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PROTEINS AND GLYCOPROTEINS OF THE MILK FAT GLOBULE MEMBRANE*

DAVID KOBYLKA** AND KERMIT L. CARRAWAY***

Department of Biochemistry, Oklahoma State University, Stillwater, Okla. 74074 (U.S.A.)

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SUMMARY

Acrylamide-gel electrophoresis and Sepharose 4B chromatography in sodium dodecylsulfate have been used to investigate the proteins and glycoproteins associated with the milk fat globule membrane. Electrophoresis followed by staining for proteins with Coomassie blue shows the presence of six major bands. These polypeptides range in molecular weight from 53000 to 240000. Periodate–Schiff staining indicates the presence of six carbohydrate containing bands in the glycoprotein region. Three of these, including the major one, are clearly different from the observed protein bands. By examination of a number of membrane samples it was shown that the major protein (molecular weight 66000) and the major glycoprotein are the most invariant components in terms of percentage composition over a series of preparations. The major glycoprotein does not stain with Coomassie blue and its calculated molecular weight on acrylamide electrophoresis in dodecylsulfate varies with acrylamide percentage. Extraction experiments with EDTA, NaCl and guanidine hydrochloride led to differential solubilization of membrane proteins, which might be useful for purification of the individual protein components. Direct solubilization of membrane proteins in dodecylsulfate can be achieved by incubating cream with the detergent. This permits studies on the changes of the membrane proteins without the necessity of preparing membranes.

INTRODUCTION

The secretion of fat from the acinar cells of mammary tissue has been proposed[1] to occur by an envelopment of the fat with plasma membrane. The membrane-surrounded fat globule is then pinched off and released from the cell. If this is true, the membrane of the milk fat globule represents a unique opportunity to study the cellular plasma membrane of a complex cell and its components without fractionating the cell into its various internal and external membranous structures. Evidence to support this hypothesis has been derived from studies on the enzymes associated with the milk fat globule membrane[2,3] and by comparisons of the lipid distributions

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** Present address: Department of Biochemistry, St. Louis University, School of Medicine, St. Louis, Mo., U.S.A.

*** Author to whom correspondence should be addressed.

of the milk fat globule membrane with those of plasma membrane isolated form mammary tissue[4]. Recently Wooding[5] has suggested that the milk fat globule is surrounded by a double membrane. The outer membrane is identical to the plasma membrane of the secretory cell and is gradually lost after secretion from the cell. The inner membrane consists of a layer of cytoplasmic material between the outer membrane and the fat.

One approach to studying the secretion process would be to purify the proteins associated with the milk fat globule membrane and compare them with proteins derived from the various fractions of the mammary cell (plasma membrane, cytoplasm, *etc.*) by immunochemical techniques. Attempts at characterization of the protein or polypeptide components of the milk fat globule membrane have met with limited success. Much of the work has been performed on lipoprotein or glycoprotein aggregates of the membrane material. These investigations have been reviewed by Brunner[6]. Keenan *et al.*[4] have shown comparative protein patterns of milk fat globule membrane and mammary cell plasma membranes subjected to acrylamide electrophoresis in a phenol–acetic acid–water–urea system[4]. The present investigation was undertaken to develop additional techniques for fractionating, identifying and characterizing the milk fat globule membrane proteins and glycoproteins using methodology which has been successful in similar studies with erythrocyte membranes[7–10]. Comparisons have been made with the bovine erythrocyte membrane, since it has been shown that the milk fat globule membrane is immunologically cross-reactive with bovine erythrocytes, but not with erythrocytes of other species[2].

METHODS

Preparation of milk fat globule membranes

Raw chilled cream was obtained from the Oklahoma State University Dairy. It was derived from a herd of mixed breeds. The cream was obtained within 2–3 h of milking and had been separated in a chilled state from the raw milk. The cream was washed by three suspensions in an appropriate medium (usually imidazole-buffered sucrose containing Mg^{2+}) at room temperature followed by centrifugation at 4000 × *g* for 20 min at 5 °C. For membrane preparations the solid wet cream was suspended to 33 % (w/v) in the preparation medium and subjected to homogenization or freezing. Homogenization was performed at 12–15 °C in a Sorvall Omni-Mixer for periods of 0–10 min at full speed. The freeze–thaw procedure involved freezing 33 % cream at −20 °C for 20 h and thawing rapidly to a temperature of 35 °C. This latter procedure was used for most of the work described. Membranes were isolated by centrifugation at 40000 × *g* for 1 h at 5 °C and washed twice in the buffer used for freezing and thawing.

Chemical analyses

Protein[11], cholesterol[12], phosphorus[13] and neutral sugar[14] were determined by standard procedures. Phospholipid content was calculated by multiplying the value for phosphorus by 25. Sialic acid was determined by the method of Warren[15] after hydrolysis with 0.1 M H_2SO_4 for 1 h at 80 °C. Values are expressed as *N*-acetylneuraminic acid. Amino acids were determined by the procedure of Spackman *et al.*[16],

on a Beckman 120 C amino acid analyzer after 22 h hydrolysis at 110 °C in 6 M HCl.

Electrophoresis and column chromatography

Polyacrylamide-gel electrophoresis was performed according to previously published procedures[7,8]. Membranes or protein fractions were solubilized in 3 % dodecylsulfate containing 1 % mercaptoethanol by overnight incubation at room temperature under nitrogen. Gels to be stained for carbohydrate by the periodate–Schiff method were washed overnight at room temperature in 7 % acetic acid–40 % methanol in a Hoefer circulating destainer to remove dodecylsulfate[17]. Molecular weights were estimated from a standard curve using myosin, β-galactosidase, bovine albumin, catalase and lactate dehydrogenase as standard proteins[8].

Column chromatography in dodecylsulfate was performed on Sepharose 4B as previously described[8]. Collected fractions were assayed for absorbance at 280 nm, combined and dialyzed against 40 % methanol to remove dodecylsulfate. The samples were lyophilized and prepared for electrophoresis or amino acid analysis.

Extraction of milk fat globule membranes

Milk fat globule membrane (1 mg/ml protein) was extracted with EDTA–mercaptoethanol by a modification of the procedure of Marchesi *et al.*[18], in which the membranes were dialyzed overnight against 0.05 M glycine, 1.0 mM EDTA and 5 mM mercaptoethanol (pH 9.5) at 4 °C. The dialyzed sample was divided into two parts. One portion was further dialyzed against distilled water for 20 h at 4 °C. The resulting suspension was centrifuged at 35000 × *g* for 1 h. Pellets and supernatants were lyophilized and prepared for electrophoresis. The second portion was brought to 50 % saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 35000 × *g* for 1.0 h. Supernatants and precipitates were dialyzed against distilled water, lyophilized and prepared for electrophoresis. Samples of 33 % cream were extracted with EDTA–mercaptoethanol using the same procedure.

The guanidine hydrochloride extraction was a modification of the procedure of Gwynne and Tanford[19]. To each ml of 33 % cream or of milk fat globule membrane (2 mg/ml protein) was added 1 g of solid guanidine hydrochloride. The suspension was made to 2 % in mercaptoethanol and to pH 8.6 with NaOH. The mixture was stirred overnight at room temperature under nitrogen and centrifuged at 4 °C for 2 h at 110000 × *g*. The supernatant was carefully removed from beneath the oily layer and was dialyzed against distilled water at 4 °C. The turbid suspension from the dialysis was centrifuged 1 h at 35000 × *g* and 4 °C. The pellet and supernatant fractions were lyophilized and prepared for electrophoresis.

Extraction with NaCl was achieved by dialyzing milk fat globule membrane and cream samples (1 mg/ml protein) overnight at 4 °C against 1.0 M NaCl solutions. The supernatant was collected and redialyzed against distilled water at 4 °C. The samples were then treated in the same manner as the guanidine hydrochloride samples.

For dodecylsulfate extractions of cream 2 ml of 3 % dodecylsulfate in 0.01 M imidazole–2 mM $MgCl_2$ (pH 7.0) was added to each g of solid washed cream. The suspensions were made to 0.2 % with mercaptoethanol and incubated under nitrogen at room temperature overnight. In some cases the suspensions were heated at 100 °C for 5 min before the overnight incubation. The extraction mixtures were centrifuged

at 35000 × *g* for 30 min at 25 °C. Aliquots of the supernatant fluid were taken for protein analyses and electrophoresis. Supernatant samples were also dialyzed against 40 % methanol and lyophilized before preparation for electrophoresis.

Density gradient centrifugation

Milk fat globule membrane (3 ml containing 22 mg/ml protein) in 35 % sucrose was layered onto a discontinuous sucrose gradient consisting of 14 ml each of 45, 55, 60 and 65 % sucrose. The sample was centrifuged 18 h at 90000 × *g* and 4 °C in SW 25.2 head of Beckman Model L2-65 centrifuge. Three fractions were collected: F-1, applied layer at top; F-2, 45 % sucrose layer; and F-3, interface at 45–55 % sucrose. Samples were dialyzed against distilled water before protein analysis and preparation for electrophoresis.

RESULTS

Preparation of milk fat globule membrane

In order to optimize the preparation of the milk fat globule membrane, several procedures were investigated. Membranes were released from the fat globule by either homogenization or by a freeze–thaw procedure. Three different media were used for these operations: 0.155 M saline, 0.25 M sucrose and 0.25 M sucrose containing 0.01 M imidazole and 2 mM $MgCl_2$ (pH 7.0). The membrane samples were dialyzed to remove sucrose or NaCl and assayed for protein, phospholipid, cholesterol, neutral sugar and sialic acid. Electrophoresis of solubilized membrane samples was performed on acrylamide gels containing 0.1 % dodecylsulfate. Based on these analyses the freeze–thaw procedure in buffered sucrose *plus* Mg^{2+} was used for further investigations, since it gave the most consistent results in terms of membrane yields and electrophoresis patterns. As a characterization of the membranes prepared by this technique, several parameters of three separate membrane preparations are compared in Table I. The yield of membrane from cream ranged from 4–9 %, based on recovery of protein, for the three preparations. The lipid values and yields are somewhat lower than those reported by some other workers[6]. The source of these differences is not known, but we used a somewhat different membrane preparation procedure than reported earlier. The milk source may also be an important factor in these results. The amino acid composition of the isolated membranes, expressed as an average of the three preparations, is shown in Table II along with the amino acid content of the bovine erythrocyte membrane.

TABLE I

MILK FAT GLOBULE MEMBRANE COMPOSITION

Values are expressed as mg/mg Lowry protein[11]. The sialic acid value was obtained from a combined sample of the three preparations.

Preparation	*Cholesterol*	*Phospholipids*	*Neutral sugar*	*Sialic acid*
Milk fat globule membrane 1	0.072	0.20	0.050	0.014
Milk fat globule membrane 2	0.069	0.23	0.055	
Milk fat globule membrane 3	0.068	0.20	0.050	
Bovine red blood cell membrane	0.25	0.52	0.10	

TABLE II

AMINO ACID COMPOSITIONS OF MILK FAT GLOBULE MEMBRANE AND BOVINE ERYTHROCYTE MEMBRANE

Values are expressed as mole % of amino acids *plus* amino sugars. Tryptophan and half-cystine were not determined. Milk fat globule membrane values are for an average of three preparations with a S.D. of less than ± 3% for each value.

Amino acid	*Mole %*	
	Milk fat globule membrane	*Erythrocyte*
Lysine	5.63	5.1
Histidine	1.85	2.1
Arginine	4.60	4.9
Aspartic acid	9.60	9.6
Threonine	6.02	5.5
Serine	8.51	8.0
Glutamic acid	12.04	13.6
Proline	6.59	6.1
Glycine	7.47	6.1
Alanine	7.20	7.6
Valine	6.87	5.8
Methionine	1.71	1.4
Isoleucine	4.25	4.3
Leucine	9.31	11.6
Tyrosine	2.45	2.2
Phenylalanine	4.19	3.6
Glucosamine	1.12	2.1
Galactosamine	0.50	0.5

Electrophoresis of milk fat globule membrane proteins

The proteins of the milk fat globule membrane can be fractionated by electrophoresis on acrylamide gels in dodecylsulfate after solubilization in dodecylsulfate *plus* mercaptoethanol. Our milk fat globule membrane preparations routinely show a pattern of six major bands, as shown for the three preparations described previously (Fig. 1). The bands have been numbered I–VI and are compared to the proteins of the bovine erythrocyte membrane, which have been characterized previously by electrophoresis and are numbered according to a scheme used routinely in our laboratory[8,20]. The membrane proteins do not represent contamination by the major proteins of milk plasma, since it was shown that the major proteins of whole milk run more rapidly in this system than the membrane proteins. Molecular weights for the major membrane proteins can be estimated by comparisons with the migration distances of standard proteins and are shown in Table III. It is not possible to determine by the electrophoretic method if most of these bands represent single polypeptide chains or mixtures of chains with similar molecular weights. Component VI does appear as a doublet in some preparations and must represent more than one polypeptide.

The distribution of glycoproteins in the acrylamide gels was determined by staining by the periodate–Schiff method[8,17]. Particular care was taken to remove dodecylsulfate before staining in order to prevent adventitious staining due to dye adsorption to dodecylsulfate–protein complexes[17]. Fig. 2 shows the pattern of carbohydrate staining as a densitometer scan compared to the protein pattern determined with Coomassie blue. Six different staining species are shown which correspond to the

molecular weights expected for glycoproteins rather than glycolipid. The broad band at the bottom of gel apparently represents lipid which is stained by the periodate–Schiff method[7,21]. The six glycoprotein bands do not all correspond to the six proteins stained with Coomassie blue, although Components I and Glycoprotein 1, III and Glycoprotein 5, and V and Glycoprotein 6 overlap and may represent the same species. The major glycoprotein Glycoprotein 2 is also the most invariant in terms of the amount present between different membrane preparations. It does not stain well with Coomassie blue, a phenomenon which has been demonstrated with other membrane glycoproteins[8,17,22]. Molecular weights of membrane glycoproteins cannot be

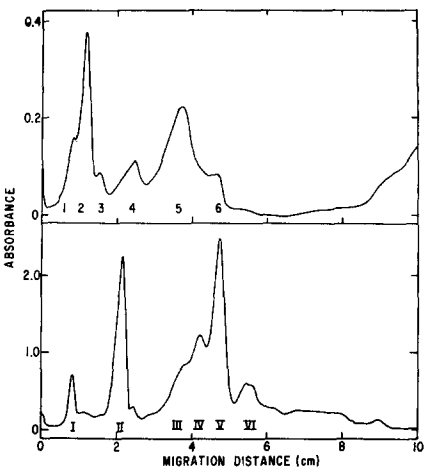

Fig. 1. Acrylamide-gel electrophoresis patterns of milk fat globule membrane showing six major bands. Three gels on left are from different bovine milk fat globule membrane preparations. Gel on right is bovine erythrocyte membrane control. Approximately 80 μg of protein was applied to each gel. Gels were stained with 0.05% Coomassie blue in 10% methanol–7% acetic acid and destained in 7% acetic acid. Numerals to left and right of gels are to designate major milk fat globule membrane and erythrocyte membrane proteins, respectively.

Fig. 2. Gel scans of carbohydrate (top) and protein (bottom) patterns for dodecylsulfate acrylamide gels of milk fat globule membrane. Protein bands are labeled as in Fig. 1. Carbohydrate bands stained by the periodate–Schiff method as previously described.

TABLE III

MOLECULAR WEIGHTS OF MAJOR MILK FAT GLOBULE MEMBRANE PROTEINS

Component	*Molecular weight*
I	240 000
II	155 000
III	92 000
IV	80 000
V	65 000
VI	53 000

determined directly by dodecylsulfate electrophoresis as for other proteins due to their inadequate ability to bind detergent[13]. For erythrocyte membrane glycoproteins the molecular weight is dependent on the acrylamide concentration in the gels[8,23,24]. This dependence is also observed for Glycoprotein 2 and Glycoprotein 5 of the milk fat globule membrane. The apparent molecular weight of Glycoprotein 2 changes from about 200000 on 5% gels to about 80000 on 12% gels, while the molecular weight of Glycoprotein 5 varies from 92000 to 56000 in going from 5 to 12% gels.

Chromatography of membrane samples in dodecylsulfate

Further characterization of the milk fat globule membrane proteins and glycoproteins was accomplished by fractionation by chromatography in dodecylsulfate on Sepharose 4B. As shown in Fig. 3 the bulk of the protein is eluted in one peak which corresponds to a molecular weight of about 66000. This value agrees well with the molecular weight estimated for Component V, the major membrane protein, by electrophoresis. Fractions from the column were combined as noted in Fig. 3, dialyzed to remove detergent and subjected to electrophoresis. No protein was detected in Fractions 1, 2 and 7. The electrophoretic distribution of the proteins and glycoproteins for the other combined fractions is shown in Fig. 4. Both Component II and Glycoprotein 2 were found predominantly in Fraction 4. The molecular weight estimated for the major Glycoprotein 2 by chromatography is considerably lower than that found by acrylamide electrophoresis on 5% acrylamide gels. The other important Glycoprotein 5 shows approximately the same molecular weight by chromatography and on 5% gels.

Amino acid analyses of the column fractions are given in Table IV. These clearly show that there are differences in the separated proteins, indicating that the

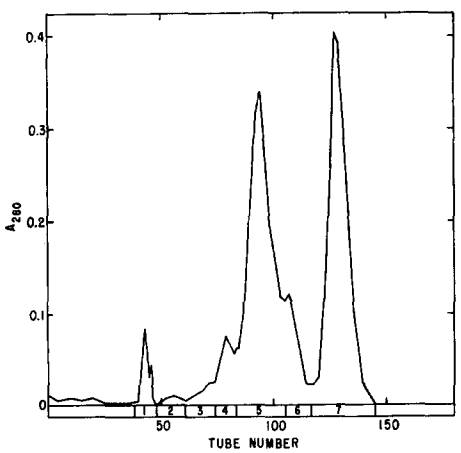

Fig. 3. Sepharose 4B chromatography of milk fat globule membrane in 1% dodecylsulfate. Samples were dissolved in 4% dodecylsulfate in 0.05 M phosphate (pH 7.8) containing 1% mercaptoethanol. After overnight incubation at room temperature under nitrogen the sample (protein concentration 10 mg/ml) was clarified by centrifugation and brought to a 20% concentration of glycerol and applied to 2.5 cm × 90 cm column. Fractions of 3.4 ml were collected and monitored for $A_{280\ nm}$. Tubes were combined to yield the fractions indicated by the bar at the bottom of the graph. Fraction 7 contains the disulfide of mercaptoethanol, but does not have a significant quantity of protein.

species observed are not aggregates of a single small unit. Fraction 3 contains primarily Component I and contains no amino sugars. Fraction 4 contains primarily Components II and Glycoprotein 1 and 2. It shows a higher content of galactosamine than glucosamine, contrary to the whole membrane. The bulk of the protein is con-

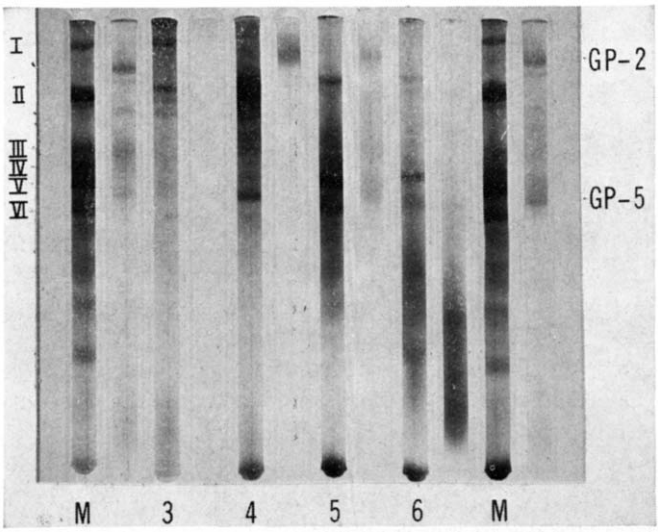

Fig. 4. Acrylamide electrophoresis of Sepharose 4B fractions. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. The number at the bottom is the fraction number for the column fractions. M indicates control membrane preparations. Fractions 1, 2 and 7 did not contain protein in significant quantities. Numerals to left and right of gels refer to protein and glycoprotein designations, respectively. GP, glycoprotein.

TABLE IV

AMINO ACID COMPOSITIONS OF SEPHAROSE 4B FRACTIONS

Values are expressed as mole % of amino acids *plus* amino sugars. Tryptophan and half-cystine were not determined. Dash indicates insufficient amount for calculation.

Amino acid	*Fraction*: 3	4	5	6
Lysine	5.5	6.6	5.8	7.7
Histidine	2.9	2.3	2.0	2.2
Arginine	7.9	5.1	6.0	4.7
Aspartic acid	8.9	9.2	9.4	9.5
Threonine	5.8	6.8	5.8	7.4
Serine	7.7	7.4	8.3	11.8
Glutamic acid	11.9	10.9	12.5	10.7
Proline	5.6	5.9	5.8	4.5
Glycine	9.0	8.6	7.5	6.9
Alanine	8.1	8.3	6.7	6.2
Valine	6.1	6.1	6.8	6.2
Methionine	1.2	1.9	2.0	1.6
Isoleucine	4.1	4.4	4.4	4.4
Leucine	10.2	9.2	9.2	8.7
Tyrosine	1.9	1.7	1.9	2.3
Phenylalanine	3.3	4.8	4.3	3.8
Glucosamine	—	0.36	1.3	1.1
Galactosamine	—	0.70	0.46	0.35

tained in Fraction 5, in which the glucosamine to galactosamine ratio is higher than the milk fat globule membrane. Fraction 6 contains smaller polypeptides *plus* lipid. The latter is indicated by the high content of serine and the presence of a large amount of ethanolamine (not shown in Table IV). Glycolipid may also be present in this fraction, since the fraction does show some carbohydrate.

Extraction of milk fat globule membrane and cream samples

Although membrane proteins can in theory be purified by repetitive chromatography in denaturing media, this approach is tedious and usually destroys the native properties of the protein. Isolation and partial purification of particular proteins can often be achieved by selective extraction methods. An example of this is the purification of spectrin from erythrocyte ghosts by EDTA extraction[18]. A similar procedure was applied to the milk fat globule membrane to investigate the solubility characteristics of its proteins. Fig. 5 shows a flow diagram of the extraction procedure. Acrylamide gels of the various fractions stained with Coomassie blue are shown in Fig. 6. Solubilization was defined operationally as the failure to sediment at 30000 $\times$ *g* for 90 min. Some discrimination in the extraction of the proteins is noted (Gel 1 *vs* 2 *plus* 3), although it is not as pronounced as with red cell membranes. Component I does not appear to extract into EDTA at all; V and VI are less readily extracted than II, III and IV. The major Glycoprotein 2 partitions between extract and pellet in approximately equal amounts, while Glycoprotein 5 is present primarily in the extract. Components II and IV are still in soluble form after dialysis against distilled water (Gel 3) and II remains in solution after bringing the EDTA extract to 50% saturation with $(NH_4)_2SO_4$ (Gel 5). Glycoprotein 2 also appears to remain partially in solution after $(NH_4)_2SO_4$ treatment. Essentially the same results in terms of extractability can be observed using washed cream rather than milk fat globule membrane for the material to be extracted.

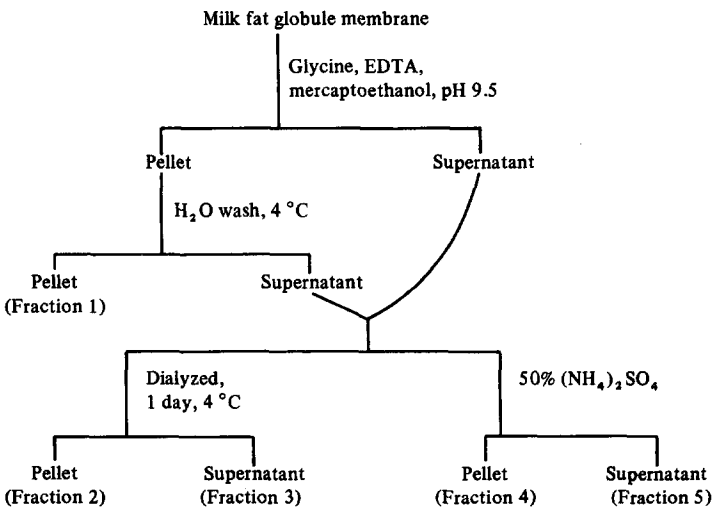

Fig. 5. Diagram of procedure used for EDTA extraction.

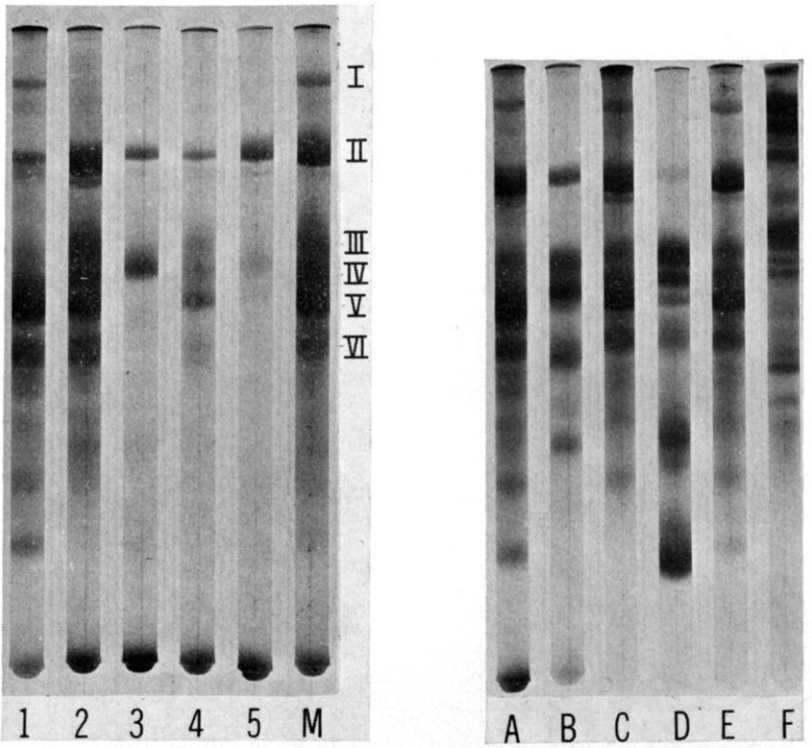

Fig. 6. Acrylamide-gel electrophoresis of fractions obtained from EDTA extraction. Numbers below gels correspond to fraction numbers in Fig. 5.

Fig. 7. Acrylamide-gel electrophoresis of water soluble and insoluble fractions of the NaCl and guanidine extracts of milk fat globule membrane. Membranes were extracted with 1 M NaCl or 6 M guanidine hydrochloride as described in text. Extracts were dialyzed against distilled water to fractionate into water soluble and insoluble fractions. Gel A, insoluble fraction of NaCl extract; Gel B, soluble fraction of NaCl extract; Gel C, insoluble fraction of guanidine hydrochloride extract; Gel D, soluble fraction of guanidine hydrochloride extract; Gel E, milk fat globule membrane control; and Gel F, bovine erythrocyte membrane.

Additional experiments were performed using 6 M guanidine hydrochloride[19] and 1 M NaCl as extracting agents. In each case the soluble material was dialyzed against distilled water. The water soluble and insoluble fractions were separated by centrifugation at 35000 $\times$ g for 1 h and were subjected to electrophoresis in dodecyl-sulfate. Fig. 7 shows the patterns obtained from these fractions. All of the milk fat globule membrane proteins can be partially extracted by either of the two salt treatments. However, only III, IV and Glycoprotein 5 remain predominantly soluble after the salt extracts have been dialyzed against distilled water. With guanidine hydrochloride about 70 % of the total milk fat globule membrane protein can be extracted, which is only slightly more than observed for the erythrocyte membrane. However, there does not appear to be the selectivity in this initial extract which has been observed with the erythrocyte membrane proteins[25]. Extraction of 33 % cream with 6 M guanidine hydrochloride or 1 M NaCl yielded essentially the same results as extraction of milk fat globule membrane.

Cream samples (10 or 33 %) can also be extracted directly with dodecylsulfate solutions. Essentially all of the protein was solubilized (110–120 % by Lowry protein analysis) in this manner. The electrophoretic patterns of these extracts differ from the milk fat globule membrane samples primarily by the addition of a smear of staining material at the dodecylsulfate front. This apparently derives from lipid material which is extracted into the detergent solution. Since it does not interfere with the remainder of the pattern, it is possible to use electrophoresis in dodecylsulfate directly on cream samples without preparing membranes.

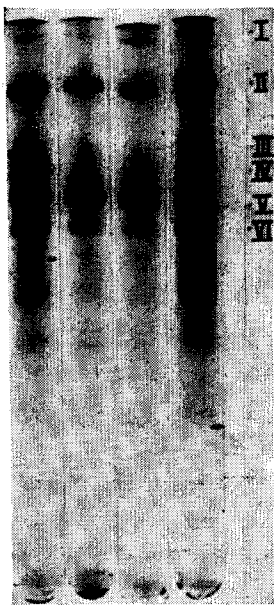

Fig. 8. Acrylamide-gel electrophoresis of density gradient fractions. Gels contain (left to right): Fraction 1, 2, 3 and milk fat globule membrane.

Density gradient centrifugation of milk fat globule membrane

Since the origin of the milk fat globule membrane is still uncertain, more than one type of membrane could possibly be represented in the isolated preparation. Therefore the membranes were subjected to a crude fractionation on discontinuous sucrose gradient. Three distinct areas of membrane material were found in the gradient: a major sharp band (F-3, 38 % of total protein on gradient) at the 45–55 % sucrose interface, a disperse band (F-2, 33 %) in the 45 % sucrose layer and a fine band (F-1, 27 %) floating at the top of the gradient which also contained considerable residual butterfat. Material from these bands was subjected to electrophoresis in dodecylsulfate and showed the patterns given in Fig. 8. Each density gradient band contained the same group of proteins, although Components III and IV were less prominent in Bands 2 and 3. These results suggest that the separated bands represent one membrane type in terms of protein components with different amounts of associated fat.

DISCUSSION

The present study was undertaken to investigate the proteins and glycoproteins which are associated with the milk fat globule membrane. Membranes were obtained by a freeze–thaw procedure from washed cream, which was derived from chilled milk. It is difficult to compare our preparation with those obtained previously because of the variability of the parameters reported and the variations in methods of reporting data. Our results do differ significantly from some of the data reviewed by Brunner[6]. Yield data, lipid content and amino acid compositions contain noteworthy disparities. These differences probably result from variations in the milk source, cream preparation and membrane isolation. The current state of the art of milk fat globule membrane isolation does not indicate what preparation techniques are optimum at the various stages. These will have to be investigated further, and the methods described in this work should be very useful in this connection.

In view of its possible derivation from the plasma membrane there was considerable interest in comparing the properties of the milk fat globule membrane with other plasma membranes. Analytical comparisons with bovine erythrocyte membranes show that our milk fat globule membrane preparation has a lower cholesterol and phospholipid content. It is not known if these differences result from the actual membrane composition or from lipid losses during preparation of the milk fat globule membrane. The amino acid compositions of the milk fat globule membrane and erythrocyte membranes are remarkably similar. Although amino acid composition of protein mixtures are of limited usefulness in characterizations, the presence of large amounts of glutamic acid, leucine and aspartic acid appear to be common to many examples of membrane or cellular structural proteins[26].

Proteins of the milk fat globule membrane were fractionated by acrylamide-gel electrophoresis as a means of physical characterization and classification. At least six major proteins can be detected by staining with Coomassie blue. These range in molecular weight from 53000 to 240000. Staining by the periodate–Schiff procedure also gives six bands, three of which are clearly different from the protein bands. Whether the overlapping protein and glycoprotein bands represent different molecular species cannot be determined at present. By examination of a large number of membrane preparations it was shown that the major protein (Component V) and glycoprotein (Glycoprotein 2) are the most invariant of the proteins or glycoproteins in terms of their occurrence in the isolated membrane. Other components are less prominent in some preparations. This was particularly true of the minor glycoproteins. To determine whether these variations result from alterations in milk or in the preparative techniques will require further investigation.

It was of particular interest to observe the properties of the major glycoprotein (Glycoprotein 2) of the milk fat globule membrane, since only a limited number of studies have been reported on membrane glycoproteins. Two noteworthy properties were its failure to stain with Coomassie blue and the variation of its calculated molecular weight with acrylamide percentage during dodecylsulfate electrophoresis. Both of the properties are exhibited by the major membrane glycoproteins of erythrocytes from a number of different animal species[8]. The failure to stain for protein has been reported for membrane glycoproteins of rat liver and kidney brush borders[17]. The anomalous staining and electrophoretic migration apparently result from the

high carbohydrate content of these proteins and may represent common features of the major glycoprotein components of cell surface plasma membranes.

The results of various extraction procedures indicate differences in the milk fat globule membrane proteins which might be exploited for the isolation of individual proteins. The initial extraction with EDTA, NaCl or guanidine hydrochloride results in a rather nonselective partial solubilization or dispersion of most of the proteins from either milk fat globule membrane or cream. After dialysis against distilled water, some selectivity based on solubility is noted. Components II and IV remain soluble after EDTA treatment and dialysis against water. Component II is not precipitated by $(NH_4)_2SO_4$ from the EDTA extract. Components III and IV are partially soluble after dialysis of the salt extracts against distilled water. These results may indicate a peripheral association of these water soluble components with the membrane. Whether the soluble fractions are molecularly dispersed is not known. Further investigations will be required to determine the aggregation state and composition of solubilized fractions. The results of this study do suggest that selective extraction procedures will be useful as a first step in purification of the membrane-associated proteins.

The question of the origin of the components of the milk fat globule membrane in the secretory cell has still not been completely resolved. Our milk fat globule membrane preparations show activities of 5'-nucleotidase, a plasma membrane marker, similar to those reported earlier[3] (D. Kobylka, unpublished observations). Martel-Pradal and Got[27] have recently reported the presence of marker enzymes for the Golgi apparatus and endoplasmic reticulum, as well as the plasma membrane, in milk fat globule membrane preparations from human colostrum. Our attempts to fractionate milk fat globule membrane preparations into different membrane types were not successful, as judged by the protein compositions of the fractions. Such fractionation attempts are complicated by neutral fat adhering to the membrane. Although our results favor the presence of a single type at the globule surface, they do not necessarily conflict with the observation of the presence of enzymes from several cellular membranes. It is possible that components from the different cellular membranes could be attached to the globule surface and integrated into a single membrane during or after secretion. It is also possible that the major proteins of the milk fat globule membrane, which may serve a structural rather than enzymatic role, may be primarily derived from one membrane type. The possibility of purifying these components should offer the opportunity to investigate their origins by immunological techniques and answer some of these questions concerning the secretory process.

The extraction and electrophoresis methodology described should be useful in several contexts. (1) The establishment of definitive, reproducible protein patterns will aid in isolation of individual protein components. (2) Studies of the organization of the membrane proteins is feasible using dodecylsulfate electrophoresis as a monitoring system. For example, proteolytic digestion of the globules or membranes can be assayed by electrophoresis (D. Kobylka, unpublished observations). (3) The direct extraction of cream with sodium dodecylsulfate to give the entire complement of milk fat globule membrane proteins in virtually quantitative yields is a particularly attractive method for studying changes in cream associated proteins without the necessity of membrane isolation, which is a rather artificial technique at best. The current study and other evidence do indicate that the milk fat globule membrane

is a promising system for the study of membrane proteins and protein–lipid interactions.

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REFERENCES

1 W. Bargmann and A. Knoop, *Z. Zellforsch. Mikrosk. Anat.*, 49 (1959) 334.
2 R. M. Dowben, J. R. Brunner and D. E. Philpott, *Biochim. Biophys. Acta*, 135 (1967) 1.
3 S. Patton and E. G. Trams, *FEBS Lett.*, 14 (1971) 230.
4 T. W. Keenan, D. J. Morre, D. E. Olson, W. N. Yunghans and S. Patton, *J. Cell Biol.*, 44 (1970) 80.
5 F. B. P. Wooding, *J. Ultrastruct. Res.*, 37 (1971) 388.
6 J. R. Brunner, in E. Tria and A. M. Scanu, *Structural and Functional Aspects of Lipoproteins in Living Systems*, Academic Press, New York, p. 546.
7 K. L. Carraway and D. Kobylka, *Biochim. Biophys. Acta*, 219 (1970) 238.
8 D. Kobylka, A. Khettry, B. C. Shin and K. L. Carraway, *Arch. Biochem. Biophys.*, 248 (1972) 475.
9 J. Lenard, *Biochemistry*, 9 (1970) 1129.
10 G. Fairbanks, T. L. Steck and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2606.
11 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
12 A. Zlatkis, B. Zak and A. J. Boyle, *J. Lab. Clin. Med.*, 41 (1953) 486.
13 P. S. Chen, Jr, T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
14 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
15 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971.
16 D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
17 H. Glossman and D. M. Neville, *J. Biol. Chem.*, 246 (1971) 6339.
18 S. L. Marchesi, E. Steers, V. T. Marchesi and T. W. Tillack, *Biochemistry*, 9 (1970) 50.
19 J. T. Gwynne and C. Tanford, *J. Biol. Chem.*, 245 (1970) 3269.
20 K. L. Carraway and B. C. Shin, *J. Biol. Chem.*, 247 (1972) 2102.
21 K. L. Carraway, A. Lam, D. Kobylka and J. Huggins, *Anal. Biochem.*, 45 (1972) 325.
22 K. L. Carraway, D. Kobylka and R. B. Triplett, *Biochim. Biophys. Acta*, 241 (1971) 934.
23 J. P. Segrest, R. L. Jackson, E. P. Andrews and V. T. Marchesi, *Biochem. Biophys. Res. Commun.*, 44 (1971) 390.
24 M. S. Bretscher, *Nature*, 231 (1971) 229.
25 T. L. Steck, *Biochim. Biophys. Acta*, 255 (1972) 553.
26 D. Mazia and A. Ruby, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1005.
27 M. B. Bartel-Pradal and R. Got, *FEBS Lett.*, 21 (1972) 220.