explained. The insoluble structural matrix, which resists the solubilizing action of DOC, can continue to keep the individual globules from coalescing (see steps 3 and 7, procedure I); however, it is released from the triglyceride core when the triglycerides are melted and then coalesced by centrifugation.

During the churning of the cream (procedure II), it is probable that parts of the membrane, containing both soluble and insoluble lipid–protein complexes, are fragmented and dispersed in the aqueous phase to form buttermilk. In this process, some of the triglycerides from the core of the globule are enmeshed in the fragments and increase their water insolubility. However, there is evidence that the process of churning does not completely randomize the membranous material but causes a fractionation. Jenness and Palmer (1945) observed that when washed cream was churned the membranous material found in the "free buttermilk" differed from that in the "butter serum"; the mass ratio of proteins to phospholipids in the butter serum, which contained about 35% of the total membranous proteins, varied from 1.0 to 2.0, whereas that in the free buttermilk varied from 2.4 to 3.8. Since the butter serum fraction had a greater affinity for the fat phase than for the aqueous phase, it is reasonable to compare this fraction with the residue (step 7) of procedure I. The residue (see Table I) contained 32% of the total membranous proteins and the calculated mass ratio of the proteins:phospholipids is 1.4; these values are similar to those for the butter serum fraction obtained by Jenness and Palmer (1945). Thus, a possibility exists that the membranous fraction in the butter serum may be related closely to that which remains on the fat globule after the release of lipoproteins by DOC. If this is true, it is reasonable to expect that the ratio of proteins to phospholipids for the free buttermilk fraction of Jenness and Palmer (1945) would be comparable to that for the sum of the fractions released by DOC. However, this ratio calculated from Table I is 1.4, which is considerably less than the 2.4–3.8 observed by Jenness and Palmer (1945). We attribute this difference to greater losses of water-soluble lipoproteins in their washing procedure. It has been shown that losses of membranous material occur with each successive washing (Zittle *et al.*, 1956), and greater losses occur when distilled water is used rather than sucrose–saline solution (Erickson *et al.*, 1964).

The results of this study cannot be explained simply on the basis of the model of the membrane of the milk fat globule proposed by King (1955). The presence of substantial amounts of phospholipids in the soluble lipoproteins is not consistent with the hypothesis that all the phospholipids are oriented neatly at the surface of the triglyceride core. The observation that DOC releases the water-soluble lipoproteins, leaving the water-insoluble membranous material still associated with the globule, indicates that these lipoproteins may occur as an adsorbed layer around the globule, somewhat in accordance with Morton's concept that microsomes form an adsorbed layer around the globule (Morton, 1954). However, in Morton's preparative procedure (Bailie and Morton 1958), the "milk microsomes" were found, together with insoluble proteins, in the sediment obtained by centrifuging buttermilk at 50,000*g* for 90 min. In our procedure, the soluble lipoproteins were released from the fat globule by DOC and were found in the supernatant fraction after centrifugation at 50,000*g* for 90 min. Also, the sedimentation coefficient ($s_{20,w}$) of the DOC-released particles of *ca.* 13 S indicates that the particles were smaller in size than the microsomes (Keller *et al.*, 1963; Hess and Lagg, 1963; Dickman *et al.*, 1962), whose $s_{20,w}$ generally lies between 50 and 100 S. Since the size of DOC-released particles resembles that of water-soluble lipoproteins of blood (Lindgren *et al.*, 1951) more closely than those of microsomes, we will consider the DOC-released particles simply as lipoproteins until they are characterized further.

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Membranous Material of Bovine Milk Fat Globules. II. Some Physical and Enzymic Properties of the Deoxycholate-Released Lipoproteins*

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ABSTRACT: Water-soluble particles of lipoprotein are released from the membrane of milk fat globules by sodium deoxycholate (DOC), leaving a layer of water-insoluble complexes composed of lipids and proteins, still attached to the globules. The lipoproteins released by DOC have a wide range in ultracentrifugal heterogeneity. Therefore, the population of lipoproteins was fractionated by ultracentrifugal flotation into three density classes: <1.13, 1.13–1.21, and >1.21 g/ml. Lipoproteins in each of these classes were analyzed ultracentrifugally. Sedimentation coefficients indicated that particle interaction occurred among the lipoproteins during the flotation procedure, and that both ionic strength of the flotation medium and the amount of lipid in the lipoproteins were factors in particle aggregation. DOC-released lipoproteins were precipitated irreversibly in an acidic medium; the maximum precipitation occurred at pH 3.8. Experiments to determine the solubility dependence of these lipoproteins on ionic strength were done at pH 7.0, using ammonium sulfate. Approximately 93% of the lipoproteins were salted out at 45% ammonium sulfate saturation; the process was reversible. Xanthine oxidase and alkaline phosphatase activities were assayed in fractions isolated, by ammonium sulfate precipitation, from the DOC-released lipoproteins. There was no marked precipitation of either enzyme, and it was concluded that these two proteins occur together in the lipoprotein particles. The distribution of the above enzymes between the water-soluble and the water-insoluble membranous fractions was determined. Both enzymes were localized principally in the water-soluble lipoproteins. This finding provides additional support for our previously proposed hypothesis that the membrane of the milk fat globule consists of two types of lipid–protein complexes distinguishable on the basis of solubility in water. A model in accordance with this hypothesis is compared to those previously proposed by other investigators.

A procedure for releasing water-soluble particles of lipoprotein (deoxycholate-released lipoproteins) from the membrane of intact milk fat globules by sodium deoxycholate (DOC)[1] was described in our preceding paper (Hayashi and Smith, 1965). These lipoproteins[2] account for approximately 45% of the protein and 67% of the phospholipids present in the original membrane. The fat globules remaining after the release of these lipoproteins were found to maintain their independent existence in the aqueous medium. To explain these observations, a tentative model of the structure of the membrane was proposed. In this, the water-soluble lipoproteins are regarded as being adsorbed on a structural matrix of water-insoluble complexes composed of lipids and proteins, which is in contact with the triglyceride core of the fat globule.

This paper reports studies aimed at finding methods to isolate a more homogeneous molecular species from the heterogeneous population of DOC-released lipo-

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[1] Abbreviation used: DOC, sodium deoxycholate.

[2] In this paper the term lipoproteins refers to the water-soluble particles composed of lipids and proteins, whereas the term lipid–protein complexes is used to denote either water-soluble or water-insoluble material composed of lipids and proteins.