I. PROTEINS AND GLYCOPROTEINS OF THE ERYTHROCYTE MEMBRANE

II. PROTEINS AND GLYCOPROTEINS OF THE MILK FAT GLOBULE MEMBRANE

By

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Dedicated to my wife,

Hannelore

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PART I

NOMENCLATURE

5'-AMP	adenosine 5'-monophosphoric acid
ATP	adenosine 5'-triphosphate
BIS	N,N'-methylenebisacrylamide
CB	Coomassie Blue
CD	circular dichroism
CPM	counts per minute
DSA	diazotized sulfanilic acid
EDTA	ethylenediaminetetraacetic acid
ESR	electron spin resonance
FMMP	formylmethionyl sulphone methyl phosphate
GP	glycoprotein
IR	infrared
LIS	lithium diiodosalicylate
MFGM	milk fat globule membrane
MW	molecular weight
NMR	nuclear magnetic resonance
ORD	optical rototary dispersion
PAS	periodate-Schiff
PEA	phosphatidylethanolamine
POPOP	1,4-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
PS	phosphatidylserine
SDS	sodium dodecylsulfate

TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
TTC	2,3,5-triphenyltetrazolium chloride

PART ONE

PROTEINS AND GLYCOPROTEINS OF THE ERYTHROCYTE MEMBRANE

CHAPTER I

INTRODUCTION

During recent years, the consensus among the majority of biologists as to the molecular organization of membranes has changed from a state of substantial agreement to one of almost total disagreement. The major areas of controversy concern the nature of membrane proteins, their interactions with membrane lipids, and their orientation in the membrane system. The following will review some of the main areas of membrane research currently under investigation and the most salient results and conclusions derived from them.

Structurally, membranes are generally examined on the basis of their major components of protein, lipid and carbohydrate which can combine to yield four components, protein, glycoprotein, lipid and glycolipid, since the carbohydrate moiety is covalently linked to either protein or lipid. The first group of these, the membrane proteins and glycoproteins, are usually studied by two methods, fractionation or extraction. Acrylamide gel electrophoresis, either in the presence of sodium dodecylsulfate (SDS) or acid-urea, are most commonly used for one step fractionation of membranes. The former was introduced by Maizel (1) and Shapiro *et al.* (2) and first applied to membranes by Berg (3). The method was further developed and modified by Lenard (4) and Carraway and Kobylka (5) to yield a technique which can separate erythrocyte membrane proteins from lipid and fractionate the proteins

into a number of bands according to their molecular size. The resultant chromatograms are seen to be representative and reproducible for a given membrane system. In the acid-urea method reported by Takayama et al. (6) the hydrophobic proteins of the mitochondrial electron transfer chain were found to be completely soluble in a solvent system consisting of phenol-acetic acid-water. Subsequent zone electrophoresis of the solubilized complexes revealed many protein bands characteristic for each enzyme complex. Demus and Mehl (7) have reported a variation of this procedure by using formic acid in place of acetic acid. Results obtained by using such techniques led to the general conclusion that there exist a large number of different protein species in a variety of systems, including mitochondrial and microsomal membranes (8), envelopes of Gram-negative bacteria (9) and Mycoplasma membranes (10). However, as rapid and reproducible as these techniques are, one still must be aware of several inherent problems which may exist.

The first is the possibility that aggregation of a small number of polypeptide chain components gives rise to the heterogeneous patterns observed by different analytical methods. Several groups have stated that their procedures produce complete disaggregation. Lenard (4) disaggregates by heating at 100° for three minutes in 2-3% SDS before electrophoresis in 1% SDS and observes band patterns ranging in molecular weight from 22,000 to 255,000 daltons. This method does not eliminate the possibility of reaggregation after heating or of intermolecular disulfide bond formation during the heating process. Demus and Mehl (11) using phenol-formic acid-water in polyacrylamide gels reported that pig erythrocyte membranes contained proteins predominantly between 27,000 and 65,000 daltons with only a small number of components above

100,000 daltons. Carraway et al. (12) have found the distribution of molecular weights in red blood cell membranes to be from 25,000 to 220,000 daltons. The largest component appears to be identical to spectrin (13) since it is extractable by exposing membranes to EDTA in the presence of mercaptoethanol. Several groups have indicated that spectrin may be an aggregate. These results are based on the salt induced disaggregation (3), protein distribution in phenol-formic acid-water (11) and immunological cross reactivities (14). In all of these instances the conclusions reached may have resulted from experimental difficulties due to insolubility of lipid-free proteins, loss of protein during membrane preparation or incomplete purification of antigenic protein, respectively. Evidence for the heterogeneity of the membrane proteins and the existence of the high molecular weight protein was presented by Gwynne and Tanford (15) who report that human erythrocyte membranes contain very large polypeptide chains of molecular weight about 200,000 daltons, detected by extracting and chromatographing the proteins in 6M guanidine hydrochloride. In addition they showed that the extracted proteins were in random coil conformation by ORD analysis and the range of molecular weights to be from 14,000 to 200,000 daltons. The study supports the hypothesis of the heterogeneity of the erythrocyte membrane proteins and of the existence of a major high molecular weight protein. A report by Laico et al. (16) that a major portion of the membrane protein is composed of a small "miniprotein" (molecular weight approximately 5,000 daltons) has been disputed by Carraway et al. (17) (18) who showed that a fast moving protein staining band on SDS-gels (which was coincident with the "miniprotein" band) actually consists of a complex of lipid, glycolipid and SDS by ^{14}C-labeled choles-

terol exchange with membranes (17) and electrophoresis of purified phospholipids (18). In addition, chromatography in SDS has been used to resolve this species to show that it represents less than 5% of the total membrane protein as determined by amino acid analysis (19).

The second area of difficulty concerning the distribution of membrane proteins involves the isolation technique employed to prepare membranes. Lenard (20) has reported that the distribution of polypeptide chains is similar in red cell membranes obtained from man, pig, sheep, rat and dog and that the proteins of red cell membranes from the different species show extensive similarities in over two-thirds of their total protein. Using a similar technique, but with more extensive washing of the isolated ghost material, Carraway and Kobylka (5) found no apparent common pattern of proteins from human, cow and horse erythrocyte membranes, when compared to dog and cat membranes, to suggest a common membrane structure based on similar proteins. Zwaal and Van Deenen (21) examined erythrocyte membrane proteins of human, rat, rabbit, pig, ox and sheep by butanol fractionation of aqueous ghost suspensions and also found that the protein composition of the erythrocyte membrane from these mammalian species is markedly different as demonstrated by sedimentation in an analytical ultracentrifuge and electrophoresis on polyacrylamide gels.

Recently, it has been recognized that a major difficulty giving rise to these significant differences in the protein distributions results from the proteolytic digestion of the membrane proteins which are fragmented during preparation. Kobylka et al. (22) observed that failure to remove white blood cells from membrane preparations was a significant source of the problem with proteolytic digestion of mem-

branes. Similarly, Fairbanks et al. (23) reported that the incubation of erythrocyte ghosts with low levels of SDS and high levels of salt produced diffuse bands of low average molecular weight on polyacrylamide gels and that this degradation is attributed to proteinases primarily due to leukocyte contamination.

Many methods have been introduced for extracting certain proteins from membranes. Marchesi and Steers (13) used ATP-mercaptoethanol at low ionic strength to extract a high molecular weight protein spectrin from guinea pig red blood cell ghosts. Subsequently, Marchesi et al. (24) extended their study of spectrin to other species including horse, sheep, rabbit and human as systems extracted with EDTA-mercaptoethanol. Treatment of hemoglobin-free human erythrocyte stroma with hypertonic sodium chloride solutions (0.2-1.4 M) effects a partial solubilization of stromal lipids and acetylcholinesterase plus other proteins without complete disruption of the underlying stromal structure (25). Rosenberg and Guidotti (26) (27) have sequentially combined various methods to extract eight fractions from erythrocyte membranes, which differ in amino acid composition and size. The glycoprotein of erythrocyte membranes can be specifically extracted using phenol (28) (29) (30). Solubilization in pyridine followed by chromatography in pyridine has been used to extract glycoproteins and glycolipid components from erythrocyte membranes (31) (32) (33). Maddy and Kelly (34) used acetic acid solutions to solubilize ghost proteins yielding a heterogeneous mixture which may contain protein aggregates. Other workers have used butanol (35) (36) (37), pentanol (38) and dilute aqueous solutions (39) (40) (41) to solubilize protein components of erythrocyte membranes.

The glycoprotein of the human erythrocyte membrane has been the

most extensively studied of all membrane components. However, significant controversy as to its apparent molecular weight has become increasingly apparent as more investigators apply various fractionation and isolation techniques to the examination of this protein. A molecular weight of 31,000 was obtained by ultracentrifugation for the disaggregated monomer when isolated by phenol extraction (29) (47). Marchesi (43) and Marchesi and Andrews (44) recently reported the extraction of the human erythrocyte membrane by use of lithium diiodosalicylate (LIS). The glycoprotein thus obtained was 60% carbohydrate and 40% protein and was found to have an apparent monomeric molecular weight of 55,000 daltons as judged by acrylamide gel electrophoresis and analysis of the tryptic and cyanogen bromide peptides. In contrast, direct SDS acrylamide gel electrophoresis of membranes followed by staining for carbohydrate indicates a single major glycoprotein for human erythrocyte membrane with a molecular weight of approximately 100,000 daltons (4) (5). A point of partial clarification of the controversy surrounding the molecular weight of the human red blood cell membrane glycoprotein has recently been made. It is now evident that the calculated molecular weight of a glycoprotein is dependent upon the acrylamide percentage in the gel used for its determination (22) (45) (46). This anomalous behavior apparently results from a decreased SDS binding capacity of the glycoprotein when compared to other proteins (45).

The human erythrocyte glycoprotein can be cleaved by trypsin either in the isolated stromal form or directly on the intact red blood cell (47) (48). In either situation a hydrophilic sialoglycopeptide is released which consists of approximately 80% carbohydrate by weight and has a molecular weight of 10,000 daltons. The glycoprotein contains at

least two different carbohydrate chains, one labile to alkaline borohydride whose structure has been studied by Winzler (48). A partial structure for the other chain has been determined by Kornfeld and Kornfeld (49) and has an apparent molecular weight of 2,000 daltons. The glycoproteins of erythrocyte membrane from other species have not been studied as extensively as in the human case. Lenard (20) reported a major glycoprotein of molecular weight 108,000 daltons. However, it appears that his observation resulted from nonspecific staining of SDS-acrylamide gels with the periodate-Schiff reagent. Kobylka et al. (22) found that for human, dog, sheep, horse and cow erythrocytes, each has a single distinct and major glycoprotein that is different in apparent molecular weight.

Physical studies of the state of membrane components are made primarily by application of infrared (IR), optical rototary dispersion (ORD), circular dichroism (CD), nuclear magnetic resonance (NMR), electron spin resonance (ESR) freeze-etch or cleave and X-ray techniques. Many workers have attempted to ascertain the conformational state of the proteins in cellular membranes. Based on IR analyses of dry films of erythrocyte membranes Maddy and Malcolm (50) observed little β-structure in the erythrocyte membrane. Wallach et al. (51) found IR evidence for β-structure in mitochondrial membranes. ORD and CD measurements have been interpreted to show the absence of β-structure and the presence of considerable quantities of an α-helix (52) (53) (54). The anomolous red shift of the CD spectra above 220 nm has been shown to arise from scattering effects of particulate membrane systems (55) (56) (57) (58). After correction for the scattering artifacts, the percentage of α-helix in erythrocyte membranes has been estimated at 40 to 50% with

little if any detectible β-structure.

Additional evidences for lipid-protein hydrophobic interactions is found in the broadened NMR signals of lipid methyl and methylene groups of lipid hydrocarbon chains (59). However, ESR studies of Hubbell and McConnell (60) using nitroxide-labeled lipids incorporated into the membrane indicated that the labels in nerve fiber are bound to a lipid bilayer very similar to that found in sonicated phospholipid dispersions. They further concluded that a large fraction of neural membrane does contain a lipid bilayer structure and that erythrocytes have a much more tightly packed bilayer. Tourtellotte et al. (61) found a major portion of membrane lipids in a semiviscous hydrocarbon environment when spin-labeled fatty acid was incorporated in vivo into the polar lipids of Mycoplasma laidlawii membranes. In addition, by freeze-etching they found particulate components in the hydrophobic region of the membrane and suggest that the mobility of the lipids in intact membranes may be influenced by their association with these particles.

The freeze-cleave and freeze-etch techniques of electron microscopy have been particularly valuable in membrane structural studies. Freeze-cleave is based on the cleavage of the membrane through its center, i.e., splitting the membrane between the halves of the bilayer and replicating the exposed surfaces (62) (63) (64). Freeze-etching removes ice from the surfaces which are not affected by cleavage, thus permitting the examination of both the exterior and interior surfaces of the membrane. Freeze-cleave patterns of erythrocytes show 85 Å particles distributed in a random manner within the membrane. These particles contain carbohydrate, probably in the form of glycoprotein and possibly lipid or glycolipid (65) (66). X-ray diffraction studies also

indicate that the bulk of the membrane lipid is in a bilayer arrangement (67).

Chemical and enzymatic studies have been performed in an attempt to localize membrane components relative to the permeability barrier. Eylar et al. (68) found that all the erythrocyte sialic acid of several animal species is present on the membrane surface and 95-100% of it can be removed by neuraminidase action. Glycopeptides containing approximately one-third to one-half of the sialic acid of human erythrocytes are released from intact cells by incubation with trypsin indicating the presence of glycoprotein at the cell surface (47). Berg (3) has used the diazonium salt of ^{35}S sulfanilic acid (DSA), which does not penetrate intact cells, as a label for outer components of the human erythrocyte membrane, showing that the majority of the membrane protein is within the permeability barrier. Phillips and Morrison (69) iodinated the surface of intact erythrocyte membranes with ^{125}I, catalyzed by the enzyme lactoperoxidase. They observed that only a single stromal protein was iodinated as determined by one peak of radioactivity when the labeled membrane preparation was electrophoresed on SDS acrylamide gels. By using both bovine and human erythrocytes with trypsin and DSA treatments, Carraway et al. (12) established that two different proteins are present at the erythrocyte surface. The glycoprotein is most readily accessible while the second protein is probably partially buried within the membrane. Bretscher (70) (71) has used ^{35}S formylmethionyl sulphone methyl phosphate (FMMP) as a nonpenetrating labeling reagent for the surface of intact erythrocytes and finds that the same two proteins on the outside surface of the cell are labeled. Pronase treatment of human erythrocytes cleaves the larger of the two proteins to yield a

smaller fragment (72). In addition, electron microscopy studies of erythrocyte membranes have been performed while ferritin-labeled antibodies to spectrin and ferritin-labeled hemagglutinins were attached to the membrane. The localization of the ferritin agrees with the view that glycoproteins are at the outer surface of the membrane while spectrin is on the inner surface (73). Many of the above techniques have recently been reviewed by Wallach (74).

The presence of lipids at the erythrocyte surface can be inferred from the work of Bruckdorfer et al. (75) (76) and Carraway et al. (17) in which it was found that cholesterol molecules readily exchange between intact erythrocytes and dispersions of phosphatidylcholine and cholesterol. Evidence from phospholipase hemolysis of red blood cells supports the concept of phospholipids being present at erythrocyte membrane surfaces (77). However, Zwaal et al. (78) (79) have shown that highly purified phospholipases do not hemolyze red blood cells. Indeed, the phospholipid molecules of the intact erythrocyte membrane cannot be attacked by phospholipase A or phospholipase C (80) (81). From these studies it appears that phosphatidyethanolamine (PEA) and phosphatidylserine (PS) are not accessible from the outside of the membrane in intact cells. Carraway et al. (17) have obtained evidence of a change in the interactions of PEA and PS when going from the intact erythrocyte to the ghost using the reactivity of the membrane toward radioactive acetic anhydride as a structural probe. Thus, the topic of phospholipid orientation in intact versus isolated membranes is still to be resolved in more detail.

From the above it can be stated that the area of membrane chemistry and structure is still in a state of ferment. A large amount of infor-

mation has been reported regarding the chemistry and organization of membrane components, some of which is contradictory. However, from this body of reports an equally impressive number of models for cell membrane structure have been proposed. These range from the lipid bilayer unit membrane model of Robertson (82) to that of the lipoprotein subunit membrane model of Benson (83). Most of the other models which have been offered represent combinations or extensions of these basic concepts. A number of these models were reviewed by Hendler (84) in an article which defends the unit membrane-bilayer model and suggests that perhaps this model needs concepts added to it, rather than abandonment. Conversely, Zahler (85) finds that recent data on membrane chemistry and physics have given rise to serious doubts about the unit-membrane theory. Most recently, Singer and Nicolson (86) and Singer (87) proposed a fluid mosaic model for the proteins and lipids of membranes in which globular molecules of the integral proteins alternate with sections of phospholipid bilayer in the cross section of the membranes. The globular protein molecules are postulated to be amphipathic as are the phospholipids i.e., they are structurally asymmetric with one highly polar end and nonpolar end. In short, much information is available concerning the structure-function aspects of plasma membranes and much has been written in terms of models to explain the various experimental observations, but more conclusive evidence is needed in all areas to more clearly understand biomembranes as functional systems.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Source of Erythrocytes

Human blood was obtained from the Dallas Community Blood Bank, Dallas, Texas and used within one week of the withdrawal date. Animal blood samples were collected by the staff of the College of Veterinary Medicine, Oklahoma State University, in citrate or acid-citrate-dextrose solution and used within 1-2 days of collection.

Polyacrylamide Gel Electrophoresis

Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Canalco. Sodium dodecyl sulfate (SDS) was a product of Sigma. The molecular weight markers for SDS electrophoresis calibration were myosin,[1] β-galactosidase, bovine serum albumin, catalase, ovalbumin, lactic dehydrogenase, α-chymotrypsinogen and cytochrome c (all products of Sigma). NCS Solubilizer was a product of Amersham/Searle. Samples of purified phospholipids were products of Applied Science Laboratories, State College, Pennsylvania.

[1] Prepared by Mr. C. Schwartz.

Radioactive Compounds

^{14}C-Cholesterol, ^{14}C-acetic anhydride, ^{3}H-acetic anhydride and ^{35}S-sulfanilic acid were all products of Amersham/Searle.

Enzymes

Trypsin (Type III), Clostridium perfringens neuraminidase (Type VI) and trypsin inhibitor (Type II-O) were all products of Sigma.

Methods

Preparation of Erythrocytes and Membranes

Erythrocyte membranes were prepared routinely from washed red blood cells according to the hypotonic phosphate procedure of Dodge et al. (88) in which solutions were prepared from reagent-grade chemicals: (a) sodium phosphate, monobasic (NaH_2PO_4), 0.155 M or 310 imOsm (ideal milliosmolar), and (b) sodium phosphate, dibasic (Na_2HPO_4), 0.103 M or 310 imOsm. The intact erythrocytes were isolated by centrifugation for 20 min at 1000 x g. The plasma and buffy coat were removed by aspiration. The cells were washed three times with isotonic phosphate buffer, 310 imOsm, pH 7.4. When appropriate, hemolysis was performed by pipetting 2 ml aliquiots of tightly packed washed red cells into 28 ml of 20 imOsm phosphate buffer, pH 7.4 (made by diluting the 310 imOsm phosphate buffer 1:15.5 with distilled deionized water). The suspension was then centrifuged for 30 min at 20,000 x g. The supernatant was carefully decanted or aspirated, and the ghost pellet was resuspended by swirling while adding sufficient 20 imOsm buffer to reconstitute the original volume of 30 ml. The ghosts were washed three times subsequent to the

hemolysis in like manner. The membrane samples were used directly for further studies or lyophilized for electrophoretic analysis. In addition to this method, erythrocyte membranes which were used for comparative studies were prepared by two different procedures: the Ca^{++}-veronal hypotonic lysis of Burger et al. (89) and the Tris-EDTA method of Marchesi and Palade (90). The Ca^{++}-veronal system used 0.155 M NaCl for the washing of intact erythrocytes in place of 310 imOsm phosphate buffer. Hemolysis and washing were accomplished (using the same ratio of 2:28, cells to buffer) by use of 25 imOsm veronal (pH 7.4) containing 3 imOsm $CaCl_2$ in place of the 20 imOsm phosphate buffer. The Tris-EDTA method consisted of washing intact erythrocytes in 0.155 M NaCl but hemolysis and washing were performed in 5 mM Tris-Cl, (pH 7.4) and 1 mM EDTA. Membranes were examined for morphological changes using a Leitz phase contrast microscope. Hemoglobin was determined by absorbance measurements at 540 nm after addition of ammonium hydroxide.

Lipids were extracted from erythrocyte membranes by Procedure I of Ways and Hanahan (91). Lyophilized membrane material was first suspended in absolute methanol for 20 min at room temperature. To the solution was added an equal volume of chloroform and the mixture was allowed to stand for 10 additional minutes. The sample was then centrifuged at 35,000 x g for 30 min. The organic supernatant was removed and concentrated under nitrogen. This procedure was repeated two more times. Cholesterol was determined by the method of Zlatkis et al. (92), phospholipid by the phosphorous procedure of Chen et al. (93), protein by the method of Lowry et al. (94), and free amino groups by the ninhydrin method of Moore and Stein (95).

Polyacrylamide Gel Electrphoresis

Gels were prepared and run according to a modification of standard procedures (1) (2) (3). The method involved the preparation of gels from fresh components each time a run was to be made. The ratio of acrylamide to N, N'-methylenebisacrylamide (BIS) was always kept constant at 1:0.027 and potassium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) were always 0.075% and 0.066%, respectively, regardless of the gel percentage desired. For example, if 30 ml of a 5% gel solution were needed, 1.4595 gm acrylamide, 0.0405 gm BIS, 0.0225 gm potassium persulfate and 0.020 ml TEMED were brought to volume using 0.1% SDS–0.10 M phosphate buffer, pH 7.2. The upper and lower chambers of the electrophoresis apparatus also contained the same 0.1% SDS–0.10 M phosphate buffer. Lyophilized membrane samples were dissolved at a concentration of approximately 10 mg/ml in 3.0% (w/v) SDS–0.10 M phosphate buffer, pH 7.8, containing 0.14 M mercaptoethanol and 20% (w/v) glycerol and allowed to stand 18–20 hr at room temperature under a nitrogen atmosphere. Typical samples applied to gels contained 100 μg of membrane protein. Gels were stained for protein using 0.025% Coomassie Blue in 7% acetic acid or 0.05% Coomassie Blue–10% methanol–7% acetic acid and destained with 7% acetic acid in a Hoefer destainer. Carbohydrate bands were visualized by a modification of the peridate–Schiff (PAS) method of Zacharias *et al.* (96) in which gels were (a) placed in a destainer containing 7% acetic acid–40% methanol overnight (b) reacted with 1% periodate–7% acetic acid in the dark for 2 hr (c) washed in a destainer containing 7% acetic acid overnight (d) stained with Schiff reagent for 2 hr in the dark and (e) finally fixed in 0.10 N HCl–1% metabisulfite solution (97). All steps were performed at room temperature. Both

Coomassie Blue and PAS stained gels were scanned at 550 nm with a Gilford 2000 spectrophotometer equipped with a Model 2410 linear transport accessory.

The standard proteins which were used for estimation of molecular weights are given below with the molecular weight value used: myosin, 212,000 (98), β-galactosidase, 130,00 (99), bovine serum albumin, 66,500 (100), catalase, 60,000 (99), ovalbumin, 43,000 (99), lactic dehydrogenase, 35,000 (98), chmotrypsinogen, 25,700 (99) and cytochrome c, 11,700 (99).

Acetic Andydride Reaction with Erythrocytes and Ghosts

Red blood cells were washed 4 times in 310 imOsm phosphate (pH 7.4) and suspended to a hematocrit of 20. Aliquots of acetic anhydride (5 mM to 1 M final anhydride concentration) were added and reaction was allowed to proceed for 15 min at room temperature. Reactions were quenched with ice cold isotonic phosphate or saline, and the cells were centrifuged. Aliquots were taken for hemoglobin determination and, in some cases, for pH measurement. Cells were then washed several times before microscopic examination. Ghosts were prepared from washed, untreated red cells by the method of Dodge *et al.* (88) and washed with 310 imOsm phosphate. An aliquot containing a number of ghosts approximately equivalent to the number of cells in a 20% suspension was reacted with acetic anhydride as in the case of the red cells. Samples of the reacted ghost suspension were taken after washing for lipid and protein analysis and for microscopic examination.

Radioactive Anhydride Labeling of Erythrocytes and Ghosts

A 20% suspension of erythrocytes (washed 5 times) in isotonic phosphate at pH 7.4 was reacted with 10 mM acetic anhydride (^{14}C- or ^{3}H-labeled) for 15 min at ambient temperature. The cells were washed with cold isotonic saline to remove unbound label and hemolyzed as described. Control samples were run with unlabeled acetic anhydride for analytical comparisons. The hemoglobin free membranes were analyzed for radioactivity incorporated, protein and cholesterol. The initial labeling and analyses of ghosts were performed in a similar manner. Paired samples of ghosts and washed red cells prepared from the same unit of blood were reacted in each case. Washing was performed in a manner such that differences between procedures for isolating labeled membranes prepared by labeling ghosts or labeling red cells were minimized. Radioactive samples were analyzed by scintillation counting using Bray's solution (101) or toluene-ethanol-PPO-POPOP (1200:800:8:0.4, v:v:w:w) solution. When necessary for comparison, samples were corrected for quenching and instrument efficiency by either the channel ratios method using sets of quenched standards (^{14}C and ^{3}H) available from Amersham/Searle or by external standarization (102). Samples of labeled membranes of each type were analyzed by electrophoresis and compared to non-acetylated controls.

In the double label experiments, samples of labeled membranes from either red cells or ghosts were solubilized in 310 imOsm phosphate containing 1% SDS and reacted with 20 mM acetic anhydride (^{3}H- or ^{14}C-labeled). The samples were dialyzed against three changes of cold water, lyophilized and prepared for electrophoresis.

Analysis of Labeled Lipids from Double Labeled Erythrocytes and Ghosts

Lipid extracts were prepared as previously described by the procedure of Ways and Hanahan (91) from lyophilized ghosts after the double labeling with acetic anhydride beginning with either the ghost or the intact erythrocyte as described above. The extracts were separated into component lipids by the thin-layer chromatographic method of Nelson (103) and Fosslien and Musil (104). Plates were spotted with the membrane lipid extracts and ^{14}C-acetylated samples of phosphatidylethanolamine and phosphatidylserine. The acetylated authentic samples were prepared by incubating a 0.10 M phospholipid-0.20 M triethylamine-50% ethanol-0.10 M ^{14}C-acetic anhydride mixture at room temperature. The degree of reaction was found to be approximately 90% as judged by ninhydrin analysis. The plates were developed in chloroform, methanol, ammonia (62:25:5, v:v:v) and radioautographed. Thus the acetylated membrane phosphatidylethanolamine and phosphatidylserine derivatives were located by comparison to authentic synthesized samples. The appropriate areas on the TLC plates were removed by scraping and counted.

Exchange of ^{14}C-Cholesterol into Red Cell Membranes

The replacement of membrane cholesterol with ^{14}C-cholesterol of both intact erythrocytes and ghosts was accomplished by the procedure of Bruckdorfer *et al.* (75) (76). A dispersion of ^{14}C-cholesterol and lecithin (1:2.4 by weight) was incubated 18 hr at 37° with the cells or ghosts in isotonic 310 imOsm Tris buffer (pH 7.4) containing penicillin G and streptomycin sulfate. After several washed with isotonic Tris to

remove excess cholesterol, the cells were hemolyzed in 20 imOsm phosphate pH 7.4 as usual. Both the labeled membranes derived from intact cells and those of the ghosts were subjected to electrophoresis as described. Gel slicing and counting is described below.

Diazotized Sulfanilic Acid Labeling of Human and Bovine Erythrocytes

Human and bovine erythrocytes were treated with diazotized sulfanilic acid according to a modification of the procedure of Berg (3). The diazonium salt of ^{35}S-sulfanilic acid was prepared by incubating 10 mg of ^{35}S-sulfanilic acid with 5 mg of sodium nitrite in 1.3 M HCl for 30 min in ice. Excess nitrite was destroyed by addition of 10 mg urea (105). The salt was then diluted to the desired concentration with 310 imOsm phosphate at room temperature. Aliquots of the diazotized ^{35}S-sulfanilic acid were immediately added to intact erythrocytes at a hematocrit of 20 to give a diazotized ^{35}S-sulfanilic acid concentration of 0.3 mM. Equivalent concentrations of reagent and membranes were used for experiments with ghosts. Incubation was performed for 20 min at room temperature. The reaction was quenched by washing with cold 310 imOsm phosphate buffer. Membranes were prepared from the reacted erythrocytes in the usual manner.

Fractionation of Labeled Ghosts

All preparations of either ^{14}C, ^{3}H or ^{35}S-labeled erythrocyte membranes were lyophilized, dissolved in SDS-mercaptoethanol and subjected to electrophoresis as previously described on either 6 or 10 cm acrylamide gels. After electrophoresis the gels were immediately sliced with

a Canalco linear gel slicer for the anhydride and cholesterol experiments. For the DSA experiments a tubular gel slicer (106) was used and found to be far more efficient. The gel slices were extracted and counted in NCS (107) using the toluene-ethanol based solution mentioned above.

In addition fractionation of double labeled membrane samples was also achieved by solubilization in 3% SDS and chromatography on a Sepharose 4B column using 1% SDS-0.05 M phosphate-0.02% sodium azide (pH 7.0) as eluting buffer (4). Column eluents were monitored by scintillation counting of both ^{3}H and ^{14}C in Bray's solution (101).

Trypsin and Neuraminidase Treatment of Erythrocytes

Washed erythrocytes were suspended to a hematocrit of 20 in 310 imOsm phosphate buffer. Trypsin in 0.155 M NaCl, pH 3.0 was added to a final trypsin concentration of 1 mg/ml. Digestion was allowed to proceed for 1 hr at room temperature.

Trypsin inhibitor was added (final concentration 3 mg/ml) and incubation continued for an additional 30 min. The mixture was then diluted with cold 310 imOsm phosphate (pH 7.4) and washed four additional times. Membranes were prepared as usual.

Samples of washed erythrocytes suspended to a hematocrit of 20 in 310 imOsm phosphate (pH 6.0) were incubated with Clostridium perfringens neuraminidase (final concentration 15 μg/ml) according to the procedure of Eylar et al. (68). Digestion was permitted to occur for 1 hr at 37°. The mixture was cooled in a salted ice bath. The samples were centrifuged and supernatants were removed. Separate samples of ghosts were

hydrolyzed for 1.0 hr at 80° in 0.1 N H_2SO_4. Supernatant samples and hydrolysates were assayed for free sialic acid by the procedure of Warren (108). Ghosts were prepared from the erythrocytes by the method of Dodge et al. (89), and both treated and control (no neuraminidase) samples were subjected to electrophoresis as described above.

CHAPTER III

RESULTS

Membrane Preparation

The variations noted in reports from different laboratories on the protein distributions of erythrocyte membranes could arise from differences in either the preparative techniques or the analytical methods. Therefore a comparison was made of the responses of erythrocyte membranes from several different species to variations in the membrane isolation conditions. Three different isolation methods were used, the phosphate procedure of Dodge et al. (88), the Tris-EDTA method of Marchesi and Palade (90) and the Ca^{2+}-veronal method of Burger et al. (89). Membranes were examined by phase contrast microscopy for morphological alterations. Table I shows the results of these studies. Examination of several samples of blood from each of the different species allows some general conclusions to be made concerning the behavior of their membranes under different isolation conditions. Human erythrocyte membranes appeared to be the most stable under all isolation conditions. Cow erythrocyte membranes tend to crenate severely in the phosphate preparation. Dog and cat membranes are particularly susceptible to fragmentation in Tris. All membranes tend to fragment or crenate during prolonged or repeated washing with hypotonic buffers. Extensive washing to remove traces of hemoglobin is consequently undesirable if an intact membrane is to be retained.

TABLE I

VARIATION OF MEMBRANE MORPHOLOGY WITH PREPARATION TECHNIQUE

Species	Preparation Method	Morphology
Human	Phosphate	Mostly biconcave
Dog	Phosphate	Some biconcave, some round
Cat	Phosphate	Round
Cow	Phosphate	Round, badly crenated
Pig	Phosphate	Round, badly crenated or fragmented
Sheep	Phosphate	Round
Human	Tris	Mostly round, some stromalytic forms
Dog	Tris	Round, many fragments
Cat	Tris	Very fragmented
Cow	Tris	Round with some spikes
Pig	Tris	Very fragmented
Human	Ca^{++}-veronal	Mostly round, some biconcave
Dog	Ca^{++}-veronal	Round
Cat	Ca^{++}-veronal	Round
Cow	Ca^{++}-veronal	Round, somewhat crenated
Pig	Ca^{++}-veronal	Round
Sheep	Ca^{++}-veronal	Round and crenated

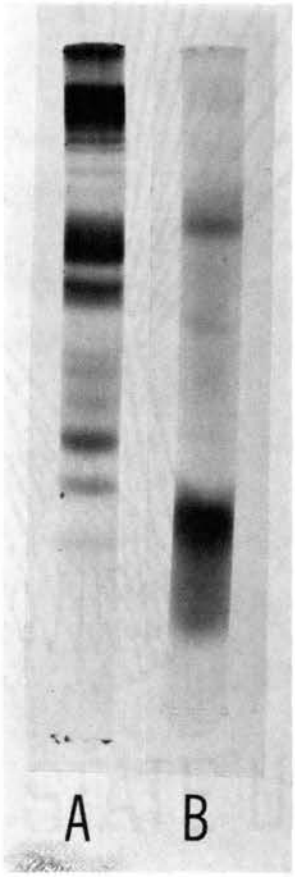
Polyacrylamide Gel Electrophoresis

In order to pursue the examination of erythrocyte and other membrane proteins, a method was sought which would effect fractionation of the membrane components in a single step. The technique of acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), as presented in this study, was developed simultaneously with other inves-

tigators of membrane systems (4) (5) (20) (23). The method was chosen because of its simplicity, speed and resolving power plus its potential for one step fractionation of membranes. To develop the basic technique, observations were made on the electrophoretic separations of membrane preparations from a number of samples of human erythrocyte membranes to try to optimize conditions of gel concentration, length of electrophoresis run and staining procedures. A typical example of the protein pattern observed by electrophoresis of human red cell membranes in the SDS-containing acrylamide gel is shown by gel A in Figure 1. At least 12 major protein bands can be counted from the gels or their photographs. The molecular weight range of the membrane proteins is quite large, varying from 25,000 to 190,000 daltons. The large size of some of the protein species suggested the possibility of aggregated polypeptide chains which had not been dissociated by SDS solubilization. In an attempt to disaggregate these larger species of proteins, the lyophilized ghosts were solubilized in an SDS solution which contained 8 M urea and were then electrophoresed in SDS acrylamide gels which also contained 8 M urea. It was found that there was no significant breakdown of the higher molecular weight proteins in favor of smaller species.

Since carbohydrate is a significant component of the human red cell membrane, the acrylamide gels were stained by a modified periodate-Schiff procedure (101) (102) in an effort to determine the carbohydrate distribution among the various protein or lipid species. The staining pattern for a typical gel is shown in Figure 1 as gel B. The pattern is surprisingly simple when compared to the protein distribution. There are only two major bands. The most noteworthy feature of the carbohy-

Figure 1. Staining Pattern Observed After Acrylamide Gel Electrophoresis of Human Erythrocyte Ghosts in 0.1% SDS–0.1 M Phosphate. Gel A was stained with Coomassie Blue. Gel B was stained by periodate–Schiff procedure.

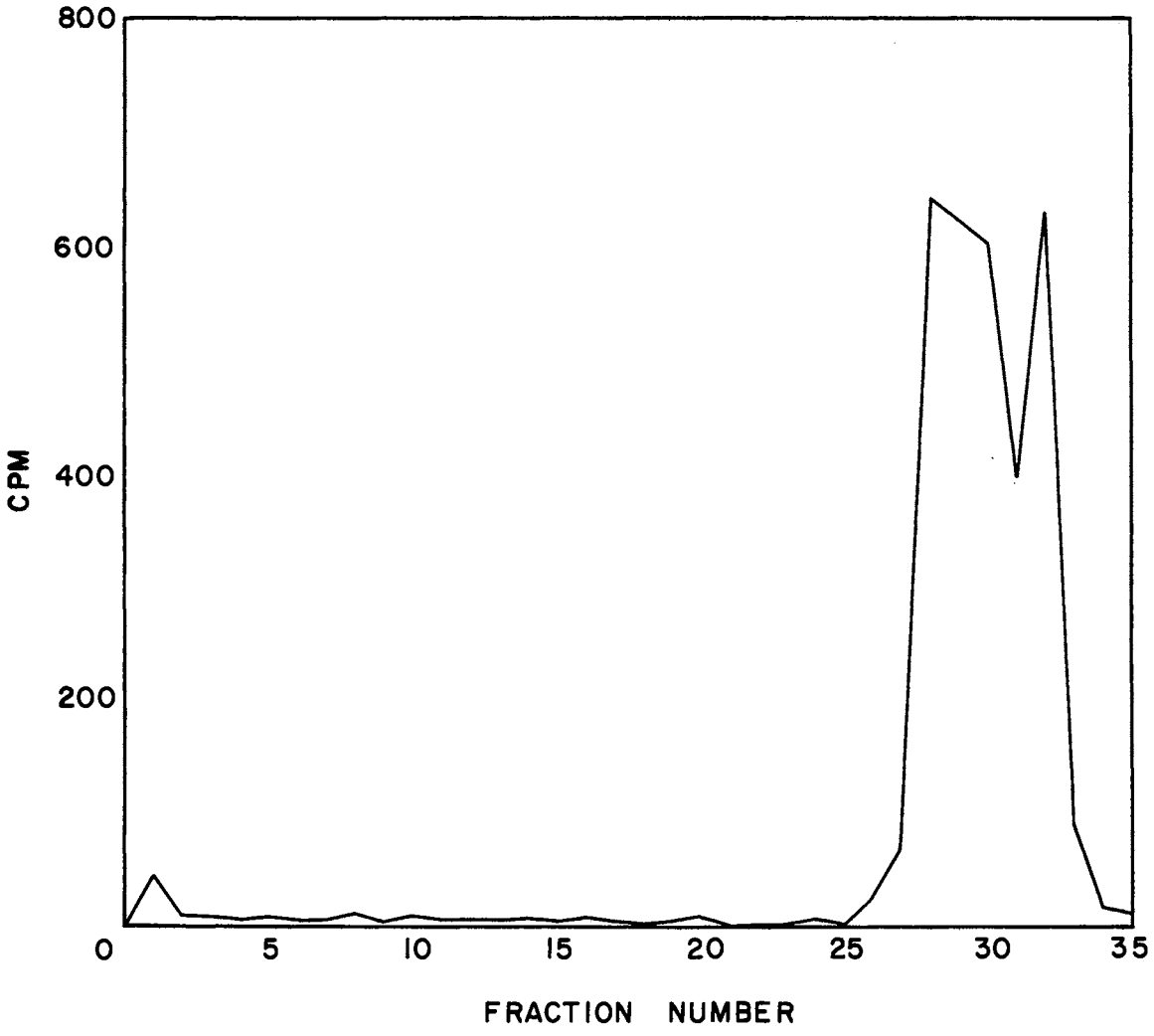

A
B

drate pattern is the fast-moving major band. Since it does not correspond to any protein band and it runs ahead of all protein species, it was presumed to be an SDS-lipid-glycolipid complex.

To establish that the fast-running carbohydrate staining band contains the lipid from the cell membrane, labeled cholesterol was exchanged into washed erythrocytes by the procedure of Bruckdorfer et al. (75). The cell membranes were isolated and subjected to electrophoresis as previously described. Figure 2 shows that the radioactivity profile obtained by slicing and counting the gel corresponds exactly to the fast-running band stained by the periodate-Schiff method. Lenard (4) has also shown that the extraction of the lipids of human erythrocyte membranes by the butanol procedure removes this fast-running carbohydrate band. Separation of protein and lipid fractions (as determined by gel electrophoresis and chemical analysis) can also be achieved by chromatography in SDS solutions on BioGel P-100 (19). Thus, these experiments strongly indicate that this fast-running band contains a mixed micelle of SDS, glycolipid and other lipid species.

Several aspects of this electrophoretic technique that have been noted during the comparative studies should be mentioned. The SDS electrophoretic method is quite versatile in its application and can be used under a variety of conditions. In some of the experiments to be described, the SDS concentration in the gel and in the electrophoresis buffer has been varied from 0.1% to 1%. The electrophoretic patterns are essentially the same under these conditions if sufficient SDS is used to solubilize the samples. Samples dissolved in 2-4% SDS can be placed directly on gels containing either 0.1% to 1% SDS without significant changes in the resultant patterns. Two advantages arise from the

Figure 2. Localization of Cholesterol on SDS Acrylamide Gel. ^{14}C-cholesterol was exchanged into human erythrocytes from a lipid dispersion. Membranes were isolated and subjected to electrophoresis as described. Gels were sliced, extracted with NCS solubilizer and counted.

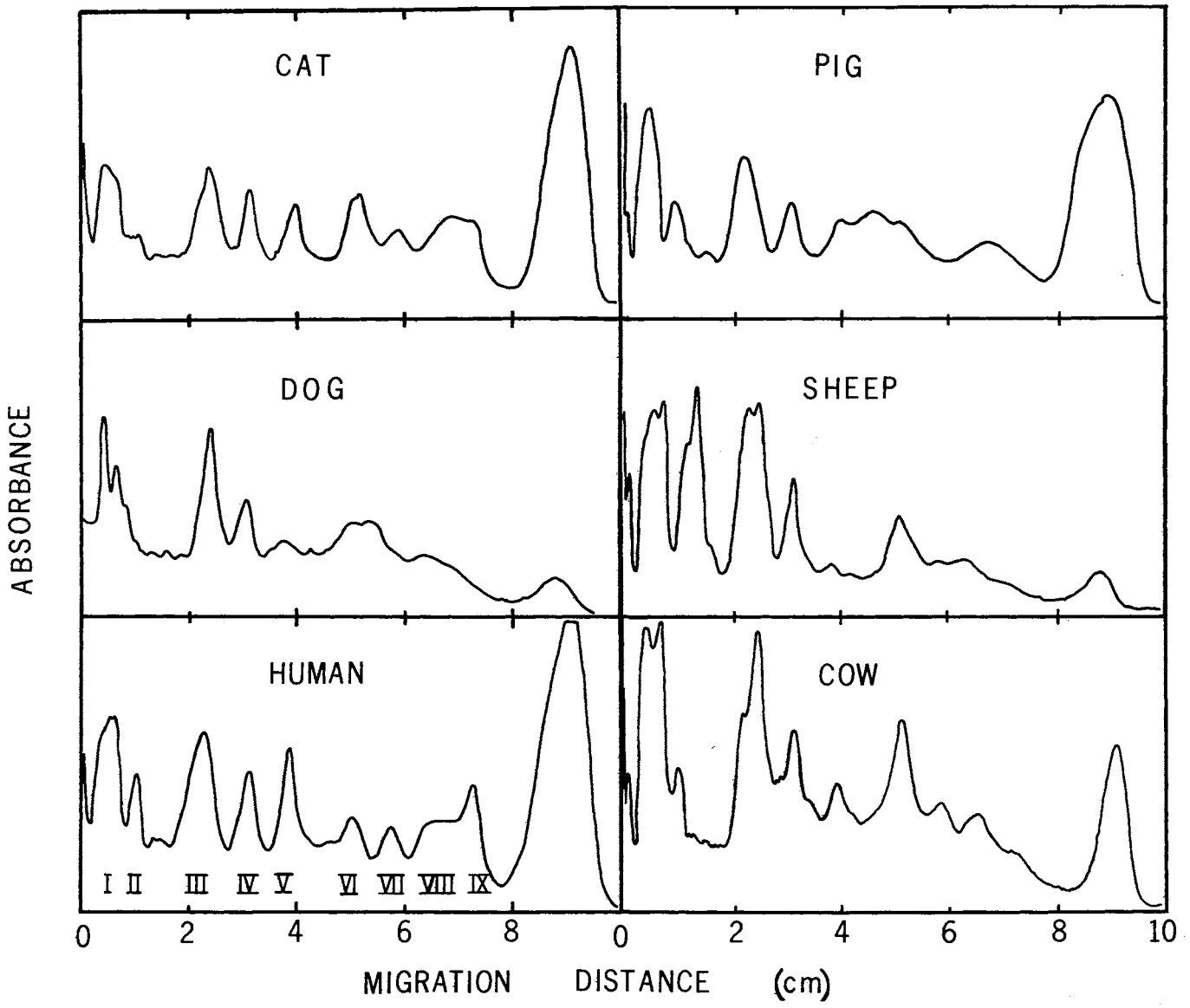

800
600
400
200
0
CPM
5
10
15
20
25
30
35
FRACTION NUMBER

use of higher SDS concentrations in sample preparation. Solubilization and disaggregation are more nearly complete, and protease activity is inhibited (23). Dialysis of the solubilized samples to remove excess SDS is therefore unnecessary and in fact undesirable, since it can result in reaggregation or proteolysis. Heating the samples to disaggregate (4) is not necessary, but does aid in destroying any protease activity present in the sample. If heating of a sample was employed to aid solubilization, fresh mercaptoethanol (1%) was added to prevent disulfide formation, even if the sample had been previously reduced. The use of 0.1% SDS in gels is preferred because of the enhanced resolving power and the facilitation of staining and destaining. Destaining in the presence of methanol hastens the removal of SDS from the gel. Failure to remove the SDS retards the uptake of stain by proteins even if they are fixed within the gel. Electrophoretic analyses were also performed using the complete protocol of Lenard (4). No significant variations from the author's results were noted in the number or distribution of bands.

Protein Comparisons

The SDS electrophoretic procedure was used to analyze the proteins from erythrocyte membranes prepared by three different methods. Figure 3 shows the patterns obtained by scanning the gels from membranes prepared by the Ca^{2+}-veronal procedure of Burger *et al.* (89). The major bands for each species are almost identical to those for the other species. Species specific bands do appear for the sheep (126,000 daltons), dog (45,000 daltons) and pig (57,000 daltons). The bands in the gels have been designated as nine separate band areas (I-IX), which can

Figure 3. Gel Scans of Coomassie Blue Stained Polyacrylamide Gels of Erythrocyte Membranes Prepared by the Veronal-Ca^{2++} Procedure (89). Approximately 100 μg of protein was applied to each 6% acrylamide gel in 3% SDS (solubilized from lyophilized materials).


CAT
PIG
DOG
SHEEP
HUMAN
COW
ABSORBANCE
I II III IV V VI VII VIII IX
0 2 4 6 8 0 2 4 6 8 10
MIGRATION DISTANCE (cm)

be identified for each species. The band at the far right on each gel scan results from residual hemoglobin in the membrane preparation. Comparable gel scans are shown in Figures 4 and 5 for the membranes prepared by the phosphate (88) and Tris (90) procedures, respectively. Although the bands have not been as clearly resolved due to shorter electrophoresis time, the band areas can be assigned as in Figure 3 by comparing molecular weights of the bands in the three different scans. Some differences can be noted in the protein patterns for membranes prepared by different methods. For example, the specific band (57,000 daltons) of pig membranes shows a greater dominance over the other bands in the phosphate and Tris preparations. Demus and Mehl (11) previously noted a predominant band in pig membranes which probably corresponds to this band. Other examples of variations in relative band intensities can be noted for the different preparations, but the most striking observation occurs with dog membranes prepared in Tris-EDTA. In this case the protein profile has been completely altered. As further evidence of the similarities between erythrocyte membrane proteins, the amino acid compositions (109) of membranes prepared in phosphate and Tris are compared in Table II. Ghosts prepared by the Ca^{++}-veronal procedure were not included due to their high hemoglobin content. The similarities between the different species are evident, even for Tris-EDTA dog ghosts, which show a grossly different gel scan profile. Variations between species are no more pronounced than variations between the two types of preparations. The results suggest that alterations in morphological integrity caused by different preparative procedures are not due to changes in major protein components of the membranes, but are more likely a result of differences in lipid components,

Figure 4. Gel Scans of Coomassie Blue Stained Polyacrylamide Gels of Erythrocyte Membranes Prepared by the Phosphate Procedure (88). Electrophoresis conditions are the same as in Figure 3 except for a shortened time of electrophoresis.

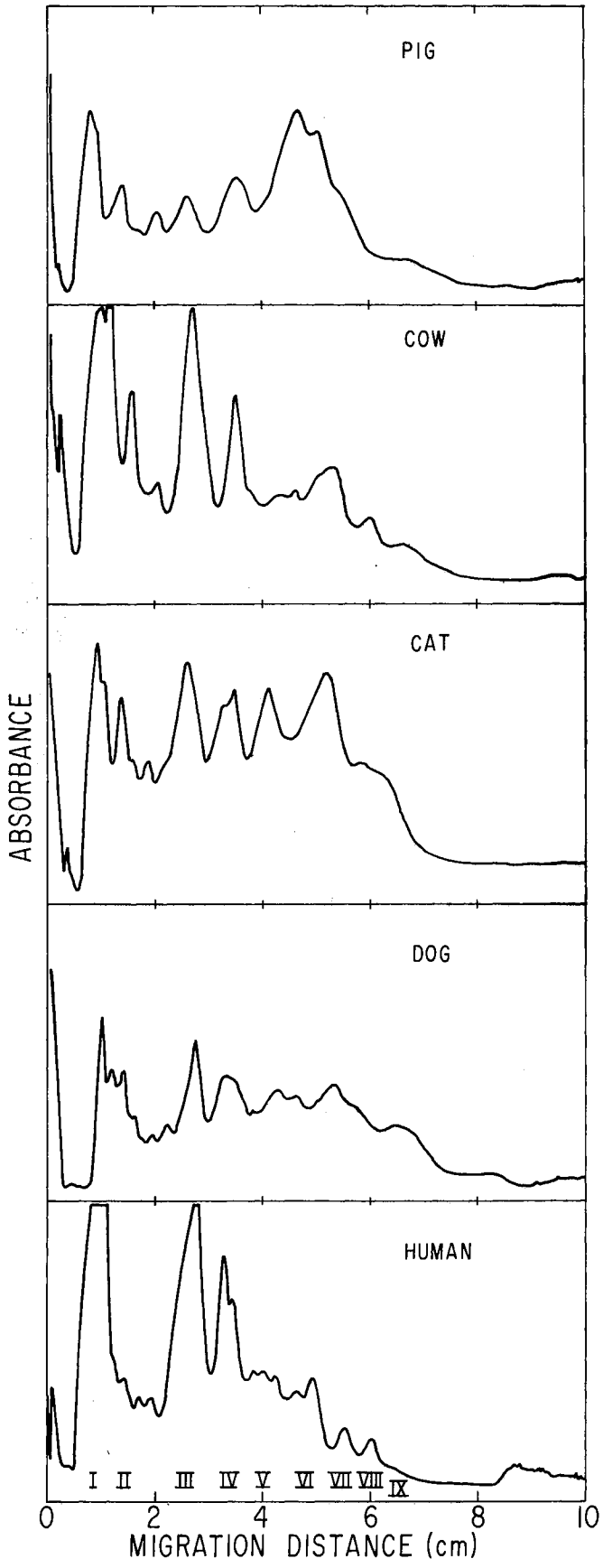
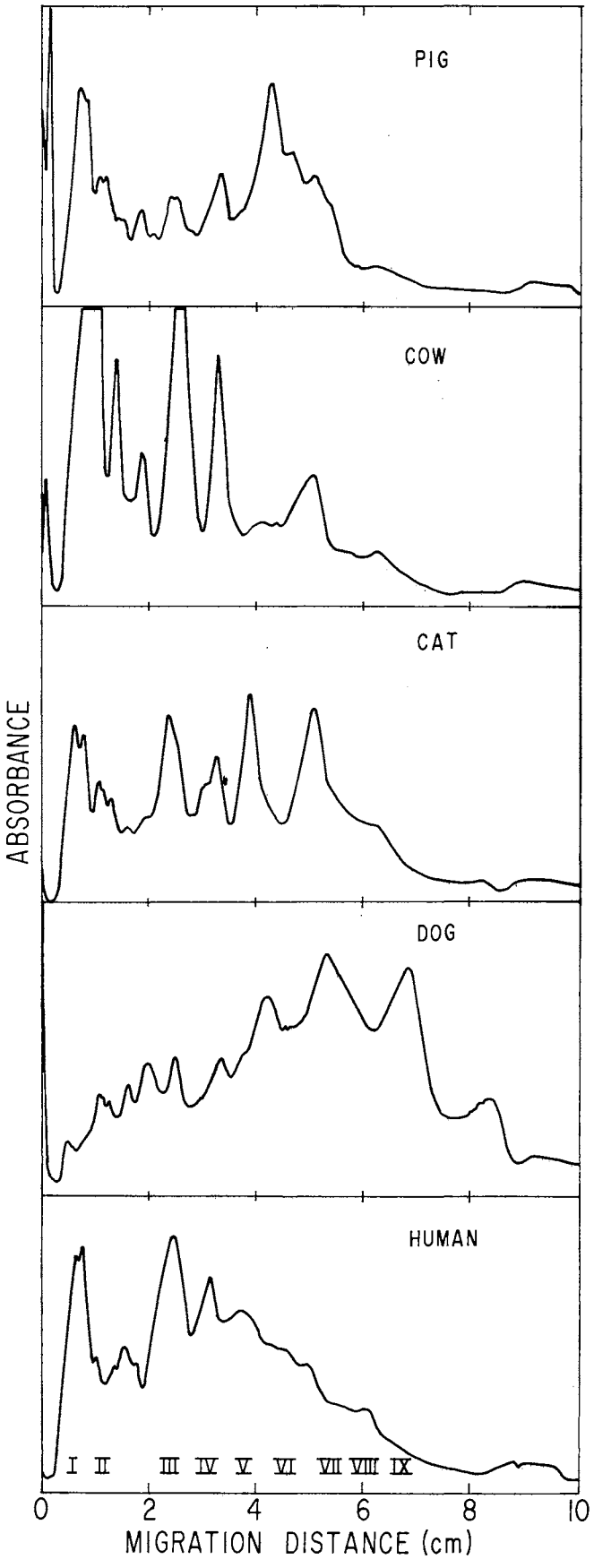

PIG
COW
CAT
DOG
HUMAN
ABSORBANCE
I II III IV V VI VII VIII IX
0
2
4
6
8
10
MIGRATION DISTANCE (cm)

Figure 5. Gel Scans for Membranes Prepared by the Tris-EDTA Procedure (90). Electrophoresis conditions equivalent to those of Figure 4.


PIG
COW
CAT
DOG
HUMAN
ABSORBANCE
I II III IV V VI VII VIII IX
0 2 4 6 8 10
MIGRATION DISTANCE (cm)

in functional activities that are not represented by the major polypeptide distribution, or in particular properties of the individual proteins.

TABLE II

AMINO ACID ANALYSES OF ERYTHROCYTE MEMBRANES

Mole Percent*

Amino Acid	Phosphate Preparation					Tris-EDTA Preparation				
	Human	Dog	Cat	Cow	Pig	Human	Dog	Cat	Cow	Pig
Asp	9.4	9.0	8.6	9.5	9.2	8.2	9.3	8.5	8.6	8.6
Thr	5.7	5.1	5.2	6.1	5.5	5.4	5.0	4.7	5.6	5.2
Ser	7.9	8.2	8.5	8.5	8.7	8.0	8.0	8.2	8.1	8.6
Glu	12.8	13.3	13.2	14.1	14.5	14.2	13.5	13.9	14.7	13.9
Pro	5.2	5.3	6.0	6.0	5.6	4.6	5.6	5.8	5.9	5.8
Gly	6.3	6.8	7.0	6.4	6.4	6.9	6.8	6.7	6.4	6.6
Ala	8.8	8.0	8.0	7.8	8.1	8.2	8.5	7.9	7.6	8.1
Val	6.4	6.1	5.6	6.0	5.7	6.9	6.7	6.9	6.0	5.9
Met	2.0	1.6	1.8	1.7	1.7	1.9	1.6	1.4	1.7	1.6
iLeu	4.5	4.5	5.2	4.6	4.4	4.9	4.2	4.4	4.9	5.2
Leu	12.4	13.7	12.8	12.8	12.6	13.1	12.8	12.9	12.1	12.3
Try	2.3	2.4	2.1	2.2	2.1	2.0	2.4	2.1	2.1	2.1
Phe	4.0	4.0	4.3	3.7	3.7	4.2	3.5	4.2	3.7	3.7
Lys	5.0	5.0	5.1	4.8	5.2	4.6	4.9	5.1	5.0	5.3
His	2.2	2.2	2.1	1.9	2.1	2.2	2.4	2.8	2.3	2.3
Arg	5.1	4.7	4.6	4.9	4.8	5.1	4.9	4.9	5.2	4.9

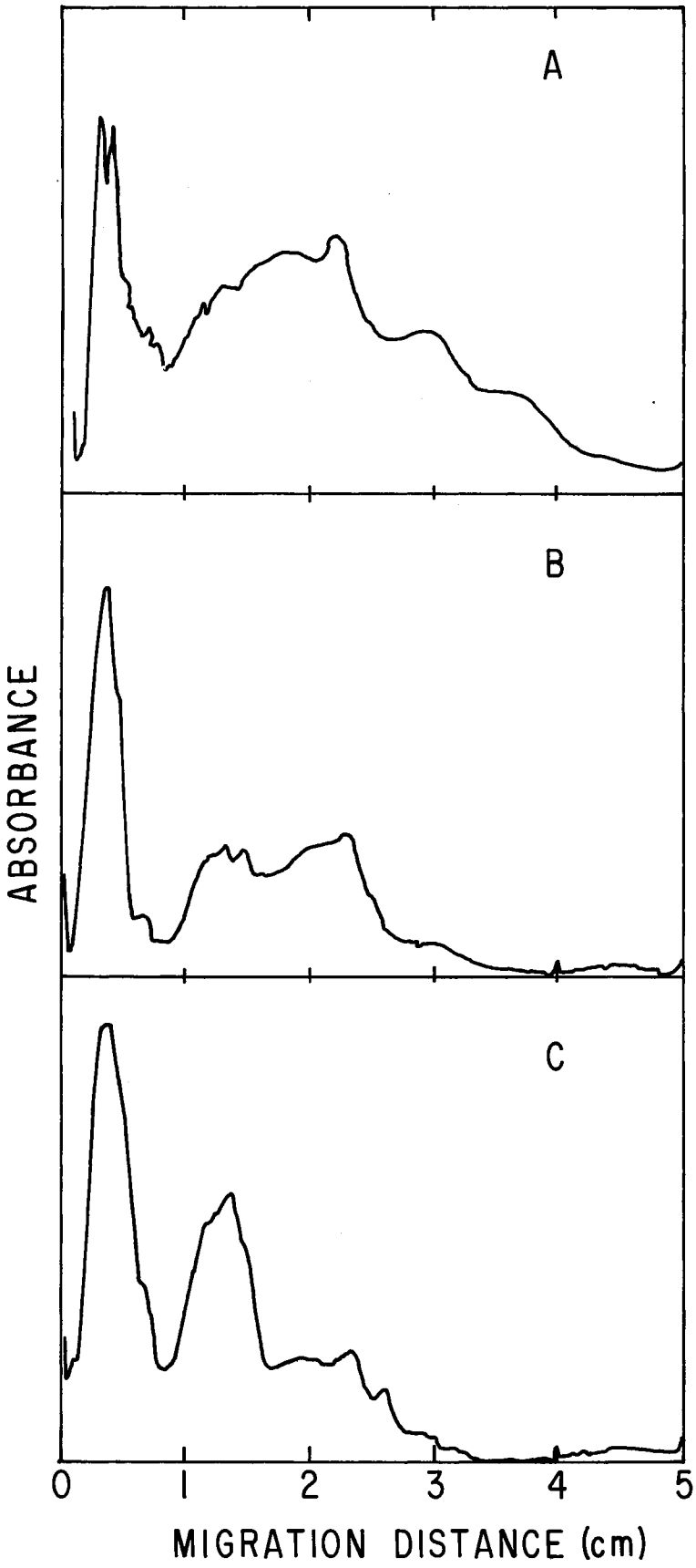
*Values are expressed as mole percent of amino acids. Tryptophan and half-cystine were not determined.

Two possibilities suggest themselves to explain the gross alteration of the Tris-EDTA dog membrane protein patterns, the loss of membrane proteins during hemolysis and washing and the degradation of

membrane proteins due to proteolysis. Several observations indicate that the latter explanation is more likely correct. 1) The altered profile shows the loss of higher molecular weight components and the appearance of new bands at intermediate and lower molecular weights. These observations would not be possible without a decrease in the yield of membrane materials, which was not observed. 2) The changes in the protein pattern are similar to those observed for intact membranes treated with low concentrations of proteolytic enzymes (12). 3) Membrane samples which are dissolved by heating in SDS still show significant amounts of band I (spectrin) of the unmodified preparation, but samples which are dissolved at room temperature in SDS show an absence of this band, as in Figure 5. This suggests the presence of a protease which is still active in the solubilizing medium. 4) No significant changes were noted in the amino acid composition of the altered membranes when compared to membranes of other species or membranes of the same species which have normal electrophoretic patterns. Complete removal of the larger polypeptide chains would be more likely to alter composition than proteolysis.

The source of the proteolytic activity must be considered if it is to be eliminated as a problem in membrane preparations. Fairbanks et al. (23) have suggested that proteases may arise from white blood cell contamination. To examine this hypothesis, human erythrocyte ghosts were prepared under three sets of conditions, described in Figure 6. Equivalent amounts of membranes were dissolved in 2% SDS and subjected to electrophoresis. The gels were scanned to give the results shown in Figure 6. Differences can clearly be seen between the membranes which were not contaminated with leukocytes (Figure 6C) and those which were

Figure 6. Effects of Retention of White Blood Cells on Erythrocyte Membrane Proteins. A, membranes prepared from cells which were not freed of white cells by removal of buffy coat. B, membranes prepared from cells washed to remove buffy coat. No precautions taken to remove white cells that remained with membrane preparation. C, membranes prepared from cells washed to remove buffy coat. Tight cell pellet removed from each membrane washing step to eliminate residual white cell contamination (23). Electrophoresis performed on 6 cm, 5% acrylamide gels.


A
B
C
ABSORBANCE
0
1
2
3
4
5
MIGRATION DISTANCE (cm)

(Figures 6A and 6B), even though the samples were run on 6 cm gels and do not show highly resolved bands. The leukocyte contaminated samples show a decreased amount of high molecular weight components (I-IV), a shift of the pattern toward lower molecular weights and a decreased resolution. These are phenomena similar to those observed with dog cell membranes prepared by the Tris-EDTA method, although the effects do not appear as severe since the membranes were not fragmented by the preparation technique.

Further confirmation of the effects of white cell proteases on erythrocyte membranes was obtained by the incubation of small aliquots of the white cell isolated from red cell preparations with intact erythrocytes. The effects were similar to those shown in Figure 6. It was also shown that the addition of low concentrations (0.1%) of SDS enhanced the extent of membrane protein degradation. These experiments clearly show that the leukocyte proteases are a source of the problem of membrane protein degradation.

No conclusive evidence has been presented to show that the bands on SDS electrophoresis are discrete polypeptide chains rather than aggregates of smaller chains. In fact, several groups have reported disaggregation of the larger chains of the membrane polypeptides into smaller components but in each case the results appear to derive from proteolysis (3) (72) and from misinterpretation of labeling (110) or staining (16) (18) patterns or combinations of these problems. Experiments which report complete disaggregation by heating or incubation with denaturing agents before SDS electrophoresis (4) (23) are suspect because the denaturants were removed before electrophoresis, thus allowing the opportunity for recombination of polypeptide chains. Experiments by the

author have shown that electrophoresis in the presence of 8 M urea (in both sample and gel) does not indicate any further disaggregation of the major polypeptide chains. Additional evidence for the components' monomeric nature is found by comparing the electrophoresis results with molecular weights obtained by chromatography in 6 M guanidine hydrochloride (15). The distribution of molecular weights is very similar (Table III) when the lower resolving power of the column chromatography method and the fact that band III is not extracted by guanidine hydrochloride (23) are considered.

TABLE III

MOLECULAR WEIGHT OF ERYTHROCYTE MEMBRANE PROTEINS

Component Band	Mol wt x 10^{-3}	
	SDS Electrophoresis[a]	Guanidine Chromotography[b]
I	225	
II	160	192
III	112	
IV	86	
V	69	74
VI	49	
VII	40	47
VIII	31	29
IX	26	

[a]Membrane weights of components I-III determined on 5% gels, IV-IX on 6% gels.

[b]Reported by Gwynne and Tanford (15).

Glycoproteins of the Membrane

The glycoproteins of the erythrocyte membrane can also be studied by polyacrylamide gel electrophoresis, but the conditions for proper detection and identification are more critical than for the proteins. Failure to remove SDS from the gels before staining by the periodate-Schiff (PAS) procedure can result in anomalous patterns. Figure 7 represents the carbohydrate staining profile of gels of erythrocyte membranes from 5 different species on 5% gels which were washed overnight in 15% acetic acid to remove SDS before staining (32). Each of these shows a single major glycoprotein band with no component corresponding to 108,000 daltons as reported by Lenard (20). The diffuse trailing edge of the bands is a characteristic of this staining procedure. It is not always reproduced and does not appear to represent a second component except in the case of the horse, where two closely spaced components are routinely observed. The diffuse nature of the band may represent heterogeneity of the glycoprotein molecules and their incomplete interaction with SDS. If SDS is not removed from the gels and they are fixed and stained by the procedure of Zacharias *et al.* (101), all bands stain initially, but extensive washing removes most of the stain until band III appears most heavily stained. Examples of this behavior are shown in Figure 8 for sheep and human membranes. The second major band in the human profile (at 3 cm migration distance) is the true glycoprotein as shown by trypsin treatment of the red cells before membrane isolation and electrophoresis. In fact, the glycoprotein does not correspond to any of the Coomassie Blue bands (12) (23).

An interesting anomaly of the carbohydrate bands was noted in comparing electrophoretic runs made on gels with varying percentages of

Figure 7. Carbohydrate Staining Profiles of SDS Acrylamide Electrophoretic Gels of Erythrocyte Membranes of Various Species. Gels (10 cm) were washed 24 hr with 15% acetic acid at room temperature before staining by PAS procedure.

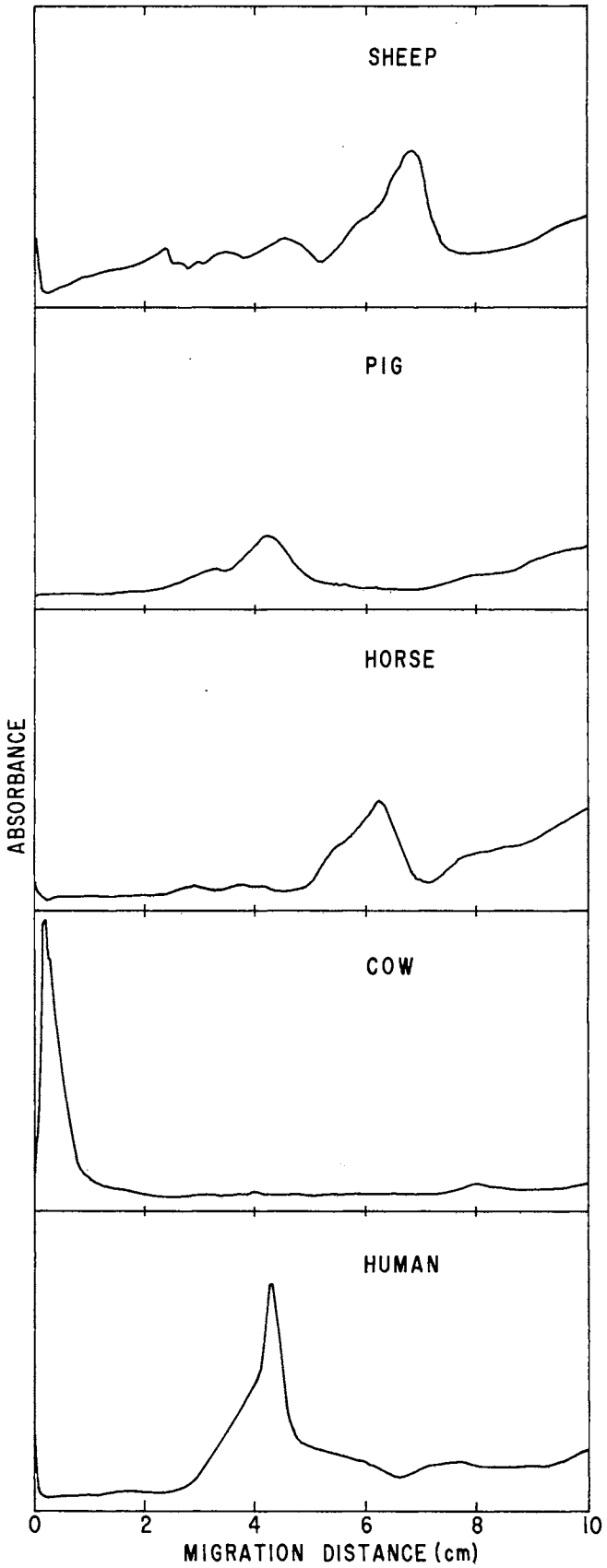
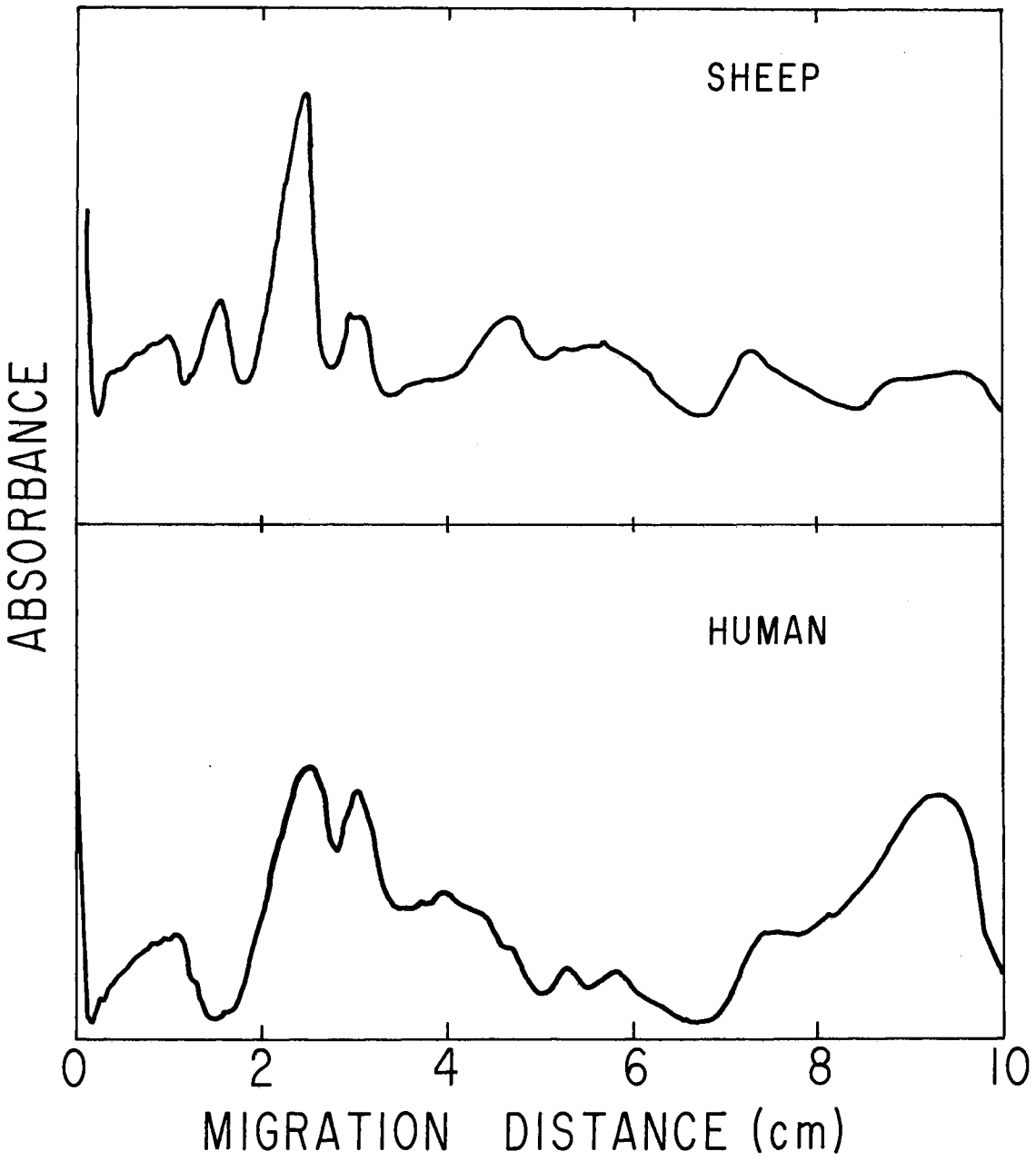

SHEEP
PIG
HORSE
COW
HUMAN
ABSORBANCE
0
2
4
6
8
10
MIGRATION DISTANCE (cm)

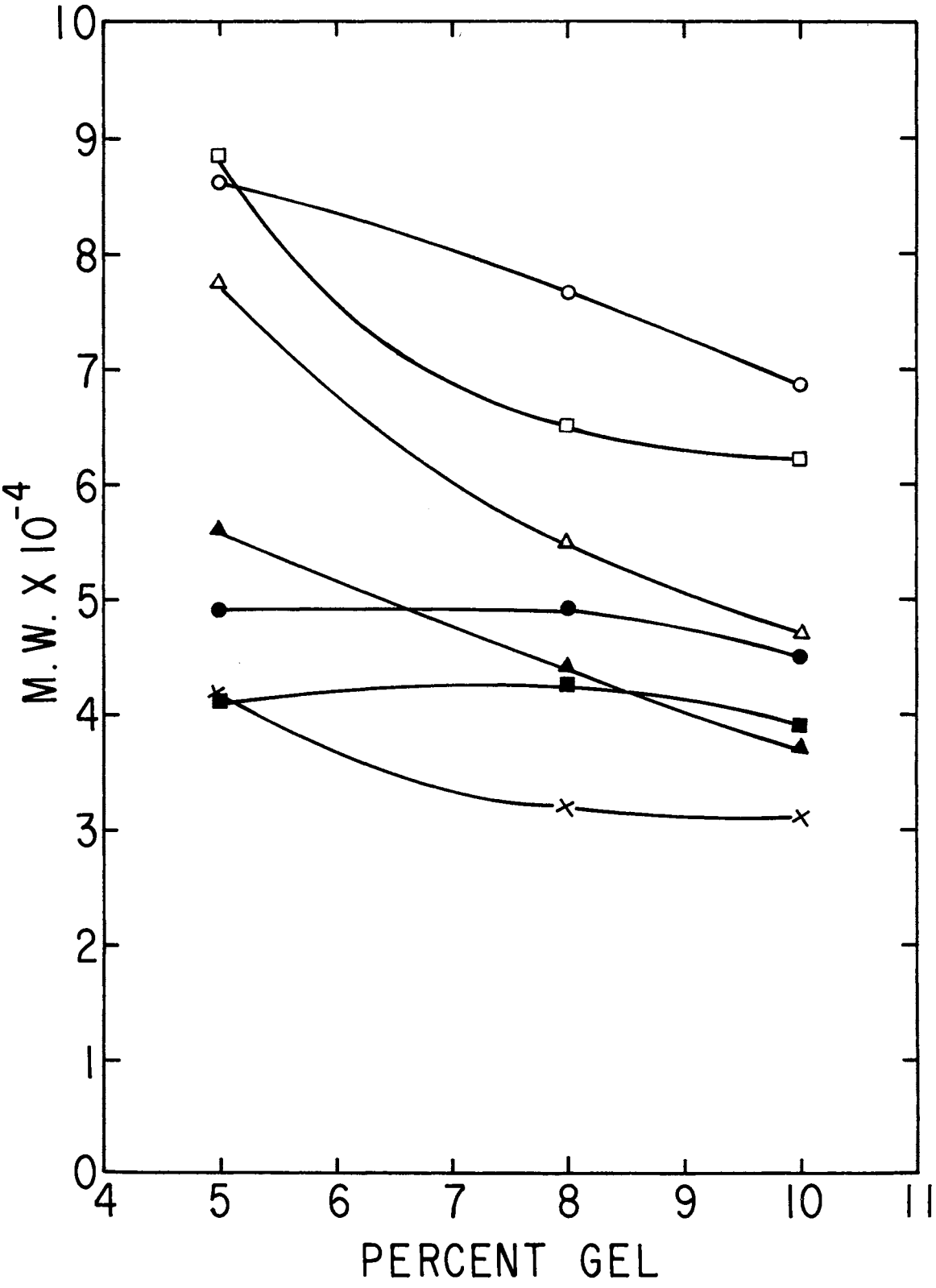
Figure 8. PAS Staining Profiles of Gels Which Were Not Washed to Remove SDS. Gels were fixed and stained according to procedure of Zacharias *et al.* (101).


SHEEP
HUMAN
ABSORBANCE
0
2
4
6
8
10
MIGRATION DISTANCE (cm)

acrylamide. The molecular weight values calculated from SDS electrophoresis for the glycopeptides in 5% gels were as follows: bovine, 285,000; human, 89,000; pig, 78,000; horse, 56,000; and sheep, 42,000 daltons. However, these do not represent the true molecular weight values for these proteins, since it was shown that the calculated molecular weights depended upon the percentage of acrylamide in the gels used. Figure 9 shows the effect of variation in gel composition on the calculated molecular weights for the glycoproteins of four different species and for selected proteins of the human erythrocyte membrane. All molecular weights were calculated from extrapolated straight lines obtained by electrophoresis of a set of standard proteins for each gel percentage. It can be seen that the molecular weights of the glycoproteins and of component IV vary with the gel percentage. The variation in the higher molecular weight components can be explained by the fact that the standard curves for 8% and 10% gels are curved in the region of these points, and the extrapolated straight line values are thus inaccurate. The variation in the calculated glycoprotein molecular weights is too extreme to be explained in this manner, and extends even to those of lower molecular weights, a region in which the other membrane proteins (VII and VIII) give reasonably constant values over the range of gel percentage used. These observations indicate that the membrane glycoproteins do not behave as normal proteins in the SDS electrophoresis system, and that the molecular weights calculated by this procedure are suspect in the absence of other supporting data. Similar phenomena have also been reported from other laboratories using the human glycoprotein and its tryptic glycopeptide (45) (46).

The ambiguities involved in the electrophoretic migration of the

ure 9. Variation in Calculated Molecular Weight with Acrylamide Percentage in Gel for Human Erythrocyte Membrane Components IV (o–o), VII (●–●) and VIII (■–■) and for Erythocyte Membrane Glycoproteins of Human (□–□), Pig (△–△), Horse (▲–▲) and Sheep (x–x).

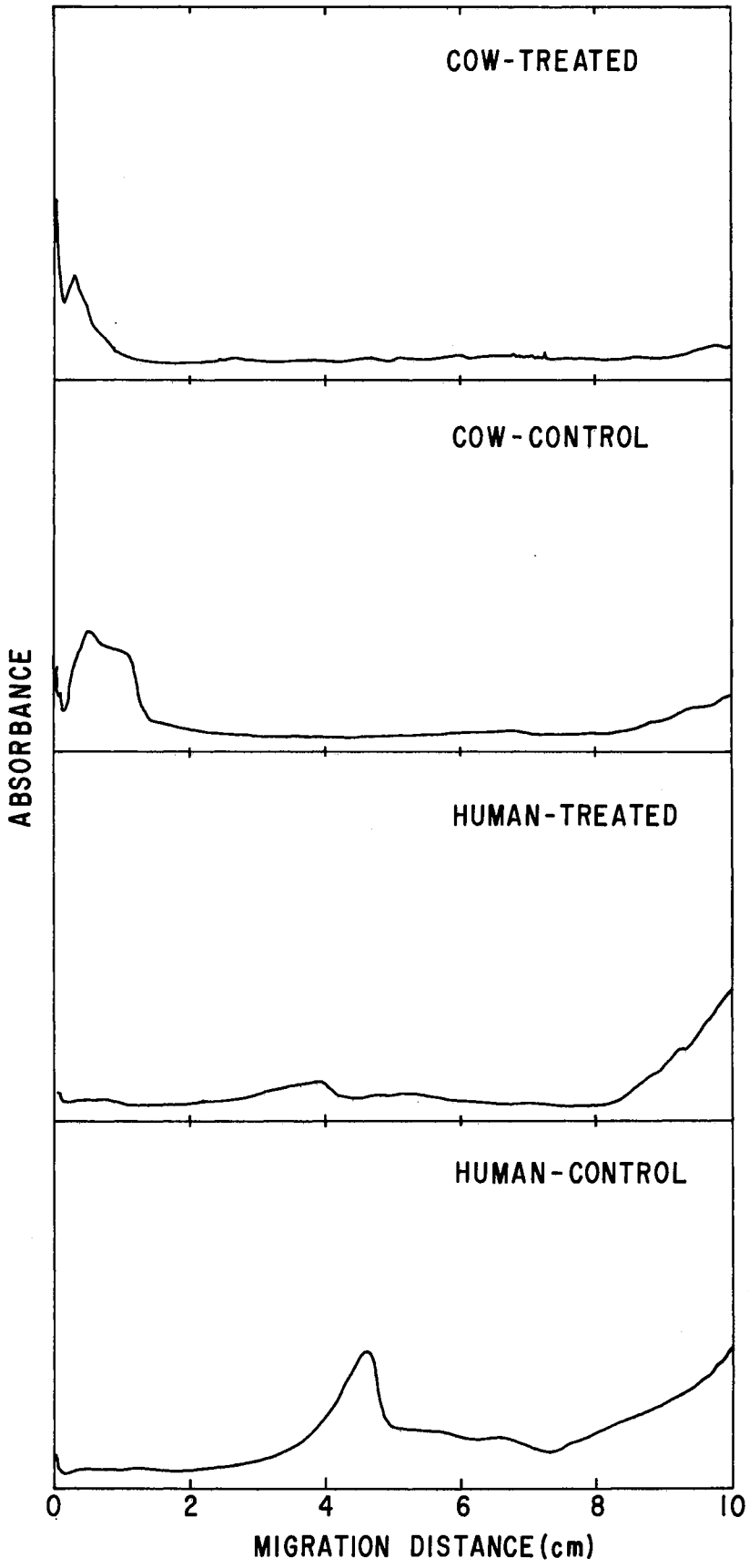

M. W. X 10⁻⁴
PERCENT GEL
0
1
2
3
4
5
6
7
8
9
10
4
5
6
7
8
9
10
11

membrane glycoproteins suggest that charge effects of the sialic acid residues may contribute to the electrophoretic behavior of the sialoproteins in the SDS system. Therefore experiments were performed on desialated membranes to examine the effect of sialic acid removal on the relative migration of glycoproteins in the SDS electrophoresis system. Both human and bovine membranes and intact erythrocytes were treated with Clostridium perfringens neuraminidase according to the procedure of Eylar et al. (68). Enzymatic removal of sialic acid was complete for membranes and cells of both species, as determined by analytical comparison of the amounts of sialic acid released by the enzyme treatment with that released by acid hydrolysis (108). The effects of sialic acid removal on electrophoretic behavior of the membrane glycoproteins are shown in Figure 10. In both cases the desialated samples show a diminished staining intensity and a decreased mobility. The latter observation is consistent with the decreased negative charge on the glycoprotein if it is assumed that SDS binding to the glycoprotein is not substantial enough to overcome charge effects from the inherent glycoprotein charge.

Preliminary Reaction Studies with Erythrocytes and Ghosts

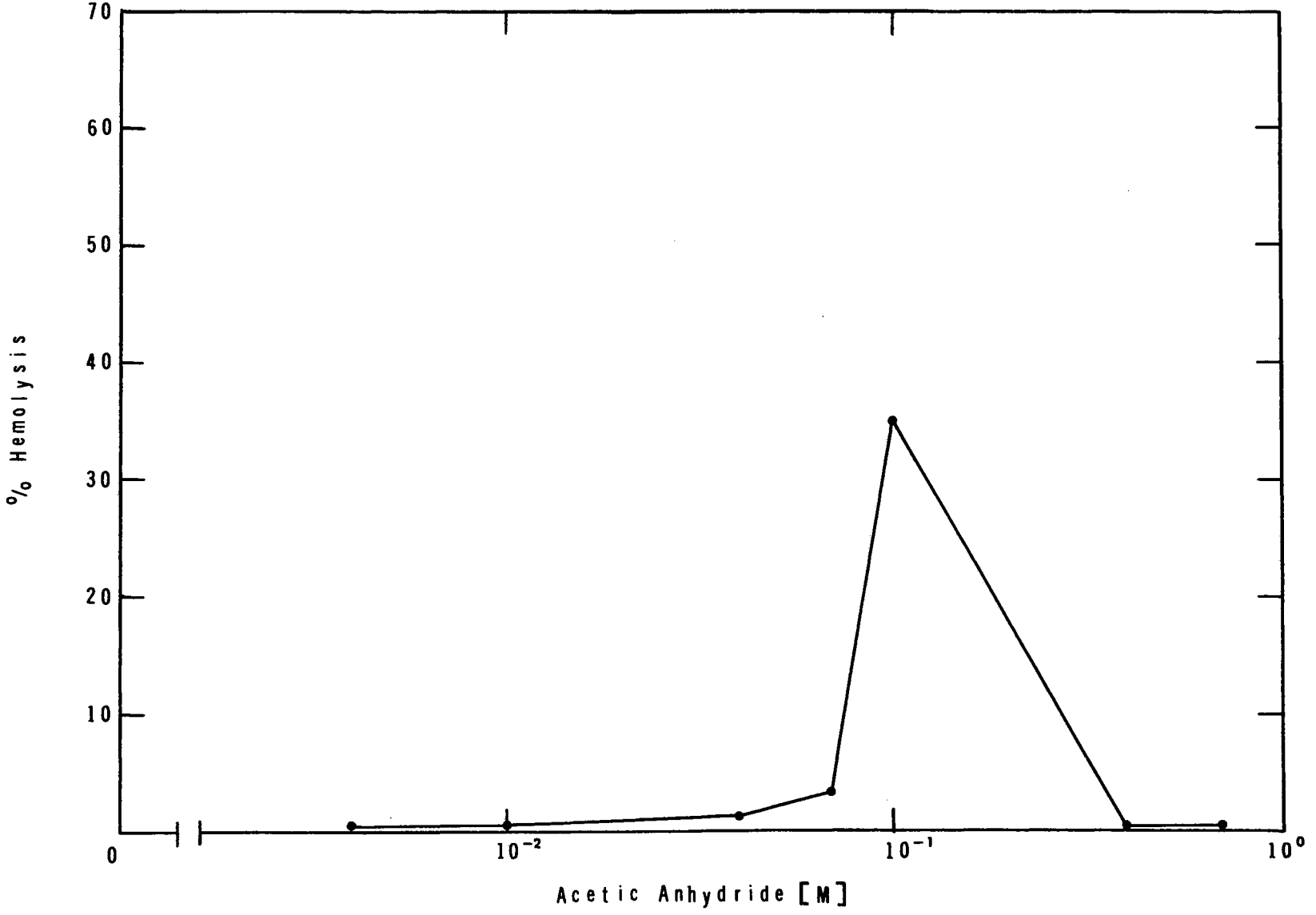
Acetic anhydride was chosen as a modification reagent because of its solubility in both organic and aqueous phases and low specific affinity for macromolecules. Its high reactivity presented a difficulty because of reagent hydrolysis, which required that fairly high concentrations of reagent be used to achieve incorporation. To estimate the effect of acetylation reactions on membranous systems, studies were

Figure 10. Effect of Neuraminidase Treatment on Electrophoretic Migration of Human and Bovine Erythrocyte Membrane Glycoproteins. Gels were stained by PAS procedure after 24 hr wash with 40% methanol-7% acetic acid. Duplicate gels stained with Coomassie Blue showed identical protein profiles for treated and control samples for each species.


COW-TREATED
COW-CONTROL
HUMAN-TREATED
HUMAN-CONTROL
ABSORBANCE
0
2
4
6
8
10
MIGRATION DISTANCE (cm)

performed with varying concentrations of anhydride which could be utilized to attain significant incorporation of reagent with minimal perturbation of membrane structure as measured by hemolysis and microscopic examination. The effect of acetylation on the erythrocyte membrane as measured by hemolysis is similar to that observed for other reagents which penetrate the cell membrane and react with the hemoglobin (3) (111) (112). Figure 11 shows that increasing concentrations of reagent caused increased hemolysis up to a concentration of about 0.4 M. At higher concentrations the cells were "fixed" and could not be hemolyzed even by suspension in distilled water. No attempt was made to control pH during these experiments, and it fell markedly at higher anhydride concentrations. The amount of hemolysis observed was dependent on the condition of the cells and the ratio of reagent to cells. Up to 70% hemolysis was observed in some experiments. Acetic acid at high concentration does not fix cells in a similar manner, but the acid may participate in the process, since incubation of fixed cells at high pH (>10) in dilute sodium hydroxide results in a slow release of hemoglobin. Microscopic examinations of cells show increased crenation at higher anhydride concentrations up to the point of fixation. Fixed cells show a mottled appearance due to hemoglobin precipitates or gels formed within the cell during reaction with acetic anhydride. However, the cells appear to maintain their biconcave shape in spite of the extreme conditions to which they have been exposed. As in the case of red cells, the ghosts show increased crenation with increasing anhydride concentration. After treatment with high concentrations of acetic anhydride, the membranes still appear to be mostly intact and retain their biconcave, rounded shape. However, the borders are ragged and ill-defined due to crena-

Figure 11. Typical Hemolysis Plot for Treatment of Human Red Blood Cells with Acetic Anhydride.


70
60
50
40
30
20
10
0
% Hemolysis
10^{-2}
10^{-1}
10^{0}
Acetic Anhydride [M]

tions.

On the basis of these results, most further studies were performed on 20% suspensions of red cells with 10 mM acetic anhydride. These conditions were chosen to give minimal pH change as well as minimal hemolytic and morphological alteration of the cells or their isolated membranes. Analyses of protein, cholesterol and phospholipid showed no significant differences when acetylated samples were compared to similarly treated non-acetylated samples. There also were no significant differences in the electrophoretic patterns of the acetylated versus non-acetylated membranes.

No attempt was made in these studies to determine a quantitative distribution of label among the various reacting functional groups. Ninhydrin analysis of anhydride treated membranes indicated that the primary site of reaction was the amino groups. Although sulfhydryl and tyrosine groups are also labeled by anhydride, solubilization of the membranes in mercaptoethanol-containing SDS solution effects the removal of the acetyl groups from these sites and they do not contribute in the electrophoretic and chromatographic analyses. Reaction of aliphatic hydroxyl groups of proteins or carbohydrates should not be a significant factor because of the competition of water and other more reactive nucleophiles for the reagent.

Anhydride Labeling of Red Blood Cells and Ghosts

Due to the lability of acetic anhydride in aqueous solutions and the difficulty of introducing exact small quantities of radioactive agent into the system, it was not possible to obtain highly reproducible results concerning the number of acetyl groups incorporated into the

membrane. For these reasons, comparative studies were performed to investigate the nature of the reactivity of the various red cell components toward acetic anhydride. In a representative experiment with erythrocytes, 30% of the reagent radioactivity was bound to the cells. Of the bound groups, 1.2% were present in the membrane fraction. Since 0.9% of the total cell protein was isolated in the membrane fraction, there appears to be little difference in the relative reactivities of the intracellular and membrane protein (after lipid labeling is considered). This was further substantiated by the close correspondence of the specific activities of isolated globin (113) and membrane protein. The labeling of isolated ghosts proceeded in a similar fashion, but because of the absence of hemoglobin to compete for the acetic anhydride, the amount of radioactivity incorporated into the membranes, i.e., the number of membrane groups acetylated, was approximately 10 times that observed in the case of the intact erythrocyte.

To characterize further the reactivity of the red cell membrane components, labeled membranes were extracted with methanol-chloroform and the radioactivity incorporated into the protein and lipid fractions compared. Of particular interest were the ratios of the amounts of radioactivity incorporated into each fraction. These ratios were found to be dependent upon whether the modification reaction was performed on the erythrocyte or the ghost. If the erythrocyte was labeled, the ratio of radioactivity in the protein fraction to that in the lipid fraction was 2.0 ± 0.17 (average deviation of three experiments). If the ghosts were modified, the ratio of radioactivity in the protein fraction to that in the lipid fraction was 0.82 ± 0.21 (three experiments). It should be noted, however, that lipid extracts did contain

small amounts of Lowry protein, but no significant amount of phospholipid was detected in the extracted proteins. To show that the reactivity differences stated above were not due to a highly reactive protein contaminant in the lipid fraction, two experiments were performed. First, material from the lipid extracts of labeled red cells and ghosts was subjected to 0.1% SDS acrylamide electrophoresis. Triplicate gels were sliced and counted, stained for protein and stained by the periodate-Schiff procedure to locate the micellar lipid band which runs with the SDS front on electrophoresis. The radioactivity in the lipid portions and protein portions of the gel were compared. Second, samples of the lipid extracts were chromatographed on thin layer plates which were analyzed for radioactivity. The phospholipid species separate cleanly from the protein by this method and only two zones of radioactivity were detected, in the protein at the origin and in the area of the phospholipids. In both types of experiments the amount of radioactivity in the protein fractions compared favorably with the amount expected on the basis of protein analysis of the lipid extracts. The radioactivity in the protein fraction was not nearly sufficient to explain the differences in reactivity observed between the red cells and ghosts.

Double Labeling of the Erythrocyte Membrane with Acetic Anhydride

Further studies on the reactivity of the erythrocyte membrane required methods of comparing reactivities that might occur during membrane isolation or as a result of disaggregation. These changes are important with respect to the arrangement of components within the membrane, since unreactive molecular species that are "buried" within the

membrane structure would become reactive when the membranes are disaggregated. Thus, reactivity changes could yield information concerning possible structural arrangements. The SDS acrylamide gel fractionation technique allows quick analysis of modification results made with radioactive reagents. But comparisons of radioactivity profiles with those obtained by staining procedures are not always satisfactory. Stains do not always yield quantitative results for all components, and reliably superimposing two profiles taken by different techniques is often difficult. The double labeling technique eliminates these problems since only one property of each fraction is being measured. In making reactivity comparisons, the problems which arise from slight differences in the size of gel slices is also less significant. Variation in slice size will only affect the shape of the peaks and not the relationship between the two curves, which is the measure of relative reactivities toward the reagent before and after disaggregation. In practice, red cells or isolated ghosts were labeled with either ^{14}C- or ^{3}H-acetic anhydride, and the membranes were isolated free of intracellular protein or unreacted label. The labeled ghosts were then dissolved in 1% SDS in phosphate buffer and exposed to acetic anhydride again containing the other label.

The double labeling procedure was applied to intact erythrocytes as represented in the following scheme:

$$\text{RBC} \xrightarrow{^{3}H\text{-}Ac_2O} {}^{3}H\text{-RBC} \xrightarrow{\text{hemolysis}} {}^{3}H\text{-ghost} \xrightarrow[\text{SDS}]{^{14}C\text{-}Ac_2O}$$

$$^{3}H,\ ^{14}C\text{-labeled ghost components} \longrightarrow \text{electrophoresis}$$

Erythrocytes were initially reacted with ^{3}H-acetic anhydride and their

membranes isolated. A second modification was performed on the disaggregated membranes with ^{14}C-acetic anhydride in SDS solution, and the dialyzed, lyophilized membrane sample was subjected to electrophoresis in SDS on a 10 cm 6% acrylamide gel. The radioactivity profiles obtained by counting the extracted gel slices were normalized by visual inspection to facilitate comparison. By this method, there do not appear to be greatly significant differences in the relative reactivities of the various components in the intact erythrocyte when compared to its disaggregated membrane, although there is some increase in the amount of label incorporated into the lipid fraction (the area near fraction number 50) and a few of the protein fractions in the disaggregated state (Figure 12).

A similar experiment was performed in which isolated erythrocyte ghosts were labeled initially with ^{14}C-acetic anhydride, as represented in the following scheme:

$$\text{RBC} \xrightarrow{\text{hemolysis}} \text{ghosts} \xrightarrow{^{14}C\text{-}Ac_2O} {}^{14}C\text{-ghost} \xrightarrow[\text{SDS}]{^{3}H\text{-}Ac_2O}$$

$$^{14}C,\ ^{3}H\text{-labeled ghost components} \longrightarrow \text{electrophoresis}$$

After appropriate washing and solubilization in SDS, the membranes were treated with ^{3}H-acetic anhydride and subjected to electrophoretic analysis.

The results are shown in Figure 13. Note that the order of addition of labels has been reversed in this case and that the electrophoresis gels were 6 cm in length as opposed to 10 cm in Figure 12. Again, the protein portion of the profile indicated no major reactivity changes on disaggregation. However, there appeared to be a significant differ-

Figure 12. Radioactivity Profiles of Acrylamide Gel From Acetic Anhydride Double Labeled Membranes. Erythrocytes were initially labeled with ^{3}H-acetic anhydride. Membranes were prepared and labeled with ^{14}C-acetic anhydride in SDS solution. Electrophoresis was performed on a 10 cm acrylamide gel; gel was sliced, extracted and counted as described. ^{3}H (——) and ^{14}C (– –).

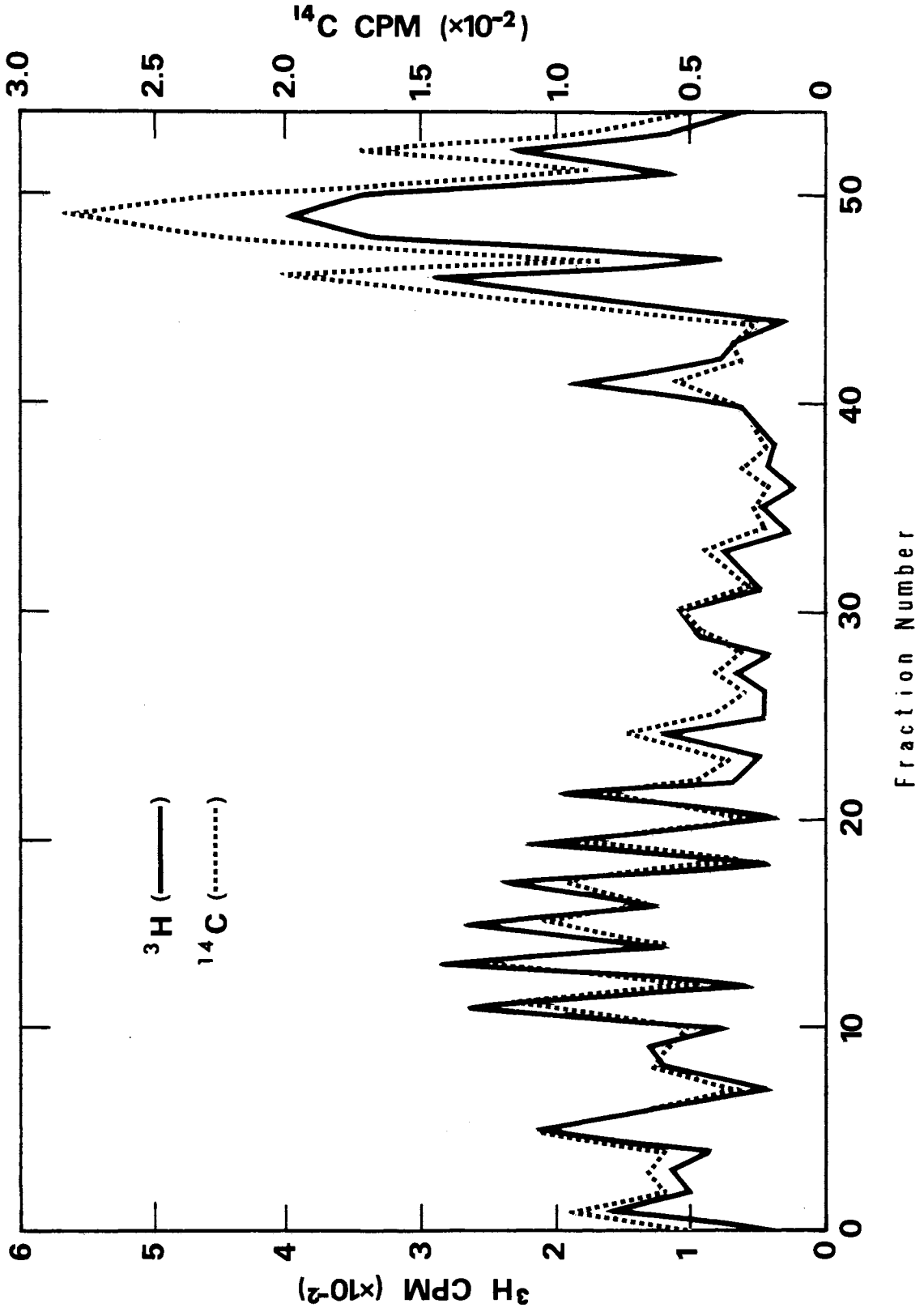
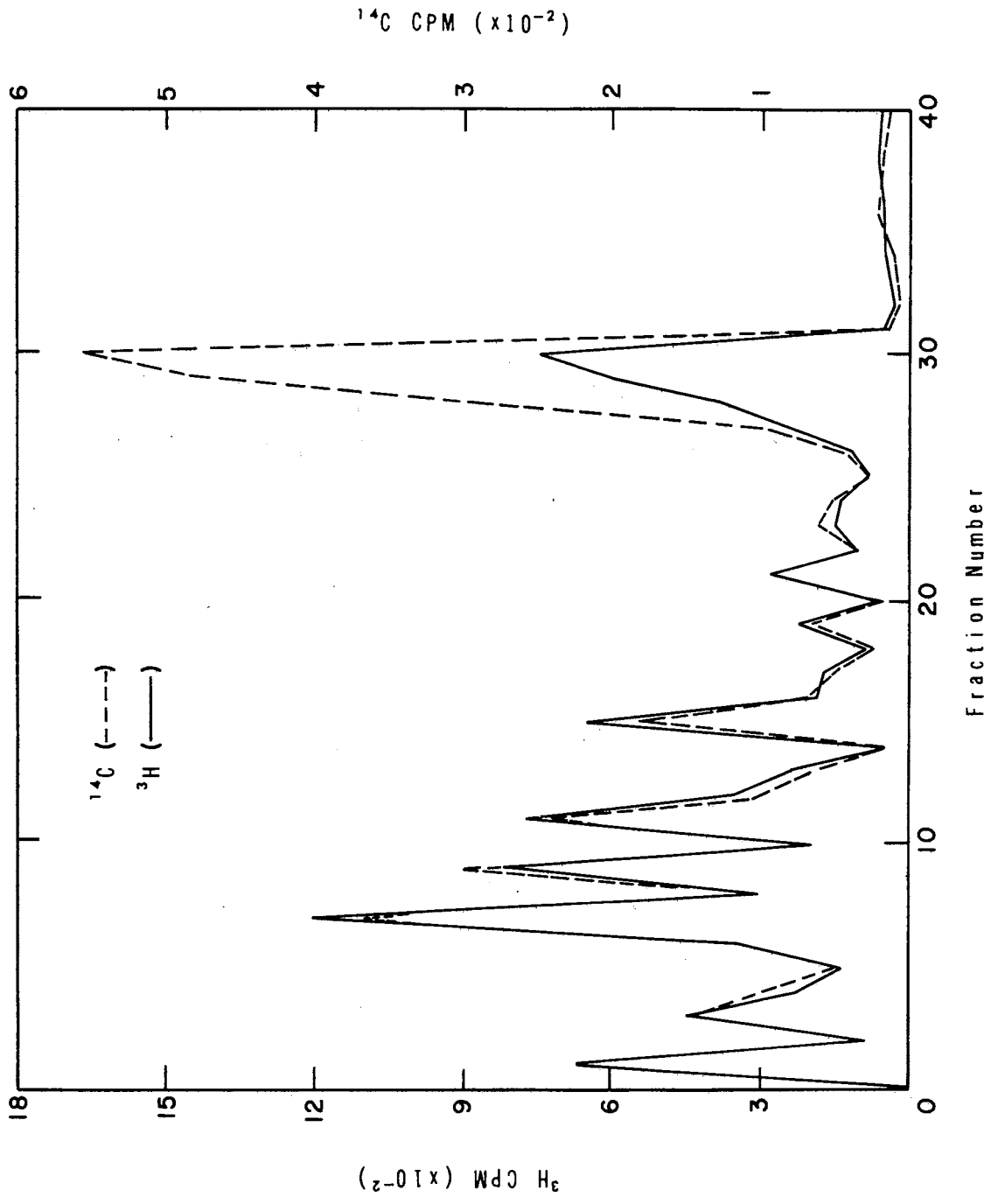

^{14}C CPM (×10^{-2})
3.0
2.5
2.0
1.5
1.0
0.5
0
^{3}H (——)
^{14}C (·····)
^{3}H CPM (×10^{-2})
6
5
4
3
2
1
0
0
10
20
30
40
50
Fraction Number

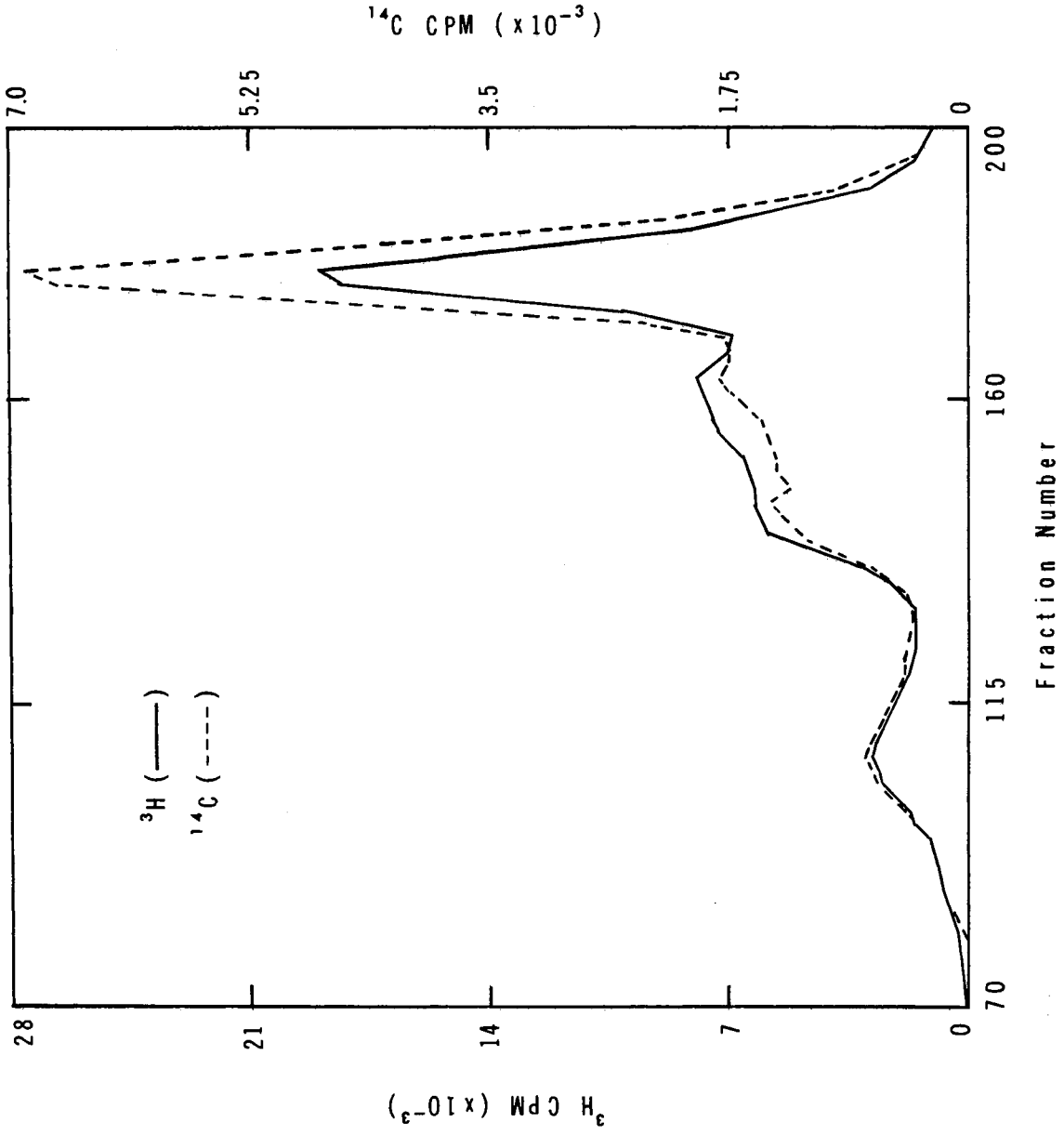
Figure 13. Radioactivity Profiles of Acrylamide Gel from Acetic Anhydride Double Labeled Membranes. Ghosts were initially labeled with ^{14}C-acetic anhydride. After washing they were solubilized in SDS and labeled with ^{3}H-acetic anhydride. Electrophoresis was performed on a 6 cm acrylamide gel; gel was sliced, extracted and counted as described. ^{14}C (– –) and ^{3}H (——).


^{14}C CPM (x10^{-2})
^{3}H CPM (x10^{-2})
^{14}C (----)
^{3}H (——)
Fraction Number
0
3
6
9
12
15
18
10
20
30
40
1
2
3
4
5
6

ence in the lipid portion of the profile (the area near fraction number 30). The incorporation of the labels into the lipid fraction suggests that there is an enhancement of reactivity of the lipid relative to the protein in the intact ghost. To obtain furthur evidence of the validity of this observation, a sample of the double labeled membrane was chromatographed according to the procedure of Lenard (4) on a column of Sepharose 4B. The results seen in Figure 14 are essentially equivalent to those seen on electrophoresis, since the significant difference between the two radioactive profiles occurs over the range where phospholipids chromatograph.

To gain additional insight into possible modes of organization of the lipids of the membrane, lipid extracts were made of ghosts from the double labeling experiments starting with either the membrane or the intact erythrocyte. The individual lipids were separated by thin layer chromatography, and the acetylated phosphatidylserine and phosphatidylethanolamine derivatives were counted to determine the ratios between their ^{14}C and ^{3}H activities. The ratios were indicative of the relative reactivities of these phospholipid components in the intact membrane structure. The results indicated that phosphatidylserine had a 20-30% greater reactivity than phosphatidylethanolamine for both the intact erythrocyte and the isolated membrane. The significance of this finding to membrane structure is difficult to evaluate in the absence of information concerning the other lipids present such as phosphatidylcholine, sphingomyelin and cholesterol.

Figure 14. Radioactivity Profile of Eluent from Sepharose 4B Column. Double labeled membrane components were prepared as in Figure 13. Column was eluted with phosphate buffered 1% SDS as described. ^{14}C (- -) and ^{3}H (——).


^{14}C CPM (x10^{-3})
7.0
5.25
3.5
1.75
0
^{3}H (——)
^{14}C (----)
28
21
14
7
0
^{3}H CPM (x10^{-3})
70
115
160
200
Fraction Number

Diazotized Sulfanilic Acid Labeling of Human and Bovine Erythrocytes

Experiments with human erythrocyte membranes suggest that the distribution of membrane proteins is not as uniform as some models suggest. Using diazotized sulfanilic acid, a protein reagent which does not readily penetrate the membrane barrier of intact erythrocytes, Berg (3) was able to obtain an enhanced specificity of labeling of certain proteins of human erythrocyte membranes but observed no labeling of the major high molecular weight protein. Phillips and Morrison (69) used the enzyme lactoperoxidase to label the exterior proteins of the erythrocyte with radioactive iodine. Only one significant band of radioactivity was noted when the membrane proteins were fractionated by acrylamide electrophoresis in sodium dodecyl sulfate. To investigate this area further, the author has used diazotized ^{35}S-sulfanilic acid (DSA) labeling alone and coupled with trypsin and neuraminidase treatment of human and bovine erythrocytes. These two species of red blood cells were chosen because 1) the protein patterns are essentially identical for the major bands of proteins observed by SDS electrophoresis, 2) the molecular weights of the glycoproteins of the two species are quite different for the gel percentages used and 3) the bovine glycoprotein, because of its high relative molecular weight, is cleanly separated on electrophoresis from the other major membrane proteins.

Human and bovine erythrocytes were treated with diazotized ^{35}S-sulfanilic acid according to the procedure of Berg (3) in isotonic phosphate buffer at pH 7.4. The reagent concentration was reduced to 0.3 mM in an attempt to enhance the specificity of the reaction by reducing the penetration of the reagent into the cell. Membranes were isolated by

the procedure of Dodge et al. (88), and subjected to electrophoresis using the sodium dodecyl sulfate-acrylamide procedure, previously described. The protein patterns observed by Coomassie Blue staining were essentially identical for the two species. The species variation in glycoproteins does not alter this observation, since the membrane glycoproteins do not stain as readily as the other membrane proteins with Coomassie Blue. For detection of radioactive labeling patterns, gels were sliced, extracted and counted as previously described. Figure 15 shows the radioactivity profile, a tracing of the gel scan from the periodate-Schiff (PAS) treated gel and a diagram of the gel stained for protein with Coomassie Blue (CB), obtained from identical 6% gels of labeled human erythrocyte membranes. One major area of radioactivity is noted; a broad band with a peak at a molecular weight of 85,000 daltons similar to that observed by Phillips and Morrison (69) after lactoperoxidase labeling. Two areas of carbohydrate stain are present in the gel, a glycoprotein band centered at 85,000 daltons and a band corresponding to the SDS front which contains the membrane lipid. The radioactive band corresponds closely to the glycoprotein, but the broadness of the band suggests that other proteins in this area may also be labeled. This question was resolved by experiments on the bovine erythrocytes. The radioactivity profile of the bovine erythrocyte membrane on 6% acrylamide gels shows two peaks, a major one at the origin corresponding to the glycoprotein and a smaller, broad peak in the 100,000 dalton range, which does not stain for carbohydrate. A clearer picture of the labeling was obtained by using 4% gels as shown in Figure 16. This technique permits clean separation of the bovine membrane glycoprotein from the origin and from the other membrane proteins

Figure 15. Labeling Patterns for Membrane Proteins of Human Erythrocytes Labeled with Diazotized ^{35}S-Sulfanilic Acid. Membranes were solubilized in 3% SDS and subjected to electrophoresis on 6% acrylamide gels as described. The radioactivity across the gel (- -) and the densitometer tracing of the duplicate gel stained for carbohydrate (——) are shown. The bar graph at the top of the figure represents the protein staining pattern with Coomassie Blue.

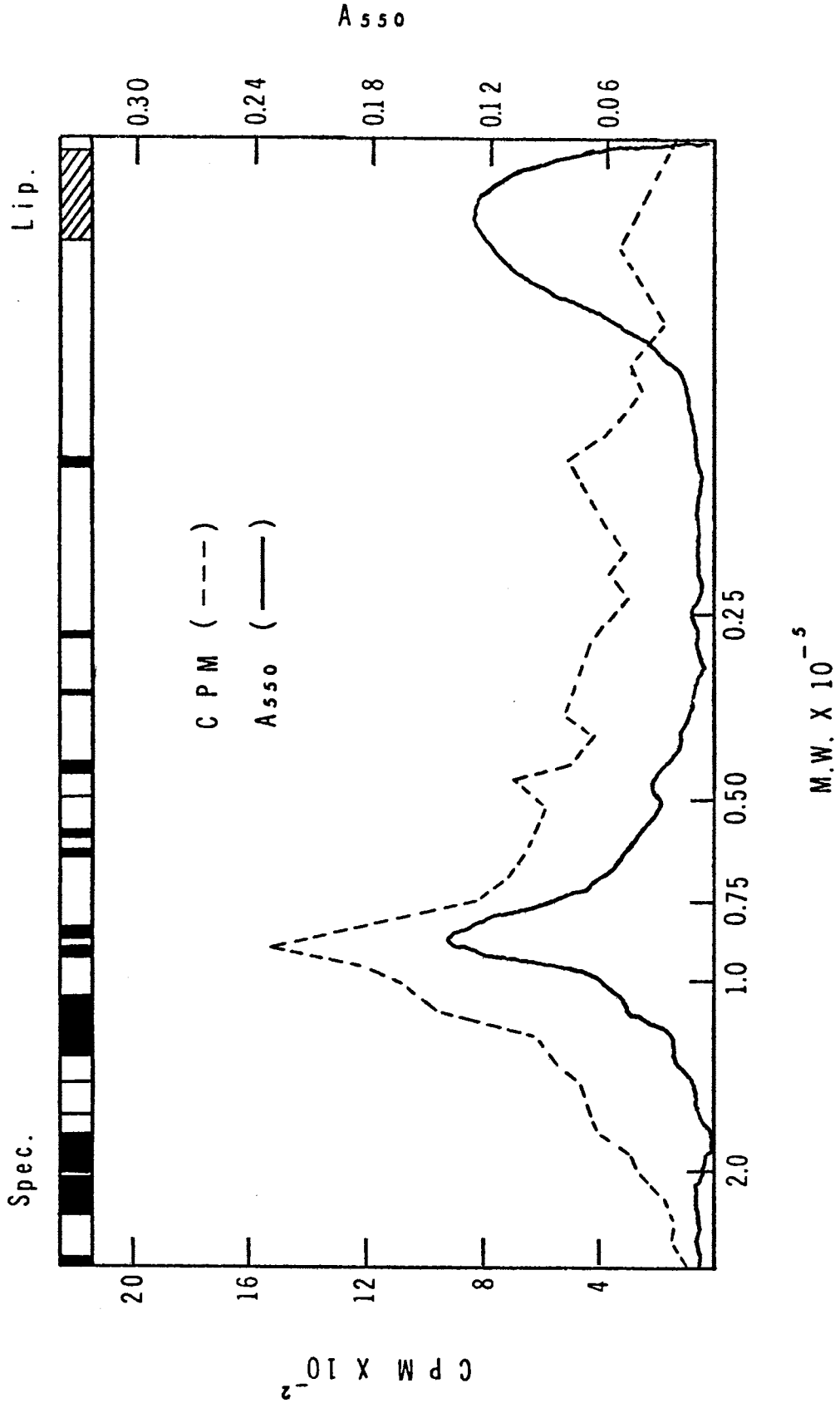
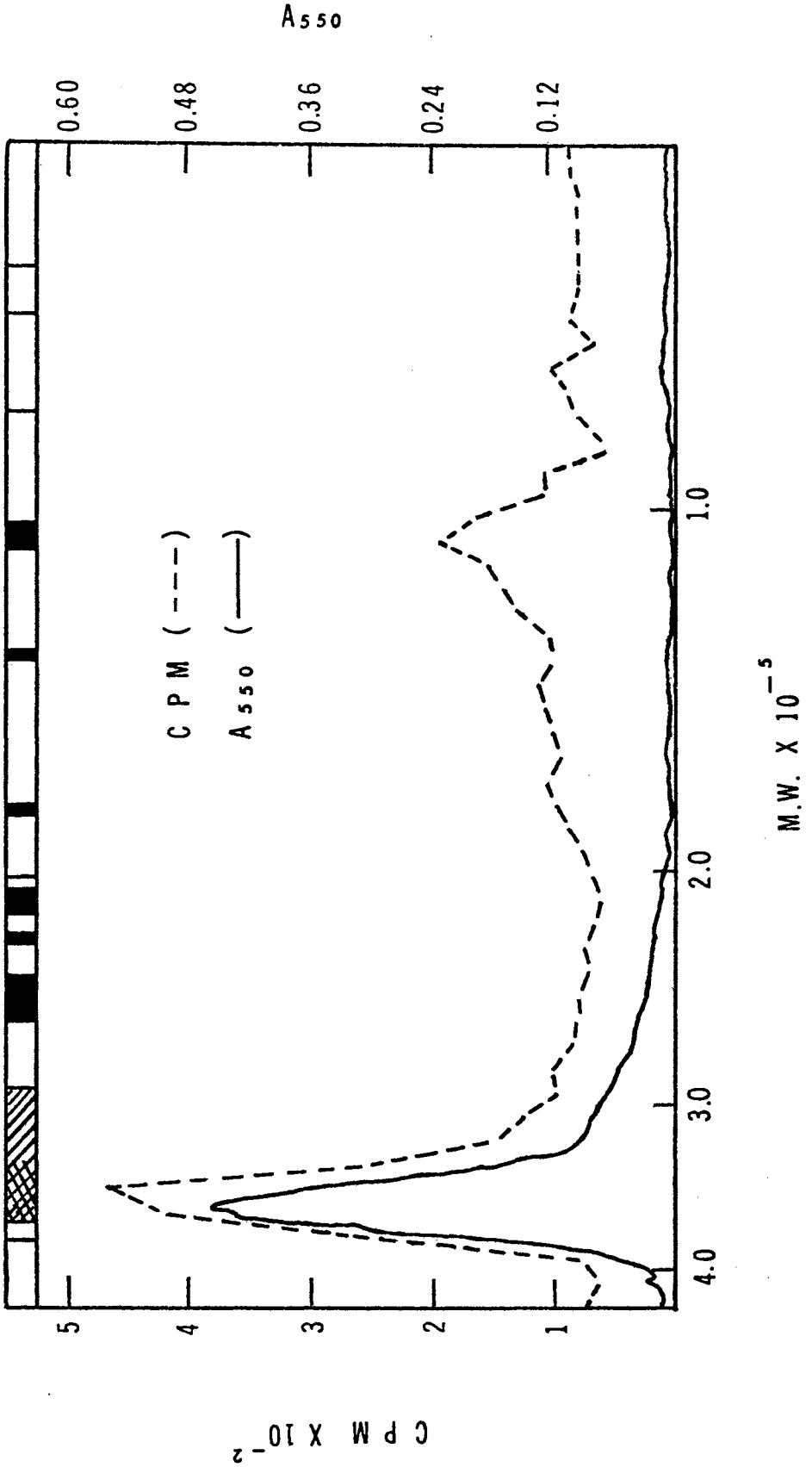

Asso
0.30
0.24
0.18
0.12
0.06
Lip.
Spec.
CPM (---)
Asso (——)
20
16
12
8
4
2.0
1.0
0.75
0.50
0.25
M.W. X 10⁻⁵
CPM X 10⁻²

Figure 16. Labeling Patterns for Membrane Proteins of Bovine Erythrocytes Labeled with Diazotized ^{35}S-Sulfanilic Acid. Electrophoresis was performed on 4% gels. Radioactivity distribution (- -) and carbohydrate staining (——) as in Figure 15.

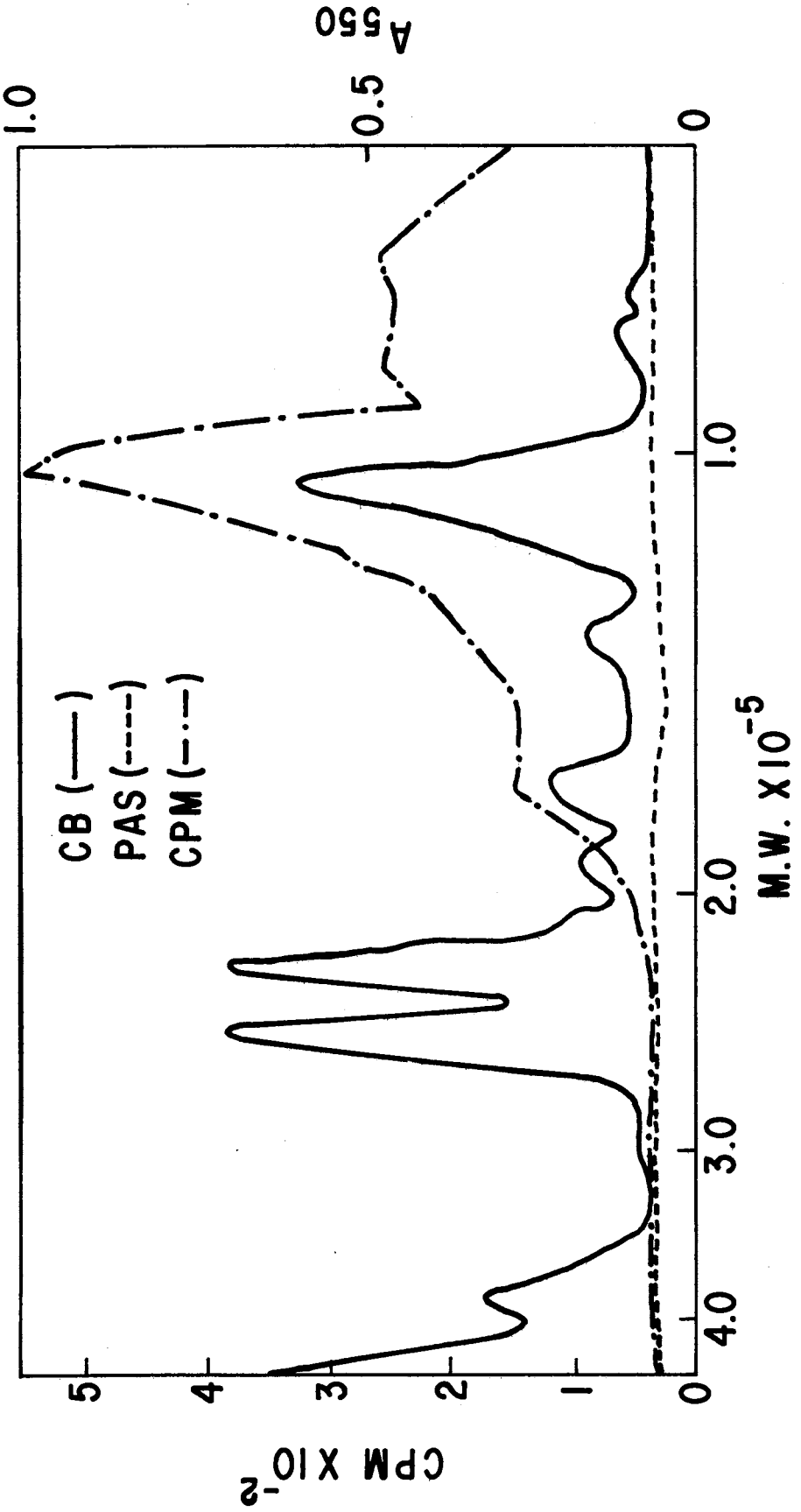

A550
0.60
0.48
0.36
0.24
0.12
CPM (---)
A550 (——)
1.0
2.0
3.0
4.0
M.W. X 10⁻⁵
5
4
3
2
1
CPM X 10⁻²

due to its high apparent molecular weight (>200,000 daltons). The labeling and staining patterns of 4% gels show that the major band of radioactivity is coincident with the glycoprotein and that the smaller band corresponds to the protein of molecular weight 108,000 daltons. To rule out the possibility that the specificity of labeling was due to a decreased reactivity of certain membrane proteins rather than their relationship to the permeability barrier, similar experiments were performed on isolated membrane ghosts, which are more freely permeable to the reagent. In these cases, the radioactivity was spread over the entire range of proteins, including the high molecular weight protein doublet spectrin (13).

In addition, when bovine erythrocytes were first treated with trypsin and then labeled with the ^{35}S-DSA reagent, an enhancement of label incorporation was observed for the 108,000 molecular weight species. Figure 17 shows the results of such an experiment where the solid line represents the Coomassie Blue stain, the dashed line periodate-Schiff, and the dash-dot line that of radioactive incorporation on a 4% gel. Note that the approximately 350,000 dalton molecular weight glycoprotein is completely absent as indicated by loss of PAS positive staining material and ^{35}S incorporation in this area, but that the 108,000 dalton protein has selectively taken up more label than in Figure 16. This observation indicates that the 108,000 molecular weight species is also present at the erythrocyte surface but is less readily accessible than the glycoprotein.

To further investigate these two surface proteins, bovine erythrocytes were first treated with neuraminidase to remove all of the membrane sialic acid and then labeled with the ^{35}S-DSA reagent. The results

Figure 17. Labeling Patterns for Membrane Proteins of Bovine Erythrocytes Labeled with Diazotized ^{35}S-Sulfanilic Acid after Treatment with Trypsin. The radioactivity across the gel (-•-) and the densitometer tracing of duplicate gels stained for Coomassie Blue (——) and carbohydrate (- -) are shown.


CPM X10^{-2}
A$_{550}$
M.W. X10^{-5}
CB (——)
PAS (----)
CPM (-·-·)
0
1
2
3
4
5
0
0.5
1.0
4.0
3.0
2.0
1.0

showed that the glycoprotein staining band was reduced in intensity and relative migration as depicted in Figure 10, but that the incorporation of ^{35}S label was essentially as shown in Figure 16, where both the glycoprotein and 108,000 dalton protein were labeled. This observation provides additional evidence for the surface orientation of the high molecular weight glycoprotein and 108,000 dalton molecular weight protein species.

CHAPTER IV

DISCUSSION

A number of investigators have reported that the erythrocyte membrane contains a heterogeneous collection of polypeptide chains, which vary in molecular weight from approximately 25,000 daltons to over 200,000 daltons (4) (5) (15) (23). Three different separation and fractionation methods, SDS polyacrylamide electrophoresis, chromatography in SDS solution and chromatography in 6 M guanidine show similar results for the polypeptide chain distribution. A common group of at least nine polypeptides with similar individual molecular weight has been demonstrated for erythrocyte membranes of a number of different animal species. Effects of variations in membrane preparative procedures have led to the observation of variation in the distribution of polypeptides by molecular weight. Different preparative methods can also lead to substantial changes in membrane morphology, but the distribution of major polypeptide chains does not appear to be very sensitive to morphological changes in the absence of other effects. The primary contributor to the alteration of polypeptide distributions appears to be proteolytic degradation of the membrane polypeptides. In the presence of proteolytic enzymes, the isolated, fragmented membranes are rapidly digested (23). Therefore a combination of fragmentation by preparative procedures and proteolysis results in extensive membrane protein degradation. The results presented above report the effects of such degrada-

tion. Evidence has been presented to show that contaminating white cells represent an important source of degradative enzymes. Whether erythrocyte membrane proteases also contribute to this phenomenon remains to be demonstrated. The results emphasize the necessity for removing protease activity from membrane preparations if any meaningful protein studies are to be done. The question of protease contamination must be resolved for preparative procedures for all membrane types before significant protein studies can be performed.

The glycoproteins of the erythrocyte membranes are not so similar for different species as are the other proteins. Each species investigated appears to have a single major glycoprotein with somewhat different characteristics, although the possibility that there is more than one protein of a similar electrophoretic mobility for each species has not been eliminated. Investigation of the membrane glycoproteins has a number of inherent experimental difficulties. Staining artifacts can lead to misidentification on SDS electrophoresis gels. The problem of determining the molecular weights of the human membrane glycoprotein is illustrated by comparing values obtained by SDS acrylamide electrophoresis in gels with varying percentages of acrylamide. These values range from 62,000 to 90,000 daltons. The problem with SDS electrophoresis derives from the failure of glycoprotein to bind SDS in the same stoichiometric proportions relative to its molecular weight as a normal protein (45). This fact is not unreasonable when it is recognized that about 60% of the total weight of this glycoprotein is carbohydrate and that the protein carries a large negative charge due to its sialic acid residues. Removal of sialic acid from either human or bovine glycoproteins results in a decreased mobility of the glycoprotein. A similar

effect of sialic acid removal was shown by Segrest et al. (45), for the tryptic glycopeptide of the human erythrocyte membrane, although no change was observed in mobility on removal of sialic acid from the human membrane glycoprotein. The variations noted in the effects of sialic acid removal between different laboratories (23) (45) may result from the effect of differences in electrophoresis conditions on the binding of SDS by the glycoprotein.

The values reported for the subunit molecular weight of the human erythrocyte glycoprotein vary from 26,000 to 100,000 daltons (4) (32) (42) (46) (114). As indicated above, the values obtained only by electrophoretic analysis are questionable. It is of interest that values obtained by column chromatography in pyridine (28,000 daltons) and in SDS (26,000 daltons) are similar and are not greatly different from those reported earlier by ultracentrifugation (42) (114). But, the effect of the carbohydrate portion of the molecule on these determinations is not clear, so the validity of the absolute values remains in doubt. Jackson et al. (115), have reported a molecular weight of 55,000 daltons based on a combination of electrophoretic studies and investigation of peptides obtained from purified human erythrocyte glycoprotein. This type of approach appears to be essential both to elucidate the size and nature of this protein and to illuminate some of the problems involved in the investigation of glycoproteins in general.

Experiments were initiated with acetic anhydride to examine changes in reactivity that occur as a result of the transition from intact red cell membrane to isolated ghost to disaggregated membrane components. In these experiments the first two states (intact cell and isolated membrane) were studied by comparison to a third common state (disaggre-

gated membrane). The proteins and lipids of erythrocytes are completely disaggregated from each other in SDS solutions, as indicated by the electrophoresis and chromatography experiments. Differences between the first two states are especially significant, since the former is the physiologically significant species, while the latter is the form upon which most studies and models of membrane structure have been based. Preliminary investigations with ^{14}C-acetic anhydride showed that the relative reactivities of the protein and lipid fractions obtained by organic extraction were dependent upon whether the reaction was performed on intact cells or on isolated ghosts. These experiments indicated a difference in structure of the membrane of the intact cell when compared to the ghost, but it was not possible to ascertain whether the difference resulted from a change in lipid or in protein reactivity, since the incorporation of label into the membrane was different for the ghost and the intact cell.

The use of double labeling procedures afforded a more accurate assessment of the reactivity changes. There were no really significant differences observed in the relative reactivities toward acetic anhydride of the protein components of either the ghost or intact red cell when compared to the disaggregated state. The differences in relative reactivities between the ghost and the intact cell reside essentially in the lipid band of the fractionated membrane. An enhancement of the label incorporated into this band is noted when ghosts, as opposed to intact cells, are initially treated. This is in agreement with the earlier observation that the extracted lipid fraction has a relatively higher percentage of the radioactivity incorporated in the membrane from labeled ghosts than it does in the membrane from labeled red cells.

These results indicate that some change in the interactions of the lipid polar groups occurs during membrane isolation. Some caution should be observed in interpreting the results of any chemical modification study, however, because of the ambiguities associated with the methods. Primary among these is the possibility of a structural change (not observable morphologically) caused by the modification reaction, particularly since in this case the incorporation of reagent into the membrane is 10 fold greater in the isolated ghost than in the intact red cells when the modification reactions are performed under identical conditions. Therefore, in one experiment, the concentrations of reagent and red cells were increased such that the incorporation of reagent was only 3 fold greater in the ghost preparation. Even under these reaction conditions the difference in relative reactivity of the lipid fraction was at least as great as in previous experiments.

These differences in lipid reactivity between the intact cell and isolated ghost suggest an alteration in the interactions among lipids or between lipids and proteins resulting from the isolation procedure. This could be attributed to a significant difference in structure between the intact cell membrane and the isolated membrane. There is at least one other explanation for possible differences in membrane properties in the ghost or red cell which would not necessitate envisioning a large change in membrane structure. Such differences could be a result of a change in the stability of the membrane due to the removal of stabilizing components during hemolysis and washing. Under physiological conditions the red cell membrane exists between two protein solutions. Since both of these solutions probably interact with the membrane surface components (inner or outer), their removal could affect

membrane stability and thus give rise to relative reactivity differences toward a chemical modifying reagent.

The diazotized ^{35}S-sulfanilic acid labeling, trypsin and neuraminidase digestion studies permitted the selective examination of several components oriented toward the outer surface of the membrane. The evidence from these experiments indicates that glycoprotein is the most readily accessible major membrane protein at the surface of the cell and outside of the membrane permeability barrier. A second polypeptide of molecular weight 108,000 daltons (component III) also appears to be at least partially outside the permeability barrier based on the labeling results, but it is apparently less accessible than the glycoprotein. Fairbanks et al. (23), have commented on the fact that this protein is not extracted by the guanidine hydrochloride procedure of Gwynne and Tanford (15), but remains associated with the lipid material. This suggests that the protein is hydrophobic in nature. Bretscher (71) and Steck et al. (116), have recently presented evidence suggesting that this component extends completely through the membrane. However, both of these studies assume that no changes in membrane organization occur in preparing ghosts from intact cells. This assumption may not be justified in view of the varying results obtained from different washing buffers and fragmentation from proteolysis plus the different relative reactivity of the lipid material toward acetic anhydride in the ghost and intact cell. However, the postulation that component III may extend through the membrane is worthy of consideration because of its size, which would require that it occupy a significant volume of internal membrane space otherwise. This protein might represent a portion of the particles observed by freeze-etch electron microscopy in red cell

membranes.

Differences in either structure or stability are important to the understanding of membrane structure-function relationship since the ghost is basically a derivative of the erythrocyte membrane (25). However, many physical and chemical studies can be performed only on membranes and not on intact cells. Correlations between the properties of intact cell membranes and isolated cell membranes must be investigated in order to approach a complete understanding of membrane behavior in a physiologically significant state. It should be noted that the present investigations do emphasize the need for caution in interpreting and extrapolating results obtained with isolated membranes to structural concepts of the intact cell membrane.

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PART TWO

PROTEINS AND GLYCOPROTEINS OF THE MILK FAT GLOBULE MEMBRANE

CHAPTER V

INTRODUCTION

A mechanism by which fat is secreted from the mammary cell has been proposed by Bargmann and Knoop (1). They considered that a large fat droplet bulges out of the apical surface of the cell and in so doing becomes enveloped by the cell plasma membrane. Eventually, it is pinched off, leaving intact membranes around the droplet and on the apical surface of the cell, the process occurring with no loss of secretory cell cytoplasm. This interpretation has been accepted by many subsequent workers. The view of Bargmann and Knoop has received substantial support from Patton and Fowkes (2) based on extensive analyses of the lipids in mammary tissue and milk. These workers found that the phospholipids of milk fat are similar to those in washed mammary tissue homogenates. However, certain constituents (phosphatidylethanolamine and carotenoids) that they regarded as characteristic of mammary cell membranes were absent from tissue cream but present in milk fat. Keenan et al. (3) later prepared fractions of the plasmalemma of cow udder homogenates and of the fat globule membranes in milk secreted by the same gland. The lipid compositions of these two fractions were very similar and phosphatidylethanolamine was present in both. Furthermore, the cell membrane portion was yellow due to the presence of β-carotene. Although there were differences in chemical composition and in the electron microscopic appearance of the fractions, their work is generally

regarded as demonstrating that milk fat globules are surrounded by membranes very similar to mammary cell plasma membranes. Patton et al. (4) found only very small amounts of cardiolipin in the milk of cows and goats compared with the large amounts present in the mammary gland. Since cardiolipin is thought to be present mainly in mitochondria, they regarded this as indicative of a negligible loss of cytoplasm during fat secretion and concluded that the apocrine loss of cytoplasm is not a major part of milk secretion.

Patton and Fowkes (2) also made some theoretical calculations on the mechanism by which the fat droplet might be enveloped and pinched off by the cell membrane. Their estimate of the London-van der Waal's forces that may be operating in the secretory cell as the fat droplet approaches the plasmalemma is about one atmosphere at 20 Å separation, increasing to 100 atmospheres at 4 Å separation. Water and dissolved substances would move out when the pressure increases their chemical potential appreciably. This process would explain the envelopment and extrusion of the lipid droplet once it has made a close approach to the apical membrane. When the fat droplet makes contact with the membrane, the areas on the droplet near the point of contact are attracted to the membranes by forces resulting from the attraction of nonpolar carbon chains in the membrane for similar chains in the fat droplet. Other areas of the surface are similarly attracted until eventually the fat droplet is completely enveloped and finally pinched off from the cell. These workers also made the suggestion that as Golgi vesicles open by "reverse pinocytosis" the membrane of the vesicle becomes confluent with the apical cell membrane and thus the mechanism of protein secretion contributes to the replenishment of the apical cell membrane lost in the

process of fat secretion.

Some authors have questioned the conclusion that cytoplasm is not lost during extrusion of the lipid droplet because in sections of mammary gland some fat droplets in the lumen of the alveolus have pieces of cytoplasm attached. Such observations have led some investigators to state that it is their belief that milk fat globules are released by an apocrine mode of secretion (5) (6) (7) (8). Wooding et al. (9) have examined fresh goat's milk and found that about 5% of the fat droplets had pieces of cytoplasm ("signets") identical to those seen in the alveoli. Both "signets" and other lipid droplets were bounded by a membrane resembling the cell plasmalemma. The signets contained rough endoplasmic reticulum, mitochondria, and vesicles containing granules identical to those found in the secretory cell. It is thus possible that the lipid droplet is extruded from the cell as described above and when extrusion is almost complete, the droplet will be connected to the cell by a narrow neck of cytoplasm. If this neck separates immediately adjacent to the lipid, a milk fat droplet with no cytoplasm will be produced, as is usually the case, but if separation occurs closer to the body of the cell then a variable amount of cytoplasm will be included to produce a signet (10). Wooding (11) has extended his study of the milk fat globule membrane (MFGM) to report that the nature of the MFGM is dependent upon the length of time after the globule was secreted from the apical mammary cell. The initial MFGM consists of a plasmalemma and cytoplasmic material and is present only immediately after release from the secretory cell. Most of the initial MFGM is lost into the milk plasma by a process of vesiculation, leaving a structureless secondary MFGM as the only continuous boundary to the milk fat globule. In apparent

support of this general concept, Henson et al. (12) reported that certain components of the MFGM aggregated inside the continuous envelope surrounding the globule. At the site of aggregates, the trilaminar electron microscopic image was similar to that of a normal cytoplasmic membrane. However, most of the membrane, continuous with fat of the globule, exhibited no trilaminar structure.

The proteinaceous nature of the milk fat globule membrane was recognized as early as 1840 by Ascherson (13). The membrane protein has been described as globulin-like (14), mucin-like (15), lacto-fibrin (16), haptan (17), and casein (18). In most instances, these investigators agreed that the membrane protein differed from other milk proteins. The fact that certain enzymes are associated with the MFGM is viewed as evidence to support the proteinaceous nature of the MFGM and its derivation from the plasmalemma (19) (20). Dowben et al. (21) have reported that bovine membrane preparations contained alkaline phosphomonoesterase, acid phosphomonoesterase, phosphodiesterase, glucose-6-phosphatase, Mg^{2+}-activated ATPase, $(Na^{+}-K^{+}-Mg^{2+})$-activated ATPase, cholinesterase, xanthine oxidase and aldolase, most of which are found in the fraction of other tissues thought to contain plasma membranes. Patton and Trams (22) have shown the presence of 5'-nucleotidase, nucleotide pyrophosphatase and Mg^{2+}-activated ATPase in bovine MFGM. These are also marker enzymes for the plasma membrane. Most recently Martel-Pradal and Got (23) reported the presence in human colostrum MFGM of 5'-nucleotidase, Na^{+}, K^{+} and Mg^{2+}-ATPase and phosphodiesterase which are all marker enzymes for the plasma membrane. Other enzymes derived from Golgi apparatus and endoplasmic reticulum were also present, possibly due to a significant number of "signet" (9) containing milk fat globules in

human colostral milk.

Another approach to studying the milk fat globule membrane would be to purify the proteins associated with the MFGM and compare them with the proteins derived from the various fractions of the mammary cell (plasma membrane, cytoplasm, etc.) by immunochemical techniques. Limited experiments of this nature have been performed. Coulson and Jackson (24) have reported that a sialic acid-containing mucoprotein isolated from bovine MFGM functioned as a strong antigen. The mucoprotein preparation induced formation of antibodies in rabbits to the mucoprotein and to at least two contaminating proteins from whey. Absorption of the antiserum with a preparation of whey proteins resulted in a homogeneous antibody system specific for the mucoprotein. This mucoprotein was distinguished immunologically from other, previously recognized proteins of milk. Dowben et al. (21) have found that antisera to fat globule membranes agglutinated and hemolyzed bovine erythrocytes but not those of rabbit or human, indicating a similarity in antigenic properties between erythrocytes and MFGM.

If the milk fat globule membrane is a derivative of the mammary cell membrane, as the evidence indicates, it 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.

CHAPTER VI

EXPERIMENTAL PROCEDURE

Materials

Source of Cream

Raw chilled cream was obtained from the Oklahoma State University Dairy. It was derived from a mixed milking herd consisting of 60% Holstein, 20% Ayrshire, 10% Jersey and 10% Guernsey. The cream was obtained within 2-3 hr of milking and had been separated in a chilled state from raw milk.

Polyacrylamide Gel Electrophoresis

The chemicals and molecular weight markers were the same as those stated in Part I, Chapter II, Materials.

Enzymes

Trypsin (Type III) and Pronase (Type VI) were products of Sigma.

Enzyme Assay Substrates

Adenosine 5'monophosphoric acid (5'-AMP, type II), adenosine 5' triphosphate (ATP), xanthine and 2,3,5,-triphenyltetrazolium chloride (TTC) were all products of Sigma.

Methods

Preparation of Milk Fat Globule Membranes

Cream was washed by three suspensions in an appropriate solution (usually 0.25 M sucrose–0.1 M imidazole–2 mM $MgCl_2$ at pH 7.0) at room temperature followed by centrifugation at 4,000 x g for 20 min at 5°. 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° in a Sorvall Omni-Mixer for periods of 2–10 min at full speed. The freeze–thaw procedure involved freezing 33% cream at –20° for 20 hr and thawing rapidly to 35°. This latter procedure was used for the majority of the work described. Membranes were isolated by centrifugation at 40,000 g for 1 hr at 5° and washed twice in the buffer used for freezing and thawing.

Direct Solubilization of Washed Cream Proteins with SDS

For SDS extractions of cream, 2 ml of 3% SDS in 0.01 imidazole and 2 mM $MgCl_2$ (pH 7.0) were added to each gm of solid washed cream. The suspensions were made to 0.2% in mercaptoethanol and incubated under nitrogen at room tamperature overnight. In some cases, the suspensions were heated at 100° for 5 min before overnight incubation. The extraction mixtures were centrifuged at 35,000 g for 30 min at 25°. Aliquots of the supernatant were taken from beneath the butter–cream layer for protein analyses and electrophoresis. Supernatant samples were dialyzed against 40% methanol and lyophilized before preparation for electrophoresis.

Comparison of Cream Obtained from Chilled Versus Unchilled Raw Milk

Fresh raw milk as a composite sample was obtained from three Holstein cows. The sample was divided in half, one portion being chilled immediately to 4° (chilled sample) and the other being maintained at 25° (unchilled sample). The cream from the chilled sample was prepared by centrifuging the chilled raw milk at 5,000 g for 15 min at 4°, removing the solid floating cream from the milk plasma and suspending it in buffered sucrose at 25°. The centrifugation and washing procedure was repeated two additional times. After the initial centrifugation and each washing step, an aliquot of cream was immediately solubilized by making the mixture 2% in SDS and 0.2% in mercaptoethanol and heating at 100° for 5 min. The sample was further incubated overnight at room temperature under nitrogen. The extraction mixture was centrifuged at 35,000 g for 30 min at 25°. Aliquots of the supernatant were taken from beneath the butter-cream layer and dialyzed against 40% methanol. The dialysates were then prepared for electrophoresis as usual. The unchilled sample was treated similarly except that the cream from it was washed with buffer at 37° and centrifuged at 25°.

Chemical Analyses

Protein (25), cholesterol (26), phosphorous (27), and neutral sugar (28) were determined by standard procedures. Phospholipid content was calculated by multiplying the value for phosphorous by 25. Sialic acid was determined by the method of Warren (29) after hydrolysis with 0.1 N H_2SO_4 for 1 hr at 80°. Values are expressed as N-acetylneuraminic acid. Amino acids were determined by the procedure of Spackman *et al.* (30), on

a Beckman 120 C Amino Acid Analyzer after hydrolysis for 22 hr *in vacuo* at 110° in 6 N hydrochloric acid.

Electrophoresis and Column Chromatography

Polyacrylamide gel electrophoresis and column chromatography on Sepharose 4B, both in the presence of SDS, were performed as described in Part I, Chapter II, Methods. Fractions from the column were pooled, dialyzed against 40% methanol to remove SDS, lyophilized and prepared for electrophoresis or amino acid analysis.

Extraction of Milk Fat Globule Membranes

Milk fat globule membranes (MFGM) at a concentration of 1 mg/ml protein were extracted with EDTA-mercaptoethanol by a modification of the procedure of Marchesi *et al.* (31) 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°. The dialyzed sample was divided into two parts. One portion was further dialyzed against distilled water for 20 hr at 4°. The resulting suspension was centrifuged at 35,000 g for 1 hr. Pellets and supernatants were lyophilized and prepared for electrophoresis. The second portion was brought to 50% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 35,000 g for 1 hr. 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 (32). To each ml of 33% cream or of MFGM (2 mg/ml protein) was added one gm of solid guanidine hydrochlo-

ride. The suspension was made to 2% in mercaptoethanol and to pH 8.6 with sodium hydroxide. The mixture was stirred overnight at room temperature under nitrogen and centrifuged at 4° for 2 hr at 110,000 g. The supernatant was carefully removed from beneath the top oily layer and was dialyzed against distilled water at 4°. The turbid suspension from the dialysis was centrifuged 1 hr at 35,000 g and 4°. The pellet and supernatant fractions were lyophilized and prepared for electrophoresis.

Extraction with sodium chloride was achieved by dialyzing MFGM and cream samples (1 mg/ml protein) overnight at 4° against 1.0 M sodium chloride. The supernatant was collected as described and redialyzed against distilled water at 4°. The samples were then treated in the same manner as the guanidine hydrochloride samples.

Density Gradient Centrifugation

MFGM (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 hr at 90,000 g and 4° in a SW 25.2 head of the 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.

Enzyme Assays of Washed Cream and Isolated MFGM

The assay of 5'-nucleotidase was performed by a modification of the method of Widnell and Unkeless (33). The assay measured the release of

inorganic phosphate from 5'-AMP and was carried out in a volume of 0.6 ml containing 100 mM Tris-HCl (pH 8.5), 10 mM 5'-AMP and 10 mM $MgCl_2$ plus 0.050 ml 33% cream (2-3 mg/ml protein) or 0.005 ml MFGM preparation (20-30 mg/ml protein). After incubation at 37° for 20 min, the reaction was stopped by the addition of 1.4 ml of a solution containing 1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate in 1 N H_2SO_4 (mixed fresh daily). After incubation at 45° for 20 min and extraction into 2 ml of reagent grade isoamyl alcohol the absorbance was determined at 795 nm.

The assay of Mg^{++} and Ca^{++} ATPase was carried out by the method of Cha et al. (34). Again the reaction mixture was carried out in 0.6 ml containing 0.05 ml of 33% cream or 0.005 ml of MFGM preparation. The Mg^{++}-ATPase mixture also contained 120 mM KCl, 0.5 mM EDTA, 30 mM histidine-imidazole buffer (pH 7.0), 5 mM $MgCl_2$ and 2 mM ATP. The Ca^{++} ATPase assay mixture contained 120 mM KCl, 0.5 mM $CaCl_2$, 30 mM histidine-imidazole buffer (pH 7.0), 5 mM $MgCl_2$ and 2 mM ATP. The samples were incubated, quenched, extracted and read at 795 nm exactly as in the assay for 5'-nucleotidase. Note that it was necessary to have a non-enzyme containing blank for each time to be assayed in the case of the ATPases since non-enzymatic hydrolysis of ATP is quite significant. This precaution was not needed in the case of the 5'nucleotidase since 5'-AMP is quite stable in solution for the assay period.

The assay of xanthine oxidase was performed according to the procedure of Zittle et al. (35). To a tube was added 1 ml of 0.5 M phosphate buffer (pH 7.5), 0.2 ml of 0.005 M xanthine and enough distilled water so as to make the total volume 3.2 ml including the 0.05 ml of 33% cream or 0.005 ml of MFGM preparation. The solution was purged of

oxygen by bubbling nitrogen through it for 5 min. At this time the aliquot of washed cream or MFGM was added and the bubbling of nitrogen was continued for 5 additional minutes. Finally, 0.2 ml of 0.05 M triphenyltetrazolium chloride (TTC) in 0.01 M phosphate buffer (pH 7.5) was added and the reaction continued for 20 min. The reaction was stopped by the addition of 5.0 ml of glacial acetic acid to each tube, and bubbling of the nitrogen was stopped. To each tube was added 4.0 ml of toluene. The tubes were stoppered and shaken vigorously. The red color extracted into the toluene (reduced TTC) is diluted if necessary with more toluene and the absorbance read at 485 nm.

Digestion of Washed Cream and Isolated MFGM with Trypsin and Pronase

To 10 ml of washed cream or MFGM (1 mg/ml protein) in 0.25 M sucrose-0.01 M imidazole-0.002 M $MgCl_2$ (pH 7.0) was added trypsin in 0.155 M NaCl (pH 3.0) to a final concentration of 1-100 μg/ml. Digestion was allowed to proceed for 1 hr at room temperature (36). To the mixture was added 2.5 ml of 10% SDS and 0.025 ml mercaptoethanol. The suspension was then heated to 100° for 5 min and incubated overnight at room temperature under nitrogen. The cream samples were centrifuged for 30 min at 35,000 g and 25°. Supernatants were removed, dialyzed overnight against 40% methanol and lyophilized before electrophoresis. SDS solubilized MFGM samples were treated in the same manner as the supernatants from the cream samples.

Pronase digestion (36) was performed at 37° in an identical manner except that both the incubation buffer and the buffer used to dissolve

the Pronase were 17.3 mM Tris-130 mM NaCl-3.6 mM KCl-1.6 mM $CaCl_2$-1.2 mM $MgSO_4$ (pH 7.6).

CHAPTER VII

RESULTS

Preparation of Milk Fat Globule Membrane

Several procedures were investigated in order to optimize the preparation of the MFGM. 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% SDS. Based on these analyses, the freeze-thaw procedure in buffered sucrose plus magnesium was used for further investigations, since the system gave the most consistent results in terms of membrane yields and electrophoresis paterns. As a characterization of the membranes prepared by this technique, several parameters of three separate membrane preparations are compared in Table IV. The yield of membrane from cream ranged from 4-9%, based on recovery of protein. The amino acid composition of the isolated membranes, expressed as an average of the three preparations, is shown in Table V along with the amino acid content of the bovine erythrocyte membrane.

TABLE IV

MILK FAT GLOBULE MEMBRANE COMPOSITION[a]

Preparation	Protein	Cholesterol	Phospho-lipids	Neutral Sugar	Sialic Acid
MFGM-1	0.15	0.072	0.20	0.050	0.014
MFGM-2	0.14	0.069	0.23	0.055	
MFGM-3	0.22	0.068	0.20	0.050	
BRBCM		0.25	0.52	0.10	

[a]Protein is expressed as mg per ml of 33% cream. Diluted cream (33%) samples from which MFGM preparations 1, 2 and 3 were derived contained 2.6, 3.3 and 2.6 mg/ml protein, respectively. Other values are expressed as mg per mg Lowry protein. The sialic acid value was obtained from a combined sample of the three preparations. BRBCM, bovine red cell membrane.

Electrophoresis of Milk Fat Globule Membrane Proteins

The proteins of the milk fat globule membrane were fractionated by electrophoresis on acrylamide gels in SDS after solubilization in SDS plus mercaptoethanol as previously described. The MFGM preparations routinely show a pattern of six major bands, as shown for the three preparations described previously (Figure 18). 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 the scheme used in Part I, Chapter III of this dissertation.

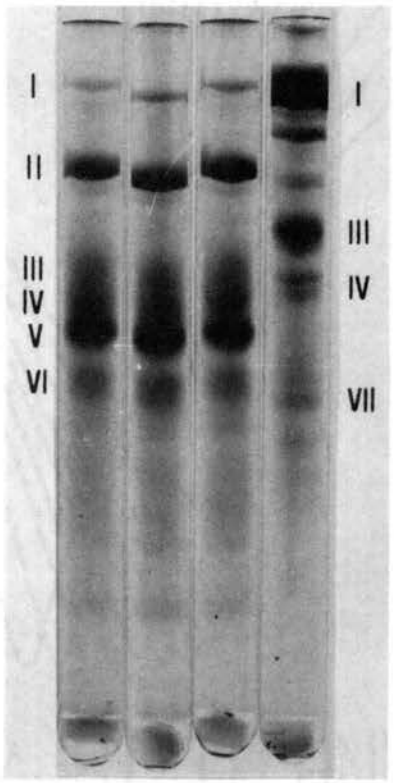
TABLE V

AMINO ACID COMPOSITION OF MFGM AND BOVINE ERYTHROCYTE MEMBRANE[a]

Amino Acid	Mole %	
	MFGM	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

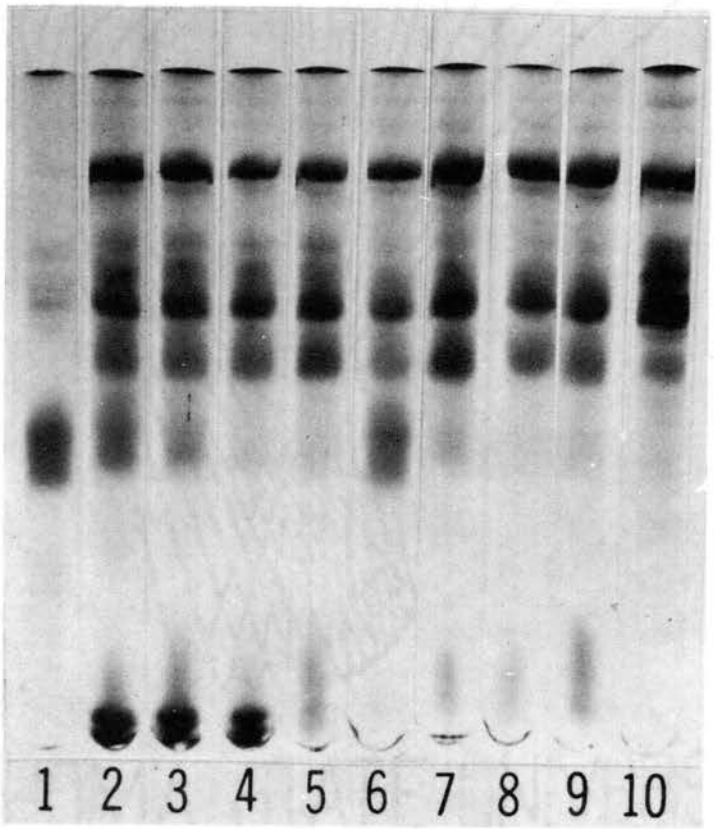
[a]Values are expressed as mole % of amino acids plus amino sugars. Tryptophan and half-cystine were not determined. MFGM values are for an average of three preparations with a standard deviation of less than $\pm$ 3% for each value.

Figure 18. Acrylamide Gel Electrophoresis Patterns of MFGM Showing Six Major Bands. Three gels on left are from different bovine MFGM 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 acetic acid. Numerals to left and right of gels are to designate major MFGM and erythrocyte membrane proteins, respectively.


I
II
III
IV
V
VI
I
III
IV
VII

Brunner (13) has noted in his review that the protein content of MFGM prepared from cream obtained from unchilled (37^{o}) fresh raw milk differs significantly from that obtained when cream from chilled (4^{o}) fresh raw milk is used to prepare MFGM. It was therefore essential to determine if this quantitative difference in protein content resulted in qualitative differences in the protein components observed by electrophoresis. Therefore an experiment was performed to compare cream from milk which had been chilled immediately after collection with that which had not been chilled. Electrophoresis of solubilized cream would then permit determining if any components were adsorbed or lost as a result of chilling. Fresh raw milk as a composite sample was obtained from three Holstein cows. The sample was divided in half, one portion being chilled immediately to 4^{o} (chilled sample) and the other being maintained at 25^{o} (unchilled sample). The cream from the chilled sample was prepared by centrifuging the chilled raw milk at 4^{o}, removing the solid floating cream from the skim milk and suspending it in buffer at 25^{o}. The centrifugation and washing procedure was repeated two additional times. After the initial centrifugation and each washing step thereafter, an aliquot of cream was immediately extracted to obtain the protein by addition of SDS, mercaptoethanol, heated and incubated as described in Part II, Chapter VI, Methods. The unchilled sample was treated similarly except that it was washed with buffer at 37^{o} and centrifuged at 25^{o}. Figure 19 shows the results obtained. The first gel on the far left is a sample of fresh raw milk prepared by the same direct SDS solubilization technique and serves as a representation of starting material. The next four gels represent the chilled cream samples while the following four are the unchilled; the last gel is an MFGM sample prepared from

Figure 19. Acrylamide Gel Electrophoresis Patterns of SDS Extracted Chilled and Unchilled Cream from Fresh Raw Milk. Gel 1 is extract of whole raw milk. Gels 2, 3, 4, 5 represent patterns obtained from SDS extraction of chilled cream after 0, 1, 2 and 3 washes, respectively. Gels 6, 7, 8, 9 are the patterns obtained from the corresponding samples of unchilled cream. Gel 10 is MFGM control pattern prepared from chilled, separated cream. Staining and destaining as in Figure 18.


1 2 3 4 5 6 7 8 9 10

freshly separated cream as usual. Note that all the patterns of the chilled, unchilled and the usual MFGM preparation are virtually the same. This observation suggests that the use of freshly separated cream as obtained and prepared in the chilled state yields the same results as cream washed from fresh raw milk without chilling. In addition, the membrane proteins do not represent contamination by the major proteins of milk plasma, since it can be seen from Figure 19 that the major proteins of whole raw milk run more rapidly in this system than the membrane proteins. This type of direct SDS extraction experiment also has shown that essentially all of the protein can be solubilized (110-120% by Lowry protein analysis) in this manner. The electrophoretic patterns of these extracts differ from the MFGM samples primarily by the addition of a smear of staining material at the SDS front near the end of the gels. 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 SDS directly on cream samples without preparing membranes.

Molecular weights for the major membrane proteins were estimated by comparisons with the migration distances of standard proteins and are shown in Table VI. 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. Components VI did appear as a doublet in some preparations and must represent more than one polypeptide.

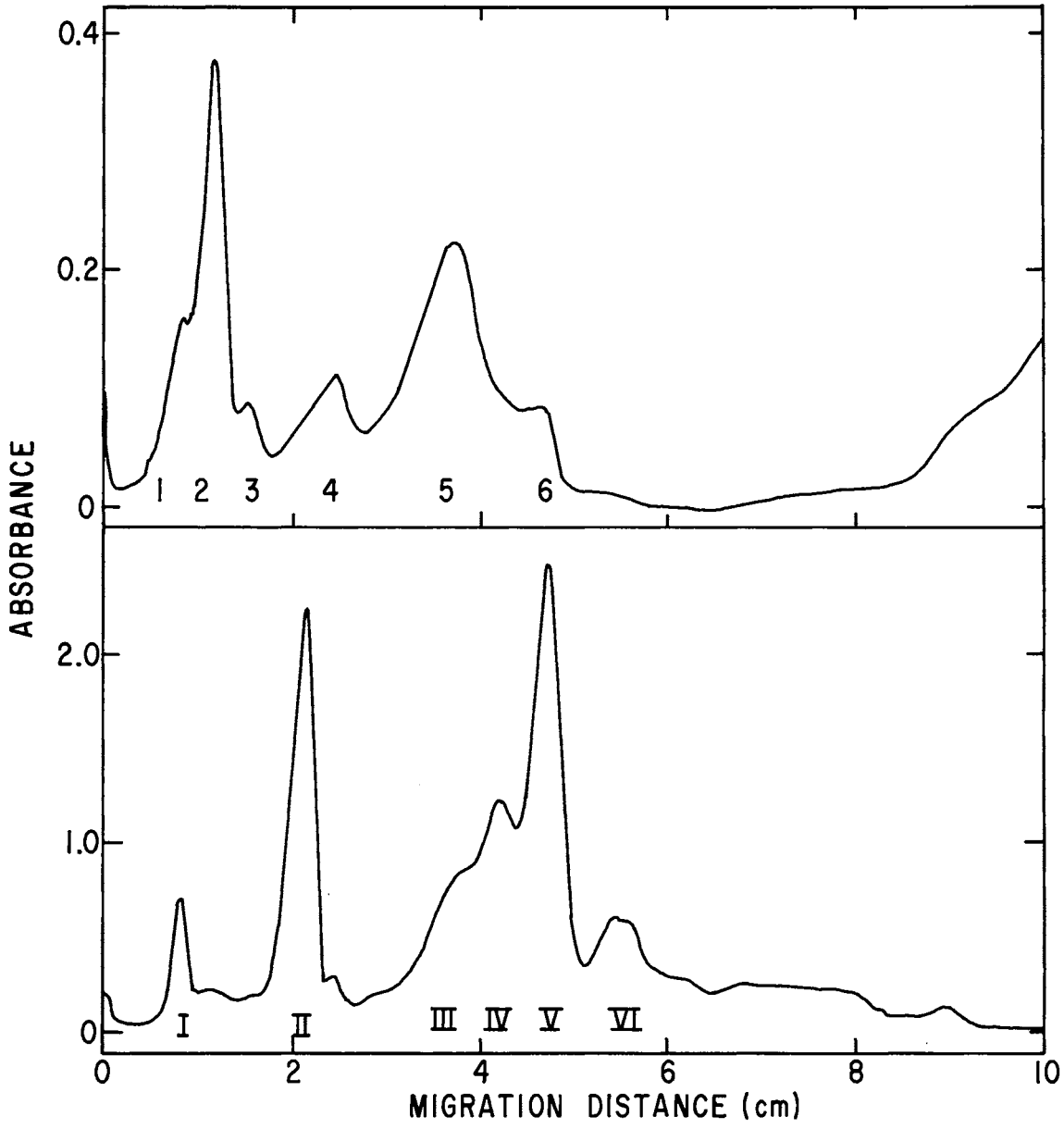
The distribution of glycoproteins in the acrylamide gels was determined by staining by the periodate-Schiff method as previously described. Figure 20 shows the pattern of carbohydrate staining as a densito-

TABLE VI

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

Figure 20. Gel Scans of Carbohydrate (top) and Protein (bottom) Patterns for SDS Gels of MFGM. Protein bands are labeled as in Figure 18. Carbohydrate bands stained by the periodate-Schiff method as described previously.

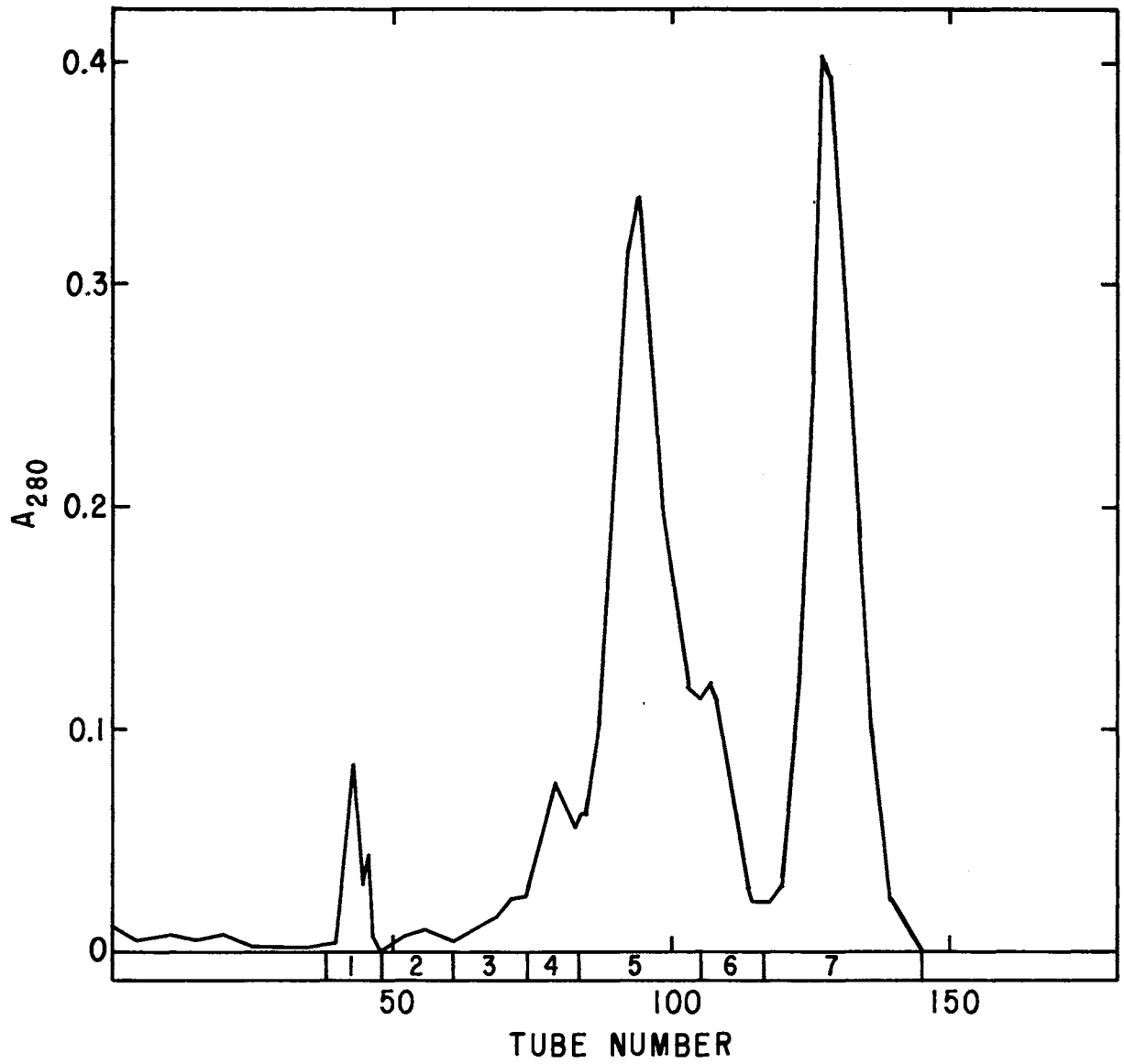

0.4
0.2
0
1 2 3 4 5 6
2.0
1.0
0
I II III IV V VI
0 2 4 6 8 10
ABSORBANCE
MIGRATION DISTANCE (cm)

meter 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 the gel probably represents lipid which is stained by the periodate-Schiff method (37) (38) (39). The six glycoprotein bands do not all correspond to the six proteins stained with Coomassie Blue, although components I and GP-1, III and GP-5, and V and GP-6 overlap and may represent the same species. The major glycoprotein GP-2 is 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 (39) (40) (41). Molecular weights of membrane glycoproteins cannot be accurately determined directly by SDS electrophoresis as for other proteins due to their inadequate ability to bind detergent (42). For erythrocyte membrane glycoproteins, the molecular weight is dependent on the acrylamide concentration in the gels (39) (42) (43). This dependence is also observed for GP-2 and GP-5 of the MFGM. The apparent molecular weight of GP-2 changes from approximately 200,000 daltons on 5% gels to about 80,000 daltons on 12% gels, while the molecular weight of GP-5 varies from 92,000 to 56,000 daltons in going from 5% to 12% gels.

Chromatography of Membrane Preparations in SDS

Further characterization of the MFGM proteins and glycoproteins was accomplished by fractionation by chromatography in SDS on Sepharose 4B. As shown in Figure 21, the major portion of the protein is eluted in one peak which corresponds to a molecular weight of about 66,000 dal-

Figure 21. Sepharose 4B Chromatography of MFGM in 1% SDS. Sample was dissolved in 4% SDS in 0.05 M phosphate (pH 7.8) containing 1% mercaptoethanol. After overnight incubation at room temperature under nitrogen, the sample (10 mg/ml protein) was clarified by centrifugation, brought to 20% glycerol and applied to 2.5 x 90 cm column. Fractions of 3.4 ml were collected and monitored for absorbance at 280 nm. Tubes were combined to yield the fractions indicated by the bar at the bottom of the graph. Fraction 7 contained the disulfide of mercaptoethanol, but did not have a significant quantity of protein.


0.4
0.3
0.2
0.1
0
A_{280}
1
2
3
4
5
6
7
50
100
150
TUBE NUMBER

tons. 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 Figure 21, dialyzed to remove detergent against 40% methanol and subjected to electrophoresis. No protein was detected in fractions 1, 2 and 7. The electrophoretic distributions of the proteins and glycoproteins for the other combined fractions is shown in Figure 22. Both component II and GP-2 were found predominantly in fraction 4. The molecular weight estimated for the major glycoprotein GP-2 by chromatography is substantially lower than that found by acrylamide electrophoresis on 5% gels. The other important glycoprotein GP-5 shows approximately the same molecular weight by chromatography and on 5% gels.

Amino acid analyses of the column fractions are given in Table VII. These clearly show that there are differences in the separated proteins, indicating that the species observed are not aggregates of a single small unit. Fraction 3 contains primarily component I and no amino sugars. Fraction 4 contains primarily components II and GP-1 and 2. It shows a higher content of galactosamine than glucosamine, which is contrary to the finding with the whole membrane. The bulk of the protein is contained in fraction 5, in which the glucosamine to galactosamine ratio is higher than in the MFGM. 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 it does show some carbohydrate.

Figure 22. Acrylamide Gel 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 preparation. 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.

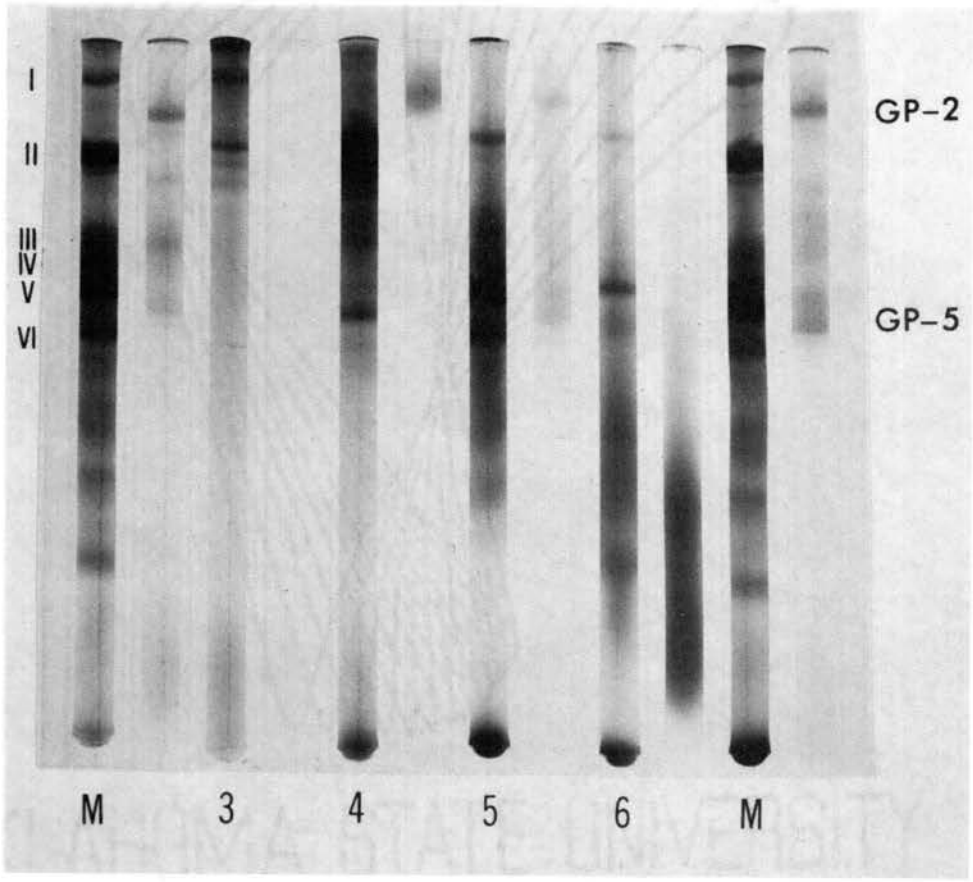

I
II
III
IV
V
VI
GP-2
GP-5
M
3
4
5
6
M

TABLE VII

AMINO ACID COMPOSITIONS OF SEPHAROSE 4B FRACTIONS[a]

Amino Acid	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

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

Extraction of Milk Fat Globule Membrane and Cream Samples

Isolation and partial purification of particular proteins can often be achieved by selective extraction methods. An example of this was the purification of spectrin from erythrocyte ghosts by EDTA extraction (31). A similar procedure was applied to the MFGM to investigate the solubility characteristics of its proteins. Figure 23 shows a flow diagram of the extraction procedure. Acrylamide gels of the various fractions stained with Coomassie Blue are shown in Figure 24. Solubilization was defined operationally as the failure to sediment at 30,000 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 blood 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 GP-2 partitions between extract and pellet in approximately equal amounts, while GP-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 ammonium sulfate (gel 5). GP-2 also appears to remain partially in solution after ammonium sulfate treatment. Essentially the same results in terms of extractability were observed using washed cream rather than MFGM for the material to be extracted.

Additional experiments were performed using 6 M guanidine hydrochloride (32) and 1 M sodium chloride as extracting agents. In each case the soluble material was dialyzed against distilled water. The soluble and insoluble fractions were separated by centrifugation at 35,000 g for 1 hr and were subjected to electrophoresis in SDS. Figure

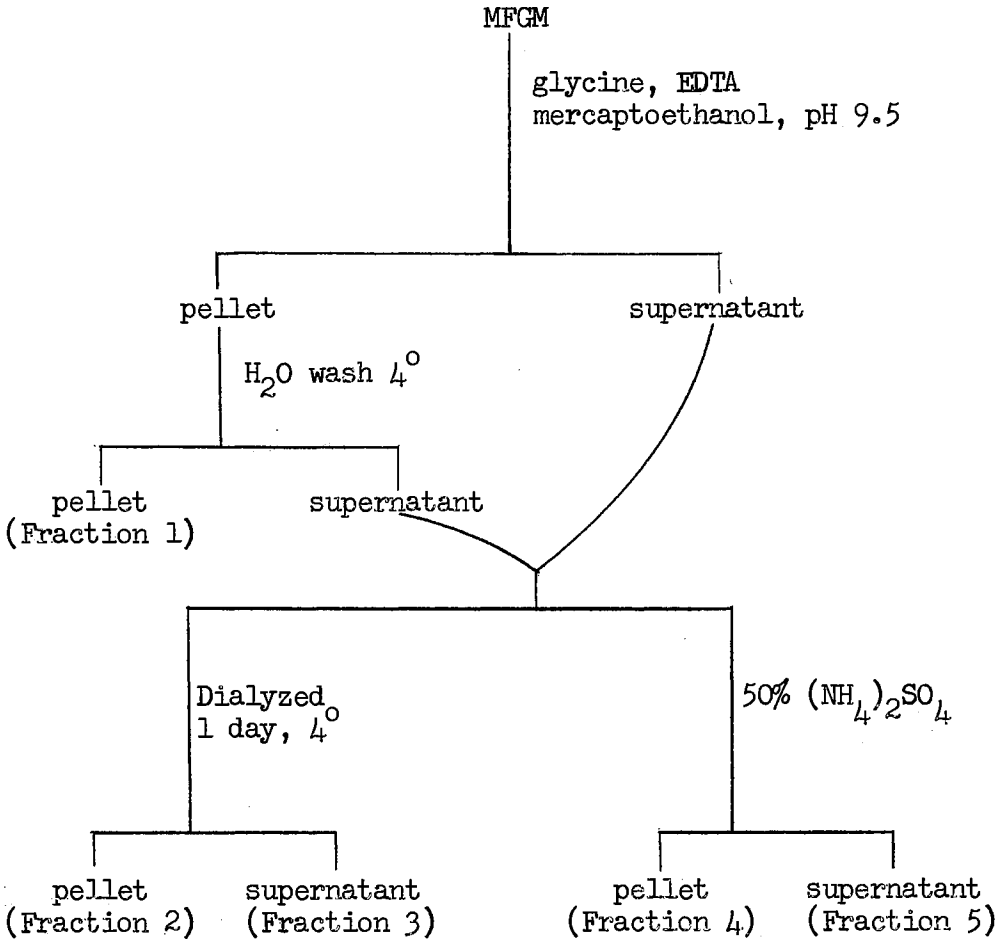
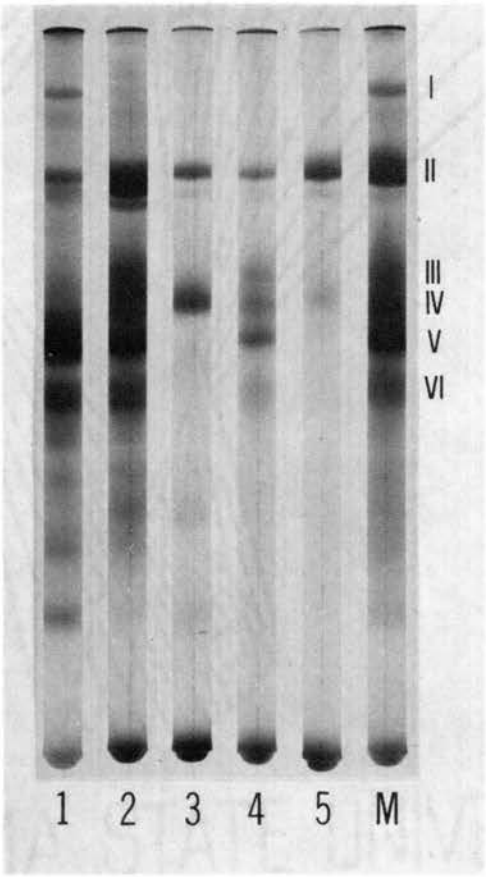


Figure 23. Diagram of Procedure Used for EDTA Extraction of Milk Fat Globule Membranes.

Figure 24. Acrylamide Gel Electrophoresis of Fractions Obtained from EDTA Extraction. Numbers below gels correspond to fraction numbers given in Figure 23.


I
II
III
IV
V
VI
1
2
3
4
5
M

25 shows the patterns obtained from these extractions. All of the MFGM proteins can be partially extracted by either of the two salt treatments. However, only components III, IV and GP-5 remain predominantly soluble after the salt extracts have been dialyzed against distilled water. With guanidine hydrochloride about 70% of the total MFGM protein can be extracted, which is only slightly more than that observed for the erythrocyte membrane. However, there does not appear to be the selectivity in this initial extract that has been observed with the erythrocyte membrane proteins (44). Extraction of 33% cream with 6 M guanidine hydrochloride or 1 M sodium chloride yielded essentially the same results as extraction of MFGM.

Density Gradient Centrifugation of Milk Fat Globule Membrane

Since the origin of the MFGM is still uncertain, more than one type of membrane could possibly be represented in the isolated preparation. Therefore membranes were subjected to a crude fractionation on a discontinuous sucrose gradient. Three distinct areas of membrane material were found in the gradient: a major sharp band (F-3, 38% of the 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 SDS-electrophoresis and showed the patterns in Figure 26. 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 compo-

Figure 25. Acrylamide Gel Electrophoresis of Water Soluble and Insoluble Fractions of the NaCl and Guanidine Extracts of MFGM. Membranes were extracted with 1 M sodium chloride or 6 M guanidine hydrochloride as described. 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, MFGM control; and gel F, bovine erythrocyte membrane. Staining and destaining as described in Figure 18.

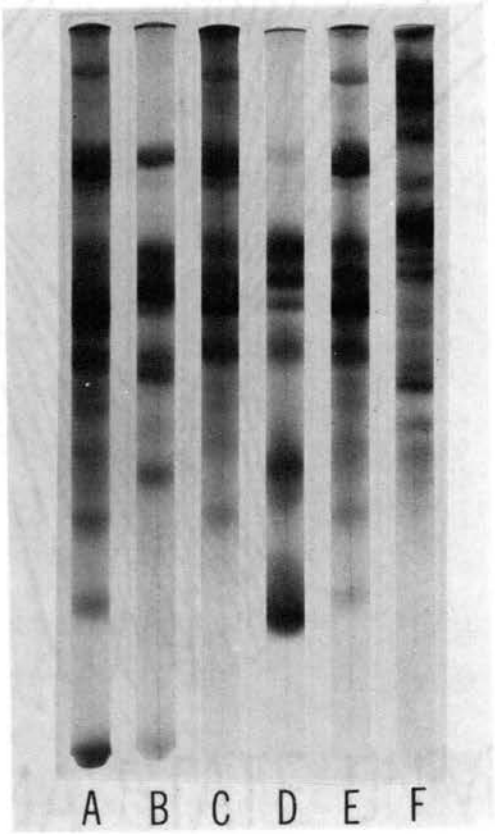
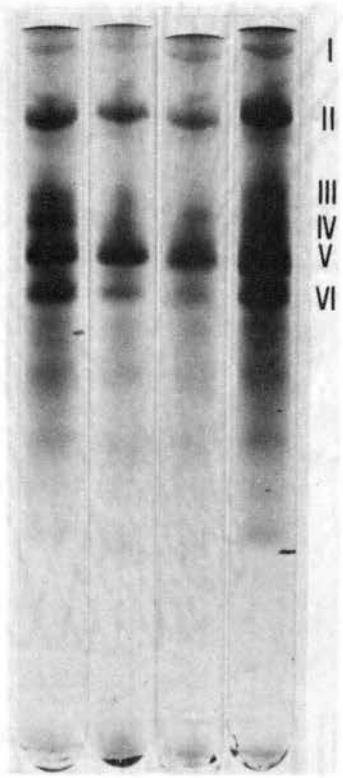

A
B
C
D
E
F

Figure 26. Acrylamide Gel Electrophoresis of Density Gradient Fractions. Gels contain (left to right): fraction 1, 2, 3 and MFGM. Stained and destained as described in Figure 18.


I
II
III
IV
V
VI

nents with differing amounts of associated fat.

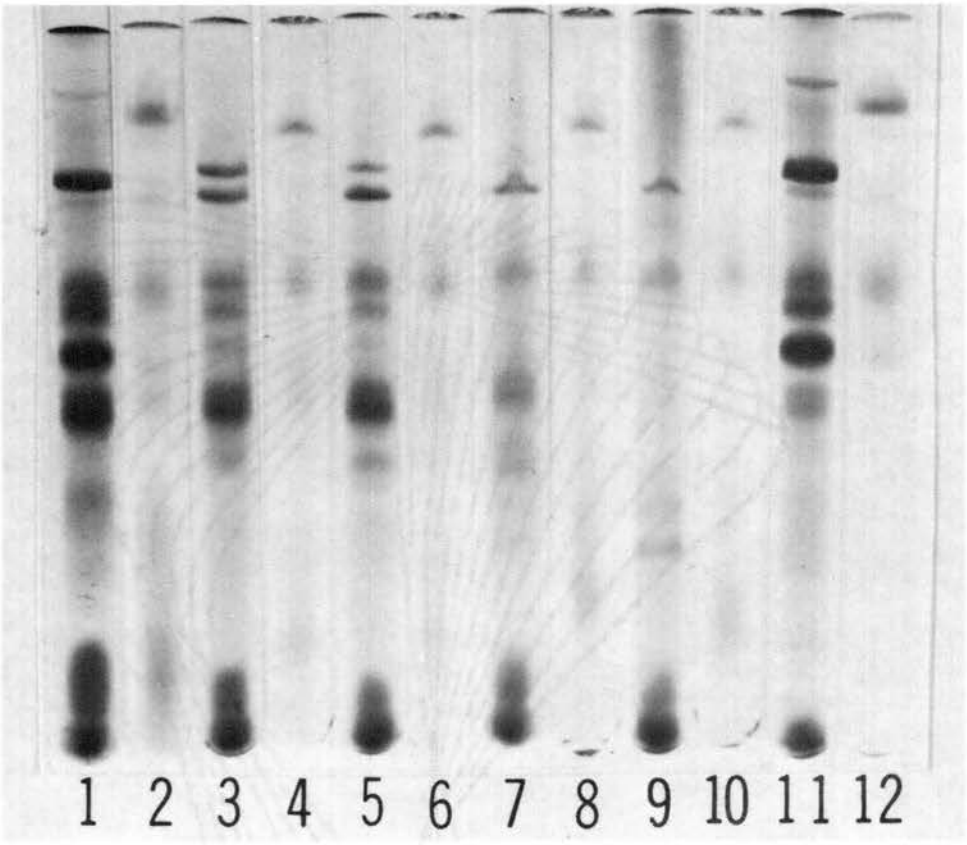
Enzyme Activities Associated with the Milk Fat Globule Membrane

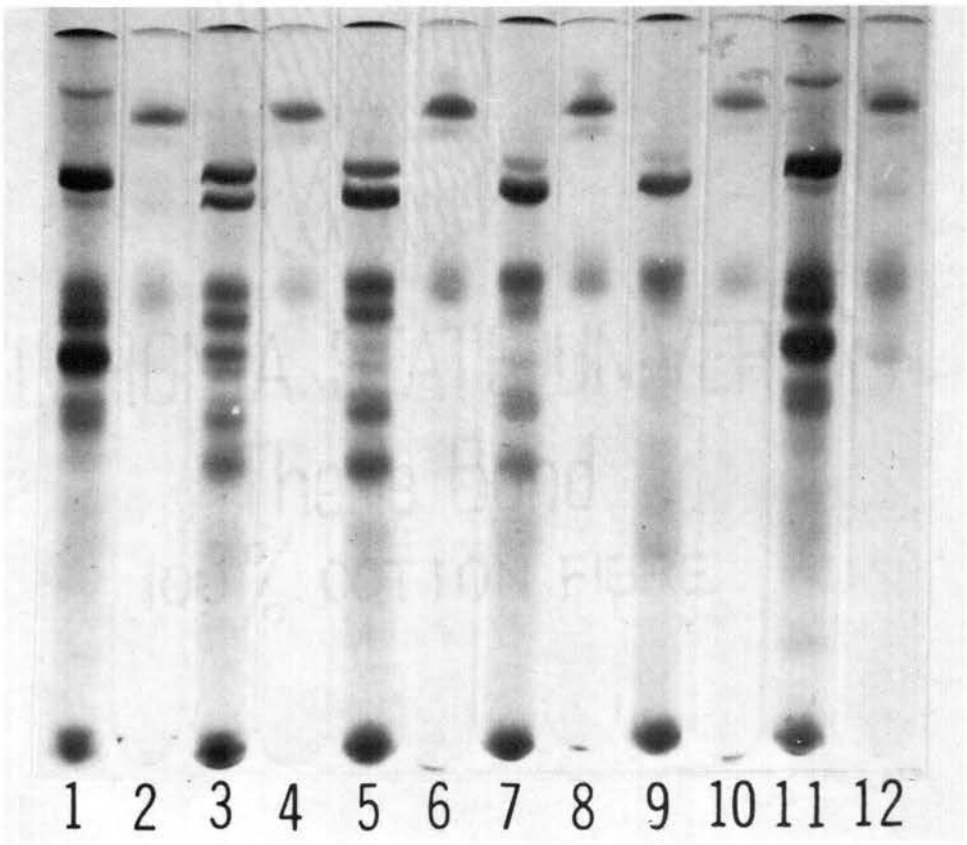
To study further the membranous nature of MFGM, samples of washed cream and isolated MFGM were assayed for the selected plasma membrane marker enzymes 5'-nucleotidase (33) and ATPase (34). Xathine oxidase (35) was also measured. Typical values obtained, when release of product versus time was linear, for 5'-nucleotidase (as nmoles phosphorous released per mg protein per min) were 300 to 350 for both the cream and MFGM. The (Na^+-K^+-Mg^{2+})-activated ATPase had values of 12-15, but the activity in the presence of Na^+, K^+, Mg^{2+}, Ca^{2+} was reduced to 7-11 for cream and MFGM (as nmoles phosphorous released per mg protein per min). The latter activity indicates that addition of exogenous Ca^{2+} to the system inhibits the total ATPase activity. The values obtained for xanthine oxidase (as μmoles TTC reduced per mg protein per min) ranged from 7 to 10 for cream and MFGM. This assay was the most variable of the three and the values obtained from it are subject to some question due to general scatter for the standard curve.

Trypsin and Pronase Digestion of Cream and Milk Fat Globule Membrane

Cream and isolated MFGM were subjected to trypsin or Pronase digestion (36) in an effort to ascertain if any obvious differences existed between the intact system versus the isolated membrane as determined by SDS-electrophoresis. Figure 27 shows the results of exposing cream and MFGM to trypsin over a range of 1-100 μg trypsin/ml incubation mixture.

Figure 27. Acrylamide Gel Electrophoresis of Cream and MFGM Digested with Trypsin. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate, respectively. Gels 1, 2 are a digestion control (no enzyme added) while 3, 4; 5, 6; 7, 8; and 9, 10 represent samples which were digested at 1 μg, 5 μg, 25 μg and 100 μg trypsin/ml, respectively. Gels 11, 12 are MFGM control. Top, cream sample; bottom, MFGM sample.

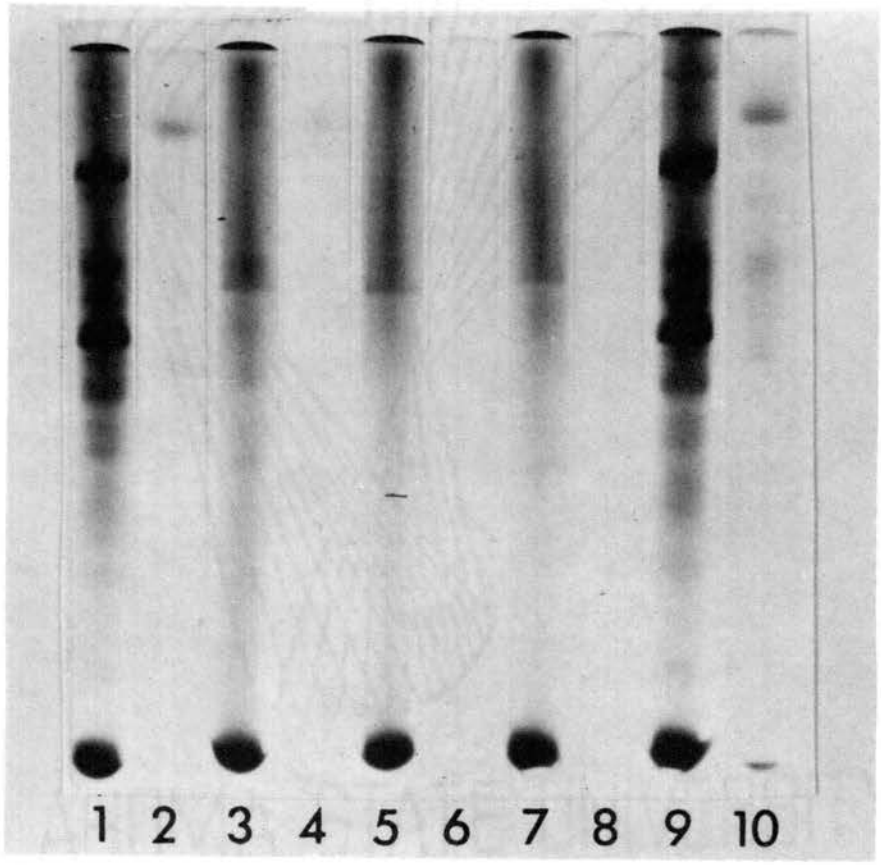

1 2 3 4 5 6 7 8 9 10 11 12


1 2 3 4 5 6 7 8 9 10 11 12

In both systems, as the concentration of trypsin is increased, component II is selectively and increasingly degraded into a component of lower molecular weight on the Coomassie Blue gel patterns. In addition, components IV and V are removed at the lower concentrations of trypsin used while component III remains relatively intact even at the highest concentrations of enzyme. The PAS (carbohydrate) gel patterns remain essentially unchanged across the range of trypsin used.

Figure 28 shows the results of the same type of experiment performed on MFGM with Pronase, which has a broader specificity than does trypsin. Even at the lowest concentration of Pronase used, all bands of both the Coomassie Blue and PAS stained gels were lost. Again the same results were obtained for both the cream and MFGM digestion studies.

Figure 28. Acrylamide Gel Electrophoresis of MFGM Digested with Pronase. Samples are arranged (left to right) with alternating gels stained for protein and carbohydrate. Gels 1, 2 are a digestion control (no enzyme added) while 3, 4; 5, 6; and 7, 8 represent samples which were digested at 5 μg, 25 μg and 100 μg Pronase/ml, respectively. Gels 9, 10 are MFGM control.


1 2 3 4 5 6 7 8 9 10

CHAPTER VIII

DISCUSSION

This study was undertaken to investigate the proteins and glycoproteins which are associated with the milk fat globule membrane. Membranes were routinely obtained by a freeze-thaw procedure from washed cream, which was derived from chilled milk. It is difficult to compare this preparation with those obtained by other investigators because of the variability of the previous parameters reported and the variations in methods of reporting data. The results reported by the author differ significantly from some of the data reviewed by Brunner (13). Yield and amino acid compositions of MFGM contain noteworthy disparities. In particular, Brunner noted differences in yield of MFGM proteins as a function of whether the cream was prepared from the chilled or unchilled state. To determine if these differences result from the state of the cream, experiments were performed on cream from chilled raw milk and cream from unchilled raw milk. After washing and extracting the protein directly from the cream with hot SDS, the electrophoretic patterns of the preparations were observed to yield virtually identical protein patterns from either chilled or unchilled cream samples. These patterns were essentially the same as the usual MFGM preparation patterns. This finding suggests that cream obtained by separation of chilled milk does not differ in its proteins from cream separated in the unchilled state. The possibility that proteins are specifically adsorbed from the whey

fraction cannot be completely excluded, although this process does not appear to be significantly temperature dependent. This type of direct SDS extraction experiment also demonstrated that essentially all of the protein could be solubilized from cream to give the entire complement of MFGM proteins and, vice versa, that all of the cream proteins are contained in the MFGM fraction. This technique presents the opportunity to study changes in the MFGM proteins without the necessity of isolating the membrane.

In view of its possible derivation from the plasma membrane, there was interest in comparing the properties of the MFGM with other plasma membranes. Analytical comparisons with bovine erythrocyte membranes show that the author's MFGM 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 MFGM. The amino acid compositions of the MFGM and erythrocyte membranes are remarkably similar. Although amino acid composition of protein mixtures are of limited usefulness in characterization studies, 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 (45).

Proteins of the MFGM were fractionated by SDS-acrylamide gel electrophoresis as a means of physical characterization and classification. At least six major proteins were detected by staining with Coomassie Blue. These range in molecular weight from 53,000 to 240,000 daltons. Staining by the periodate-Schiff procedure also gave six bands, three of which are clearly different from the protein bands. Whether the overlapping protein and glycoprotein bands represent different molecular

species was not determined although preliminary isolation studies indicate that component III and GP-5 may be identical. By examination of a large number of membrane preparations it was shown that the major protein (component V) and glycoprotein (GP-2) are the most invariant of the proteins or glycoproteins in terms of their occurrence in the isolated membrane. Other components were less prominent in some preparations. This was especially true of the minor glycoproteins.

It was of particular interest to observe the properties of the major glycoprotein (GP-2) of the MFGM, 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 SDS-electrophoresis. Both of the properties are exhibited by the major membrane glycoproteins of erythrocytes from a number of different animal species (39). The failure to stain for proteins has been reported for membrane glycoproteins of rat liver and kidney brush borders (42). The anomalous staining and electrophoretic migration apparently results 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 MFGM proteins which might be used for the isolation of individual proteins. The initial extraction with EDTA, sodium chloride or guanidine hydrochloride resulted in a nonselective partial solubilization or dispersion of most of the proteins from either MFGM or cream. After dialysis against distilled water, some selectivity based on solubility was noted. Components II and IV remained soluble after EDTA treatment

and dialysis against water. Component II was not precipitated by ammonium sulfate from the EDTA extract. Components III and IV were 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. The results 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 MFGM in the secretory cell has still not been completely resolved. The MFGM preparations used in this study show activities of 5'-nucleotidase (a plasma membrane marker enzyme), ATPase and xanthine oxidase similar to those already reported (21) (22) (35). Martel-Pradal and Got (23) have recently reported the presence of marker enzymes for the Golgi apparatus and endoplasmic reticulum, as well as the plasma membrane, in MFGM preparations from human colostrum. Attempts to fractionate MFGM preparation by sucrose density centrifugation into different membrane types were not successful, as judged by the protein compositions of the fractions. Such fractionation attempts were complicated by neutral fat adhering to the membrane. Although the author's 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, especially when one considers the possibility of significant "signet" (9) formation during the secretory process and the variability of the cream from different species.

If the membrane which surrounds the milk fat globule is intact, as it is in the erythrocyte, it could be anticipated that some of the

protein components would be more accessible than others due to the existence of a permeability barrier. To investigate this possibility, both washed cream globules and isolated MFGM were exposed to trypsin digestion. The results obtained indicate that all components of the intact globule and the MFGM are accessible to the enzyme. This observation suggests that there are no buried membrane components in the cream globule which are made more accessible after MFGM isolation. The supportive experiment performed with Pronase again gave the same kind of results as did the trypsin experiment. In both the Pronase digested cream and MFGM samples, complete elimination of all recognizable protein and glycoprotein patterns of polyacrylamide gel electrophoresis was noted. These observations are not in accord with models which suggest the presence of an intact semi-permeable membrane surrounding the fat globule. If this were the case, it would be expected that the proteins inside the membrane permeability barrier would be protected from digestion, as in the case of the red blood cell (41). These results are more consistent with the electron microscopic model of Wooding (11), in which the MFGM of isolated cream consists of a structureless secondary coat of protein at the fat surface topped by "blebs" or patches of plasma membrane, which are not continuous over the entire surface of the milk fat globule. This kind of arrangement of the MFGM could allow for the accessibility of large molecules (such as enzymes) to all of the membrane proteins, since no structural permeability barrier exists.

The studies described 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. However, until more definitive work on the characterization of the MFGM as to origin has been accom-

plished, extreme caution must be exercised in extrapolating physical, chemical and enzymatic results obtained from washed cream or isolated MFGM back to the secretory cell plasma membrane.

CHAPTER IX

SUMMARY

Erythrocyte membranes from several species were prepared by three different methods of hypotonic hemolysis and examined for variations in protein and glycoprotein content by acrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). Significant morphological variations were noted in some of the membranes prepared by the different methods. A similar pattern of nine common band areas was observed for all species on electrophoresis gels stained for protein. The common pattern was generally present even for membranes which showed morphological differences as a result of the method of preparation employed. The differences in protein pattern which were noted were attributed to proteolytic digestion of membranes which were fragmented during preparation. Failure to remove white blood cells from membrane preparations was shown to be a significant source of the problem with proteolytic digestion. Glycoproteins were analyzed by periodate-Schiff staining of SDS-acrylamide gels. Each species appears to have a different major glycoprotein. Molecular weights of glycoproteins calculated from acrylamide gel electrophoresis in SDS were found to vary with the percentage of acrylamide in the gel, indicating that these proteins do not behave in a normal fashion in this electrophoresis system. The replacement of membrane cholesterol with ^{14}C-cholesterol permitted the assignment of the lipid band obtained on SDS-acrylamide electrophoresis gels.

Acetic anhydride was used as a reagent to investigate the relative reactivities of various components within the human red blood cell membrane. The reagent penetrates the membrane rapidly and reacts with intracellular and membrane proteins as well as lipid amino groups. To determine the relative reactivities of the various components in intact and disaggregated membranes, a double labeling procedure with ^{14}C- and ^{3}H-labeled reagents was performed. Either whole red cells or intact ghosts were initially reacted with reagent containing one of the radioisotopes. After hemolysis and appropriate washings, the singly labeled membranes were treated with reagent containing the second isotope in the presence of SDS. Comparisons of the relative reactivities of the membrane components in the intact and disaggregated states were then made by fractionation of the membranes by SDS-acrylamide gel electrophoresis and analysis of the gels for the two radioisotopes. This procedure separated the membrane into a lipid fraction plus a number of protein components. The results of the double labeling indicate that there is little difference in the relative reactivities of the membrane protein components that can be attributed to their incorporation into the membrane structure. The lipid fraction did show a change in its relative reactivity toward acetic anhydride when the membrane was disaggregated which was dependent on whether whole red cells or intact ghosts were initially labeled. Column chromatography in SDS of the double labeled membrane material indicated similar results. Diazotized sulfanilic acid (a protein reagent which penetrates the membrane barrier of intact erythrocytes slowly) labeling studies of human and bovine erythrocytes indicate that the glycoprotein of each species is the only major membrane protein which is readily accessible at the cell surface. When

cells were digested with trypsin before labeling, a second less accessible protein was found to be located at the membrane surface. Neuraminidase treatment decreases the intensity of the periodate-Schiff band, but had no effect on the observed labeling pattern. All of the labeling results suggest that the structure of the membrane of the intact cell may be different from that of the isolated ghost and that caution should be exercised in interpreting studies on isolated membranes in terms of the structure of the membrane of the intact cell.

Acrylamide gel electrophoresis and Sepharose 4B column chromatography in SDS were used to investigate the proteins and glycoproteins associated with the milk fat globule membrane (MFGM). Electrophoresis followed by staining for proteins shows the presence of six major bands. 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 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 for protein and its calculated molecular weight on acrylamide electrophoresis in SDS varies with acrylamide percentage. Extraction experiments with EDTA, sodium chloride and guanidine hydrochloride led to differential solubilization of membrane proteins. Direct solubilization of membrane proteins in SDS was achieved by incubating cream with the detergent. The enzymes 5'-nucleotidase, ATPase and xanthine oxidase were found to be associated with cream and isolated MFGM. Trypsin and Pronase digestion of cream and MFGM suggest that no true permeability barrier exists around the

milk fat globule and it is therefore not an intact membrane system as had been previously suggested.

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