CONTROL OF CELL SURFACE ORGANIZATION

IN ASCITES TUMOR CELLS

By

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LIST OF ABBREVIATIONS

Å	-	Angstrom, 10^{-8} cm
ASGP	-	Asialoglycoprotein
ATP	-	Adenosine triphosphate
^{o}C	-	Degrees centigrade
cm	-	Centimeter
CPM	-	Counts per minute
Con A	-	Concanavalin A
D Buffer	-	0.7 M guanidine HCl, 0.5 M sodium acetate, 0.5 mM $CaCl_2$, 0.5 mM ATP, 0.5% Triton X-100, 0.01 mM PMSF, pH 7.6 Buffer
DOC	-	Deoxycholate
DNase	-	Deoxyribonuclease
DPM	-	Disintegrations per minute
EDTA	-	Ethylenediamine tetraacetate
F-Actin	-	Filamentous actin
g	-	Gravity
G-Actin	-	Globular actin
γ-GGTP	-	γ-glutamyl transferase
GEM	-	5 mM glycine, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 9.5 buffer
>	-	Greater than
hr	-	Hour
IOV	-	Inside-out vesicles
K	-	Thousand
<	-	Less than

M - Molar

mM - Millimolar

Mr - Molecular weight

MAT - Mammary ascites tumor

MF1 - Membrane fraction 1 (Tris extraction)

MF2 - Membrane fraction 2 (GEM extraction)

MF2P - MF2 S buffer pellet fraction

MF2R - MF2 Triton residue

MF2S - MF2 S buffer supernate fraction

mg - Milligram

ml - Milliliter

nm - Nanometer

PMSF - Phenylmethylsulfonyl fluoride

PC - Phosphatidyl choline

PBS - 120 mM NaCl, 5 mM KCl, 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 0.5 mM $MgCl_2 \cdot 6\ H_2O$ pH 7.4 buffer

PTA - Phosphotungstic acid

PAGE - Polyacrylamide gel electrophoresis

RBC - Red blood cell

rpm - Revolutions per minute

S Buffer - 5 mM Tris, 0.15 M NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythritol, 0.5% Triton X-100, 0.01 mM PMSF, pH 7.6 buffer

SEM - Scanning electron microscopy

SDS - Sodium dodecyl sulfate

TEM - Transmission electron microscopy

µCi - Microcurie

µg - Microgram

µl - Microliter

µm - Micrometer

CHAPTER I

INTRODUCTION

The organization of components at mammalian cell surfaces is believed to be important in a number of recognition processes including those involved in developmental phenomena, immune destruction mechanisms and neoplastic disease (27, 41, 42, 43). In fact, there is no phase of cell behavior that can be unequivocally stated to be free of the influence of the cell surface. Although the dynamics of this organization are clearly important, the mechanisms by which cell surface molecules are moved or are restricted in movement are still poorly understood.

Mobility of Cell Surface Receptors

The dynamic nature of the animal cell surface was demonstrated by observation of movement of cell surface antigens in fused heterokaryons (21) and in cells labeled by indirect immunofluorescence with antisera against cell surface antigens (55). This movement was consistent with the ability of motile cells to transport particles over their dorsal surfaces. This dynamic feature was incorporated into the "fluid mosaic model" of membrane structure (51) in which it was assumed that proteins and lipids have lateral mobility in the plane of the membrane.

Although the model served its original purpose to stimulate

thought about the dynamic nature of the cell surface, it was soon clear that the proposal was much too simple. Two different mobility phenomena could be distinguished: 1) patching, which involved relatively short-range movements and was energy-independent; and 2) capping, which required long-range movements and energy from cell metabolism (40). In general, receptor movements are decreased by lower temperatures or by fixation of the cells. Different antigens behave differently under conditions which cause capping. Some cap essentially completely; others do not. Caps are formed on different regions of the cell, depending upon the cell type and the particular antigen (52). Thus there is a selectivity to the surface movement phenomena, as also exhibited by differences in the uptake of cell surface components during phagocytosis (58).

Redistribution of cell surface components is not limited to antigens. Multivalent lectins, which are able to bind and crosslink cell surface carbohydrates, are also able to effect patching and capping of their receptors in some cells (7). For example, Concanavalin A (Con A) promotes patching of its receptors in transformed (but not most non-transformed) fibroblasts (45). Treatment of lymphocytes with low concentrations of Con A causes capping. However, capping of both Con A receptors and antigens is inhibited in lymphocytes in the presence of higher Con A concentrations (7, 62), except in the presence of colchicine (7, 19), which disrupts cytoplasmic microtubules (61). Such experiments led to the postulation of a colchicine-sensitive submembrane assembly which is involved in "modulation" of cell surface receptors (20). If Con A linked to an insoluble support such as nylon fibers is allowed to interact with lymphocytes, it causes immobilization of their

antigens (46). From such results it was suggested that a "global" network of submembrane elements, a cellular cytoskeleton, controls the state of cell surface receptors.

Association of Cytoskeletal Elements with the Plasma Membrane

There are several lines of evidence for a cytoskeleton-membrane interaction. The drugs, cytochalasin B and colchicine, disrupt cytoskeletal elements (18, 40, 47) and have been shown to alter the patching-capping phenomena. Cytoskeletal proteins are major constituents of plasma membrane preparations (25). Transmission electron microscopic studies visualized cytoskeletal structures associated with plasma membranes. In addition, immunofluorescence-microscopy using antibodies against cytoskeletal proteins demonstrated concomitant redistribution of cell surface receptors and cytoskeletal elements (1, 24, 57). Perhaps the best example of a cytoskeletal-membrane interaction is provided by the work done by Ji and Nicolson on the erythrocyte membranes (28, 29). Spectrin, a cytoplasmic-membrane associated protein, does not readily cross-link when normal erythrocytes are treated with dimethylmalonimidate. When cell surface molecules are induced to patch or cap, spectrin can be cross-linked. Furthermore, if spectrin is cross-linked by an antispectrin antibody, patching can be seen on the cell surface (28, 29). These results strongly suggest an interaction between the cell surface and cytoskeleton.

Composition of the Cellular Cytoskeleton

There are three types of elements which appear to contribute to the cytoskeleton: microtubules, intermediate filaments and microfilaments. Microtubules are hollow cylindrical structures approximately 24 nm in diameter (23) and they are composed of tubulin, a protein of about 55,000 daltons (48). Intermediate filaments average about 10 nm in diameter and are often localized around the nucleus (9) in non-muscle cells. The intermediate filament subunits vary in different tissues; vimenten and desmin of fibroblasts and muscle cells, respectively, are about 55 K. Prekeratin filaments of epithelial cells have polypeptides ranging from 40-70 K (9). Microfilaments can be identified as stress fibers in spread fibroblasts, as a network of filaments underneath the membrane of the dorsal surface of spread cells (36) or as filamentous core bundles in brush border micorvilli (9). Microfilaments are primarily composed of polymerized actin.

Actin is a ubiquitous protein of approximately 43,000 daltons. It is known to exist in two different states: G-actin, the globular, soluble form, and F-actin, the filamentous, polymerized form, which predominates in muscle. In non-muscle cells G-actin is found in cell extracts and can be quantitated by electrophoresis after sedimentation of insoluble actin (10) or by analysis of its inhibition of DNase (8). The insoluble actin exists in several forms, such as bundles or meshwork of filaments (2). However, actin is also found associated with membranes in an insoluble form (3, 35, 37, 44). In the case of the erythrocyte membrane actin is present as a significant component, but no F-actin-like filaments can be observed by electron microscopy (56).

It has been postulated that erythrocyte actin exists as oligomeric units (11, 17), possibly similar to short lengths of F-actin, based on the ability of actin-containing extracts to accelerate G-actin polymerization by nucleation (11). Direct observation of the actin oligomers has not been possible, because the erythrocyte actin is present as a macromolecular complex with the erythrocyte cytoskeletal protein spectrin (12).

Cytoskeletal Models

Two of the best understood cytoskeletal systems are the erythrocyte membrane and brush border microvilli.

Erythrocyte

A submembrane cytoskeleton of erythrocytes can be obtained as the residue after extraction of RBC membranes with mild nonionic detergents. The shape of the residue corresponds closely to the shape of the cell from which it was obtained (53). The major components of this residue are spectrin, actin, ankyrin (band 2.1) and band 4.1. Bands 3, 4.2, 4.9 and 7 are partially retained in the residue. (Numerical designation according to Fairbanks (22).) However, further extraction of this residue indicates that only spectrin, actin, band 4.1 and possibly 4.9 are necessary for structural integrity of the residue (53). Spectrin and actin can be eluted from RBC ghosts at alkaline pH and low ionic strength. The soluble material contains dimers and tetramers of spectrin as well as a large complex of spectrin, actin and 4.1. Spectrin is a heterodimer with an elongated structure that forms end-to-end tetramers but not higher polymers. Mixing purified spectrin

tetramer and band 4.1 with F-actin causes it to gel. Both actin and 4.1 appear to bind near the end of the spectrin molecule (59). Since actin apparently must be polymerized to complex with spectrin, and actin filaments are not found in RBC membranes, it has been suggested that actin must exist in the RBC as short filaments (33).

The mode of association of spectrin with the membrane was discovered in the course of studying the binding of spectrin to spectrin-depleted inside-out erythrocyte membrane vesicles (IOV). Chymotryptic treatment of IOV prevented spectrin binding by releasing an Mr 72,000 protein, which associated with spectrin (5). Peptide mapping and immunological comparisons indicated that the 72 K fragment was derived from band 2.1 (32), which was then named ankyrin to indicate its role as a linking element between the membrane and cytoskeleton. If spectrin-depleted membranes are extracted with detergent, ankyrin is found associated with band 3 in the detergent solution. Although only 10-20% of the band 3 molecules are associated with ankyrin, no chemical differences were observed between the associated and non-associated species. The picture of the erythrocyte membrane which emerges is of a bilayer through which are inserted glycoproteins accessible at both the inner and outer membrane faces. Some of these associate with an underlying meshwork connected to the membrane at least by the ankyrin molecule. The meshwork is composed of spectrin-actin-4.1 complexes. Other components may be involved in the meshwork, as additional linking elements and in the membrane.

Brush Border

A second system which has been often used for investigation of

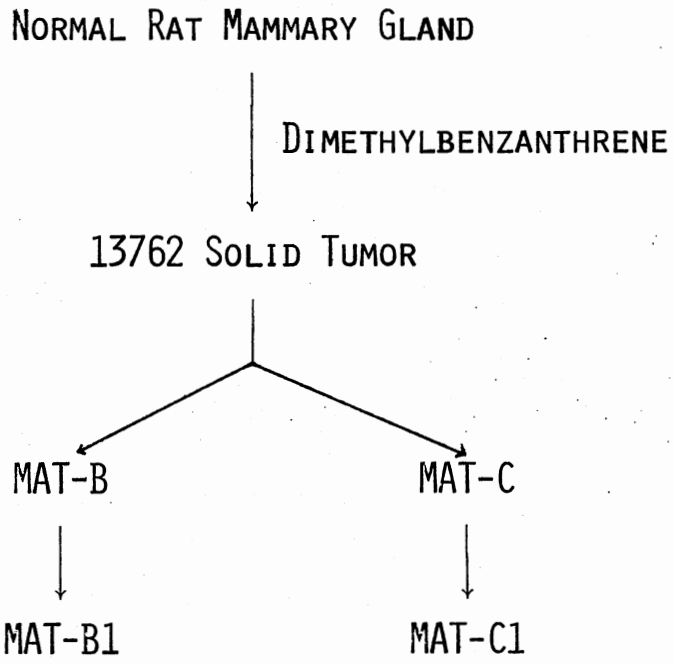
membrane-cytoskeleton interactions is the intestinal brush border. Microvilli from brush border are highly organized structures containing a core of microfilaments associated with the covering plasma membrane at the microvillus tip and by lateral cross bridges. The nature of the linking proteins has been a matter of some controversy. Early immunofluorescence studies suggested that α-actinin was the linking molecule. More recent studies have failed to verify the presence of α-actinin in the microvilli. Triton treatment removes the microvillar membrane, leaving a core composed of 5 major polypeptides: 110 K, 95 K, 68 K (fimbrin), 42 K (actin), and 16.5 K (13). Fimbrin is also found in ruffles and microvilli of other cells (13, 34). Further extractions provide evidence that polypeptides of 110 K and 16.5 K are involved as lateral arms, and a polypeptide of 95 K is involved in the formation of actin filament bundles (34).

Both the erythrocyte and brush border are highly differentiated, highly specialized systems. Their components and organization may be unique. Therefore, it is desirable to examine other systems which are less specialized.

13762 Tumor Line

The 13762 solid rat mammary tumor arose from normal gland when treated with dimethylbenzanthrene. At Mason Research Labs, this solid tumor was developed into an ascites form and several sublines were produced, including the MAT-B and MAT-C sublines. While these cell lines were being carried at Oklahoma State University, two more stable varients were obtained, the MAT-B1 and MAT-C1 sublines (Figure 1).

Figure 1. Tumor Family Tree of the MAT-B1 and MAT-C1 Sublines.


NORMAL RAT MAMMARY GLAND
DIMETHYLBENZANTHRENE
13762 SOLID TUMOR
MAT-B
MAT-C
MAT-B1
MAT-C1

The MAT-C1 subline has a unique morphology. The cell surface, observed by scanning electron microscopy, is covered with highly branched microvilli extending from the cell body (Figure 2B, C) (15). Concanavalin A (Con A) receptors on the microvilli appear immobile by fluorescence microscopy even after treatments of the cells with colchicine, cytochalasin B, hypotonic buffer or protease (15, 26). Moreover, the microvilli are not disrupted by these treatments (26) and the subline is xenotransplantable (Table I). In contrast the MAT-B1 subline has highly mobile Con A receptors, and its morphology is grossly altered by treatments with cytochalasins and hypotonic buffer, which cause a loss of microvilli (26). It has been postulated that the reduced mobility observed for MAT-C1 Con A receptors results from their association with the microvilli and that the MAT-C1 microvilli are stabilized by some cellular component.

The purpose of this work was to identify the stabilizing component and to investigate its mechanism of control. To approach this problem, microvilli have been isolated from both MAT-B1 and MAT-C1 cells. Electrophoretic comparisons show the presence of a polypeptide of Mr 58,000 present in MAT-C1 microvilli but greatly diminished or absent in MAT-B1 microvilli. This protein is associated both with membrane fractions obtained by fragmenting the microvilli and with the Triton-insoluble microvilli and MF2 cytoskeletal residues. In addition, oligomeric actin units, either small F-actin fragments or actin associated in a non-polymerized conformation, have been identified in the MF2 fraction.

Figure 2. Scanning Electron Micrograph of A) MAT-B1 and B,C) MAT-C1 Cells.

Magnifications: A) 6,000, B) 4,900, C) 17,500.
Bars = 2 μm.
(Photo by Dr. John Huggins)

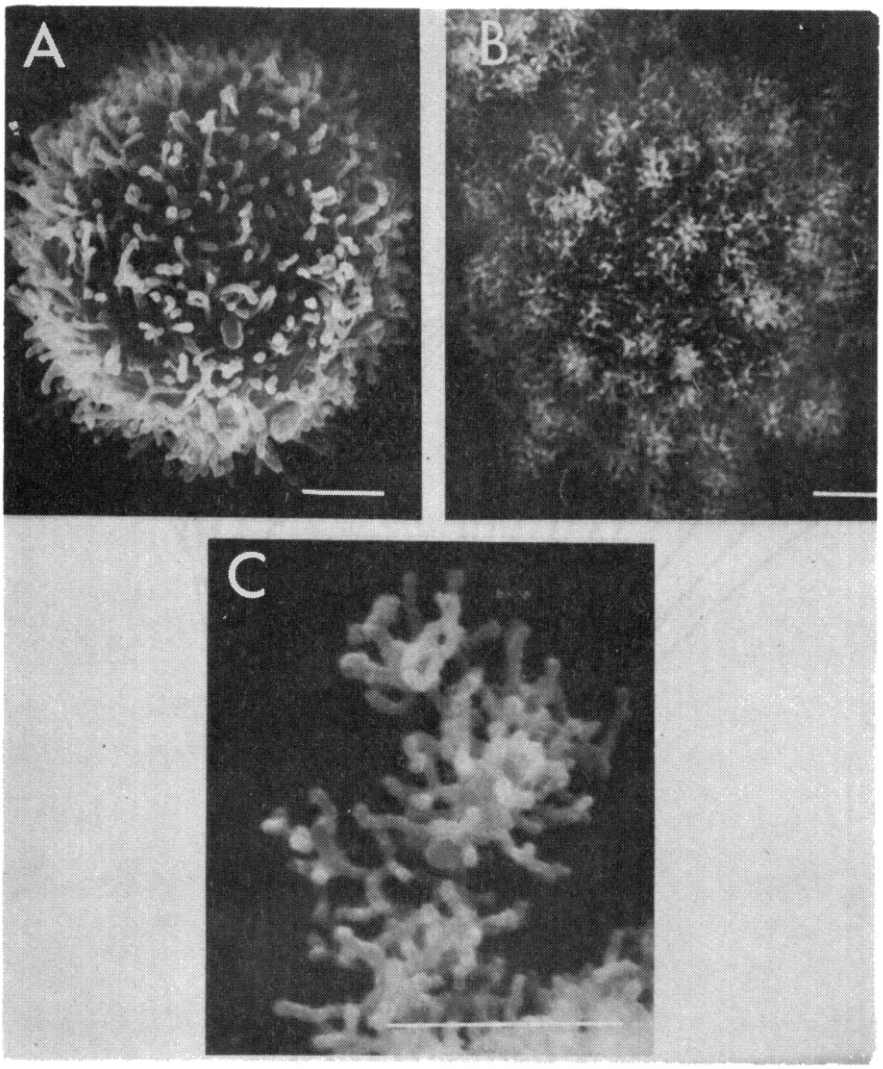

A
B
C

TABLE I

COMPARISON OF CELL LINES

	B1	C1
Morphology	unbranched	branched
Con-A Receptor Mobility	+	-
Xenotransplantability	-	+

CHAPTER II

METHODS

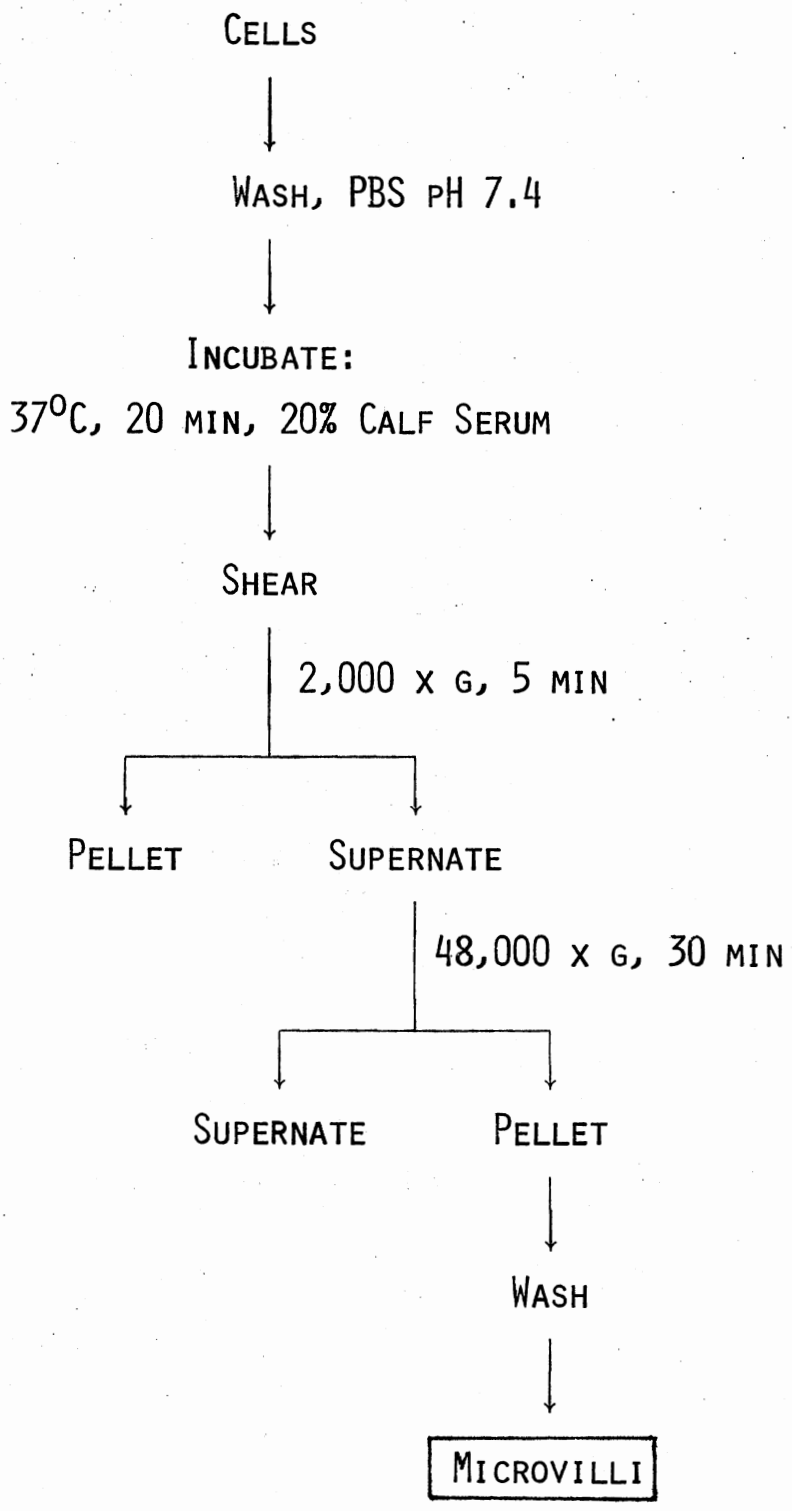
Cells

The 13762 MAT-B1 and MAT-C1 cells were obtained and maintained as previously described (50). During the course of these studies two different isolates of MAT-C1 cells were used, one from the continuously passaged subline and a second started from an early passage that had been frozen. Some variations in specific activities of the enzyme markers in intact cells have been noted between these cell subpopulations. However, these differences did not affect the isolation procedures or characteristics of the cell fractions described in this work, except for the absolute specific activity values.

Preparation of Microvilli

Microvilli were prepared as previously described (16) or by the scheme outlined in Figure 3. Cells were washed 2X in PBS buffer, pH 7.4, suspended to 5×10^7 ml^{-1} in 20% fetal calf or calf serum in PBS and incubated at $37^{\circ}C$ for 20-30 minutes. Cells were drawn (10-14 times) through a 14-gauge needle to shear the microvilli. The suspension was diluted to 3X the original volume and centrifuged at 2,500 x g for 5 minutes. The supernate was further centrifuged at 48,000 x g for 30 minutes to pellet the microvilli. The microvilli pellet was suspended

Figure 3. Outline of Microvilli Preparation.


CELLS
WASH, PBS pH 7.4
INCUBATE:
37°C, 20 MIN, 20% CALF SERUM
SHEAR
2,000 x G, 5 MIN
PELLET
SUPERNATE
48,000 x G, 30 MIN
SUPERNATE
PELLET
WASH
MICROVILLI

in PBS and washed 2X.

Preparation of Microvillar Membrane Fractions

Two different fractionation schemes were used in attempting to prepare microvillar membranes. 1) Microvilli were incubated at a concentration of 0.5 mg protein per ml in 40 mM Tris (pH 7.4) for 20 minutes at 40°C followed by homogenization in a tight Dounce homogenizer (20 strokes). The homogenate was layered onto a 42% sucrose cushion and centrifuged at 40,000 x g for 16 hr to give a soft pellet (Tris residue) and a band at the top of the cushion (MF1). This band was removed and washed twice in 40 mM Tris with centrifugation at 100,000 x g for 1 hr. 2) Microvilli (0.5 mg protein per ml) were incubated in GEM (5 mM glycine, 1 mM EDTA, 5 mM β-mercaptoethanol, pH 9.5) buffer for 30 minutes at room temperature and homogenized in a tight Dounce homogenizer. The homogenate was centrifuged at 10,000 x g for 15 minutes to give a pellet (GEM residue). The supernatant was centrifuged at 100,000 x g for 1.5 hr to give the membrane fraction (MF2).

Characterization of Microvilli and Microvillar Membrane Fractions

For transmission electron microscopy fractions were fixed overnight at 37°C in 1% glutaraldehyde, 0.1 M sodium cacodylate, 0.1 M sucrose at pH 7.4 and post-fixed in 1% OsO_4 for 10 min at 4°C. Fractions were dehydrated in a graded ethanol series and embedded in Epon 812. Silver sections were placed on 300 mesh grids and lightly stained with uranyl acetate and lead citrate (60). Negative staining was performed by floating a parlodion coated 300 mesh grid on a drop of glutaraldehyde

fixed microvilli containing 1% PTA or uranyl acetate. Scanning electron microscopy was performed as previously described (16).

5'-Nucleotidase was assayed as described previously (15); alkaline phosphatase and γ-glutamyltransferase were assayed as described in Sigma Technical Bulletins #244 and #415, respectively. Glucosamine labeling, lactoperoxidase labeling, SDS PAGE, fluorography and autoradiography were performed as described previously (49, 50).

58 K-Membrane Interaction

MF2 was extracted at 0.5 mg protein/ml in PBS containing DOC (see figure legends for concentrations) for 15 minutes at room temperature and centrifuged at 200,000 x g for 1 hr. 100 μCi of [^{14}C]-phosphatidyl choline (PC) was added to 0.5 ml of the supernate from the MF2 extract and dialysed for 24 hr at room temperature against PBS. Sucrose flotation gradient centrifugation of the dialysed DOC soluble material was done on a linear 20-50% sucrose with the extracted MF2 fraction in a 55% sucrose solution. Chromatography of MF2 DOC extract was over a 0.75 cm x 60 cm column of Sepharose 4B and eluted with GEM or PBS buffer without DOC. Exact conditions are specified in the figure legends.

58 K-Actin Interaction

Differential centrifugation of Triton-extracted microvilli was for 1 hr at centrifugal forces from 10,000 x g to 200,000 x g. Exact conditions are in the figure legends.

Chromatography of MF2 extracted in S or D buffer was performed with a 0.75 cm x 60 cm Sepharose 6B column equilibrated with S or D

buffer. Extraction of MF2 was for 15 minutes at room temperature in an actin-stabilizing buffer (S buffer, 5 mM Tris, 0.15 M NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithioerythritol, 0.5% Triton X-100, 0.01 mM PMSF, pH 7.6) (54), an actin-destabilizing buffer (D buffer, 0.75 M guanidine HCl, 0.5 M sodium acetate, 0.5 mM $CaCl_2$, 0.5 mM ATP, 0.5% Triton X-100, 0.01 mM PMSF, pH 7.6) (54), or GEM containing 0.2% Triton X-100 (G-T Buffer). Extraction of MF2R (the 0.2% Triton residue of MF2) was for 15 minutes at room temperature in 40 mM Tris pH 9.0, 0.6 M KI-GEM or 0.6 M KCl-GEM.

The Myosin Affinity Technique was performed according to Koch and Smith (31) using 0.1 mg myosin and 50-100 µg MF2 protein for assay.

State of Actin

DNase I inhibition was assayed by the method of Blickstad et al. (8). Chromatography of MF2 or microvilli extract was over a Sepharose 6B 0.75 cm x 60 cm column equilibrated with S or D buffer. Sucrose density gradient sedimentation of MF2 extracted in S or D buffer was performed on a 5-20% linear sucrose gradient centrifuged at 130,000 x g for 40 hr.

For in vitro labeling washed cells were suspended in 10 ml of McCoy's Modified Media containing 20% fetal calf serum and 350 µCi [^{35}C]-methionine at a concentration of 1 x 10^7 cells/ml for 1 hr at 37°C. [^{35}S]-Actin was isolated from labeled cells by incubating isolated microvilli in D buffer for 15 minutes at room temperature, followed by centrifugation at 200,000 x g for 1.5 hr. The supernate was chromatographed over a Sephadex G-150 column and fractions eluting between 16.5 and 21.5 grams were pooled, concentrated and dialysed against

S buffer. The dialysate was clarified by centrifugation at 200,000 x g for 1.5 hr.

CHAPTER III

RESULTS

Isolation and Characterization of Microvilli from MAT-B1 and MAT-C1 Cells

Microvilli were isolated from MAT-B1 and MAT-C1 cells using either the procedure previously reported (16) or by the procedure outlined in Figure 3. The preparations were examined by transmission electron microscopy after preparing thin sections (Figure 4) or after negative staining (Figure 5). MAT-B1 microvilli preparations contained predominantly unbranched tubular structures (Figure 4a) while highly branched structures were found in preparations of MAT-C1 microvilli (Figures 4B and 5).

Enzyme activities known to be associated at least in part with the cell surface were assayed in the preparations of microvilli. For the MAT-C1 microvilli, the enzyme activities showed an 11-fold purification of 5'-nucleotidase, a 3-fold purification of alkaline phosphatase and a 4-fold purification of γ-glutamyltransferase (Table II). Both 5'-nucleotidase and alkaline phosphatase activities were negligible in MAT-B1 cells. The γ-glutamyltransferase activity was purified relative to whole cells, about 2-fold in microvilli preparations from MAT-B1 cells compared to a 4-fold purification in microvilli from MAT-C1 cells (Table III). Although some differences were noted in specific activities

Figure 4. Transmission Electron Microscopy of Thin Sections of Microvilli and Membrane Fractions.

A) MAT-B1 microvilli, B) MAT-C1 microvilli, C) MAT-B1 MF1, D) MAT-C1 MF1, E) MAT-B1 MF2, F) MAT-C1 MF2. Magnifications: A) 40,000, B) 31,000, C) 27,000, D) 20,000, E) 29,000, F) 17,000. Bars = 0.5 μm.

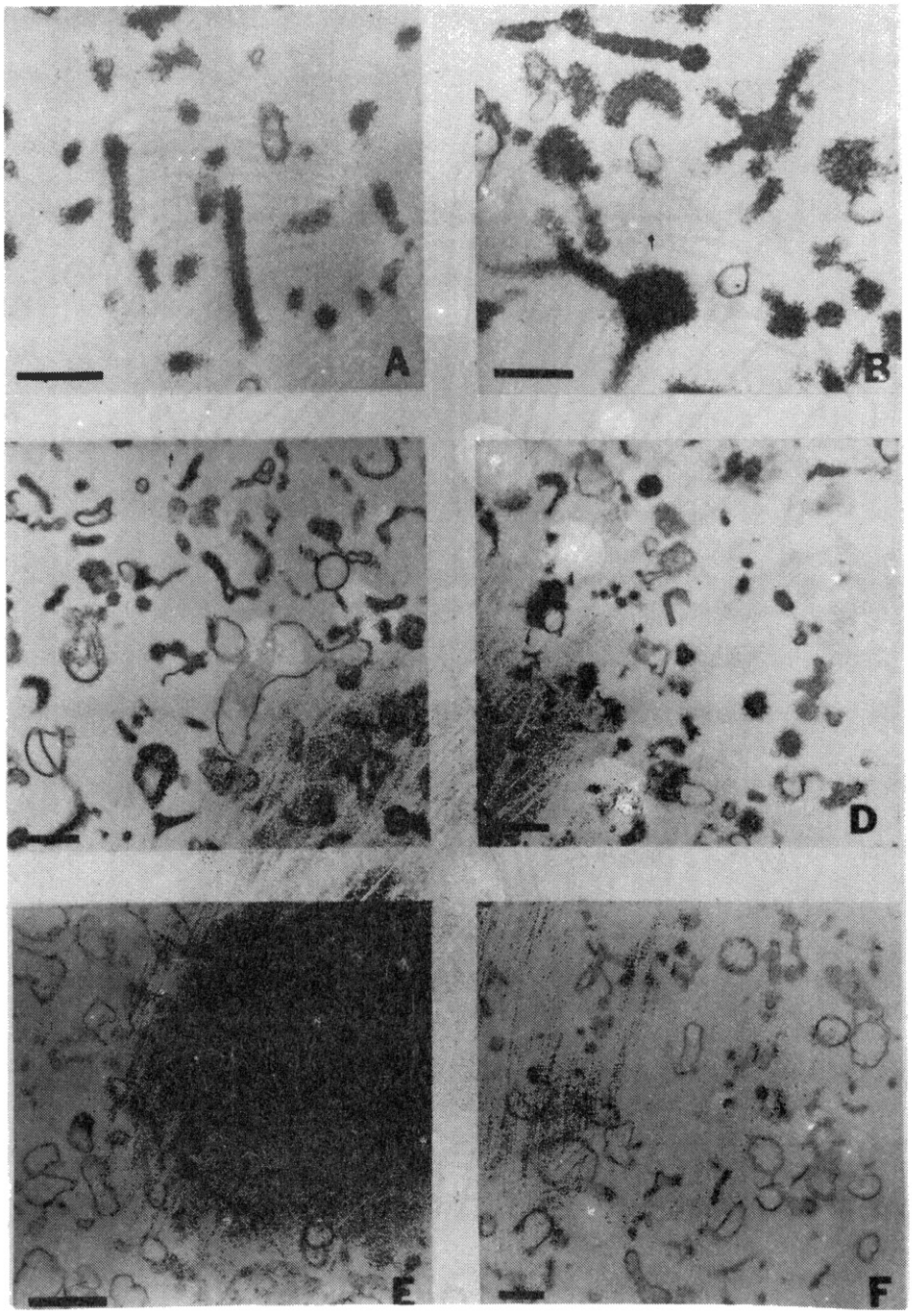

A
B
D
E
F

Figure 5. Transmission Electron Micrograph of Branched Microvillus.

Magnification 51,000 X.

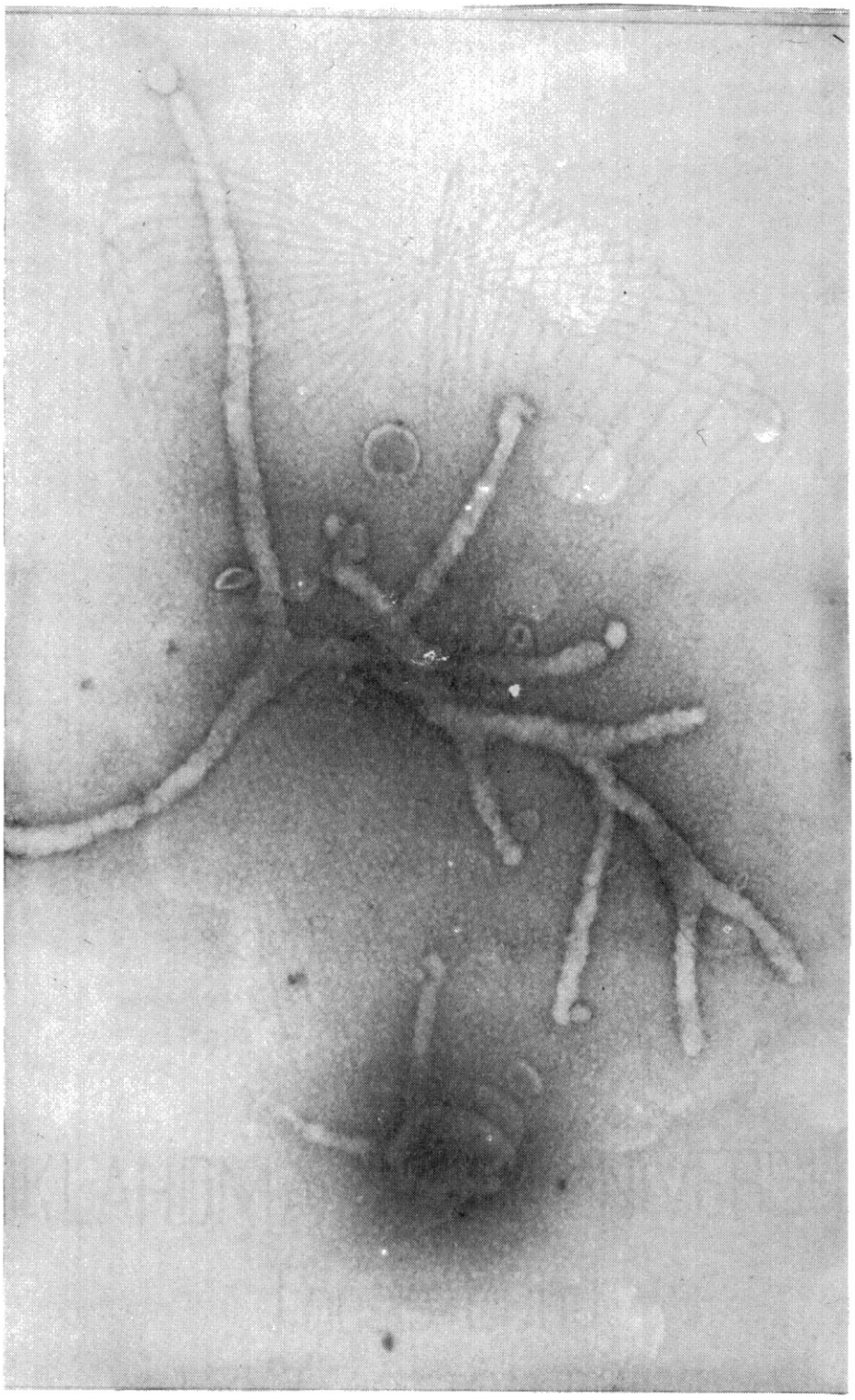


TABLE II

PLASMA MEMBRANE ENZYME MARKER ACTIVITIES OF THE MAT-C1 CELL LINE

Fraction	5'-Nucleotidase	Alkaline Phosphatase	γ-Glutamyl Transferase
		$\mu moles \cdot (hr \cdot mg)^{-1}$	
Whole Cells	4.0	8.6	6.0
Microvilli	45.8	24.4	23.3
MF1	66.3	44.7	36.8
Tris Residue	55.6	28.5	25.6

TABLE III

γ-GLUTAMYL TRANSFERASE ACTIVITIES AND ^{3}H-GLUCOSAMINE INCORPORATED IN ISOLATED MICROVILLI AND FRACTIONS

Fraction	γ-Glutamyl Transferase		[^{3}H]-Glucosamine incorporated	
	MAT-B1	MAT-C1	MAT-B1	MAT-C1
	μmoles•(hr•mg)$^{-1}$		CPM•μg^{-1}	
Whole Cells	5.92	3.29	59	63
Microvilli	12.45	11.7	276	588
MF1	11.4	15.1	204	747
MF2	17.03	22.21	551	953

of different cell preparations, as noted in Experimental Procedures, the degree of enzyme purification obtained when activity in microvilli was compared to that in intact cells was reproducible and indicative of purification of a cell surface fraction for both sublines.

Previous studies have shown that labeled glucosamine is incorporated predominantly (>70%) into a single cell surface glycoprotein in MAT-B1 and MAT-C1 cells (49). Isolated microvilli from MAT-B1 and MAT-C1 cells had approximately 5- and 9-fold increases, respectively, in specific activity of glucosamine label (cpm/mg protein) relative to those of the corresponding intact cells (Table III). That the label was incorporated predominantly into a single sialoglycoprotein present in both whole cells and microvilli preparations was demonstrated by SDS PAGE followed by fluorography, as shown for MAT-B1 cells in Figure 6. Similar results were obtained for MAT-C1 cells and microvilli preparations.

SDS PAGE was performed to investigate polypeptide similarities and differences between MAT-B1 and MAT-C1 microvilli (Figure 7). Polypeptide patterns for the intact cells were essentially identical and those from the microvilli preparations were very similar. The most notable difference in the latter was the presence of a polypeptide of Mr 58,000 in the MAT-C1 microvilli (Figure 7, lane E, note position of upper arrow) which was greatly decreased or absent in MAT-B1 microvilli.

Fractionation of Isolated Microvilli

Microvilli contain two primary structural features, a plasma membrane comprising their surface and a core of microfilaments. It seems likely that the element(s) responsible for the morphological

Figure 6. SDS PAGE and Fluorography of Microvilli and Fractions from Glucosamine-labeled MAT-B1 Cells.

A) cells, B) microvilli, C) MF1, D) MF2. The top bar shows the position of ASGP-1, the predominant labeled component of the cells. The other bar shows the position of actin. A second labeled component is barely visible about half-way between the bars. A similar experiment with MAT-C1 cells showed essentially the same labeling pattern for all fractions.

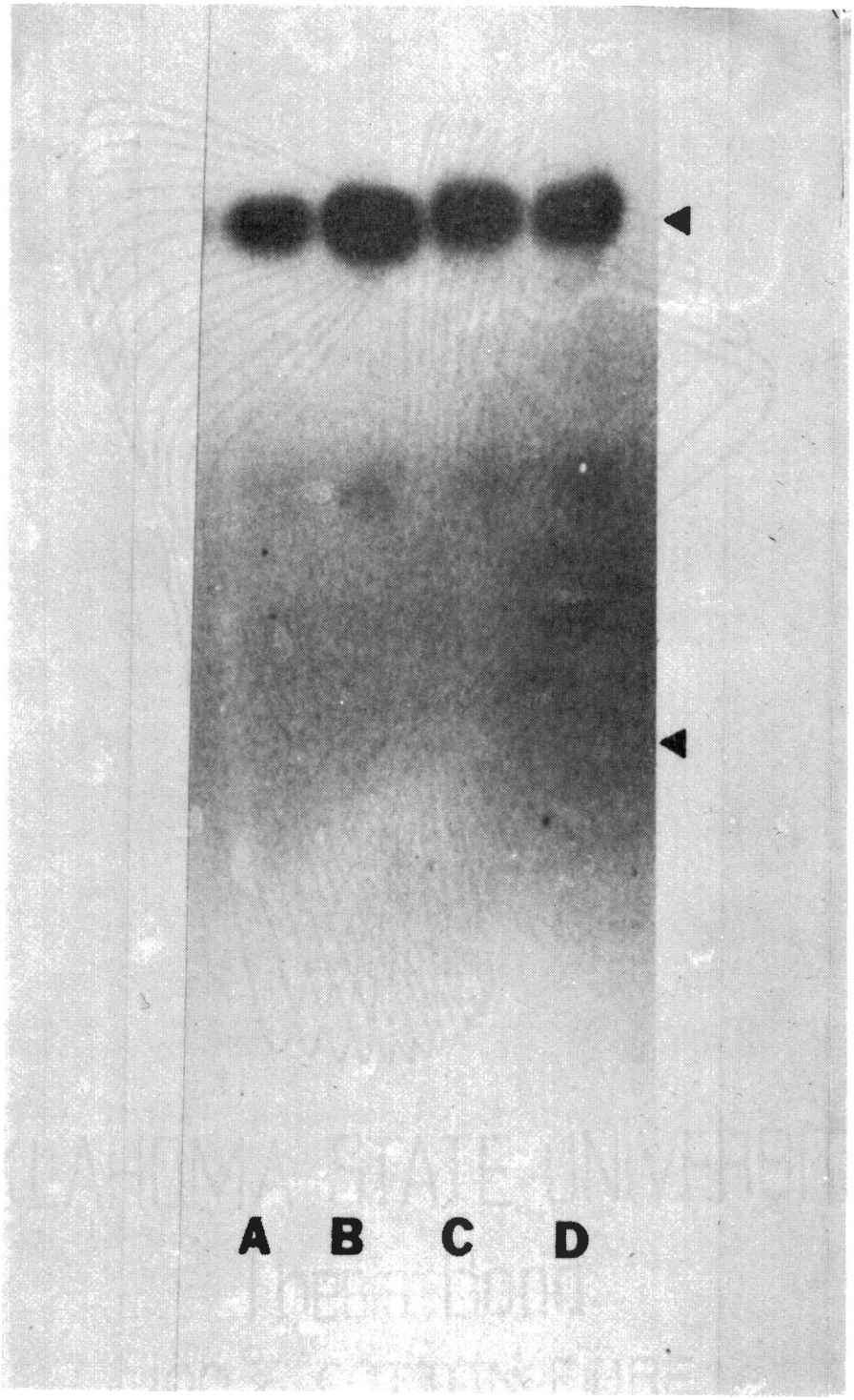
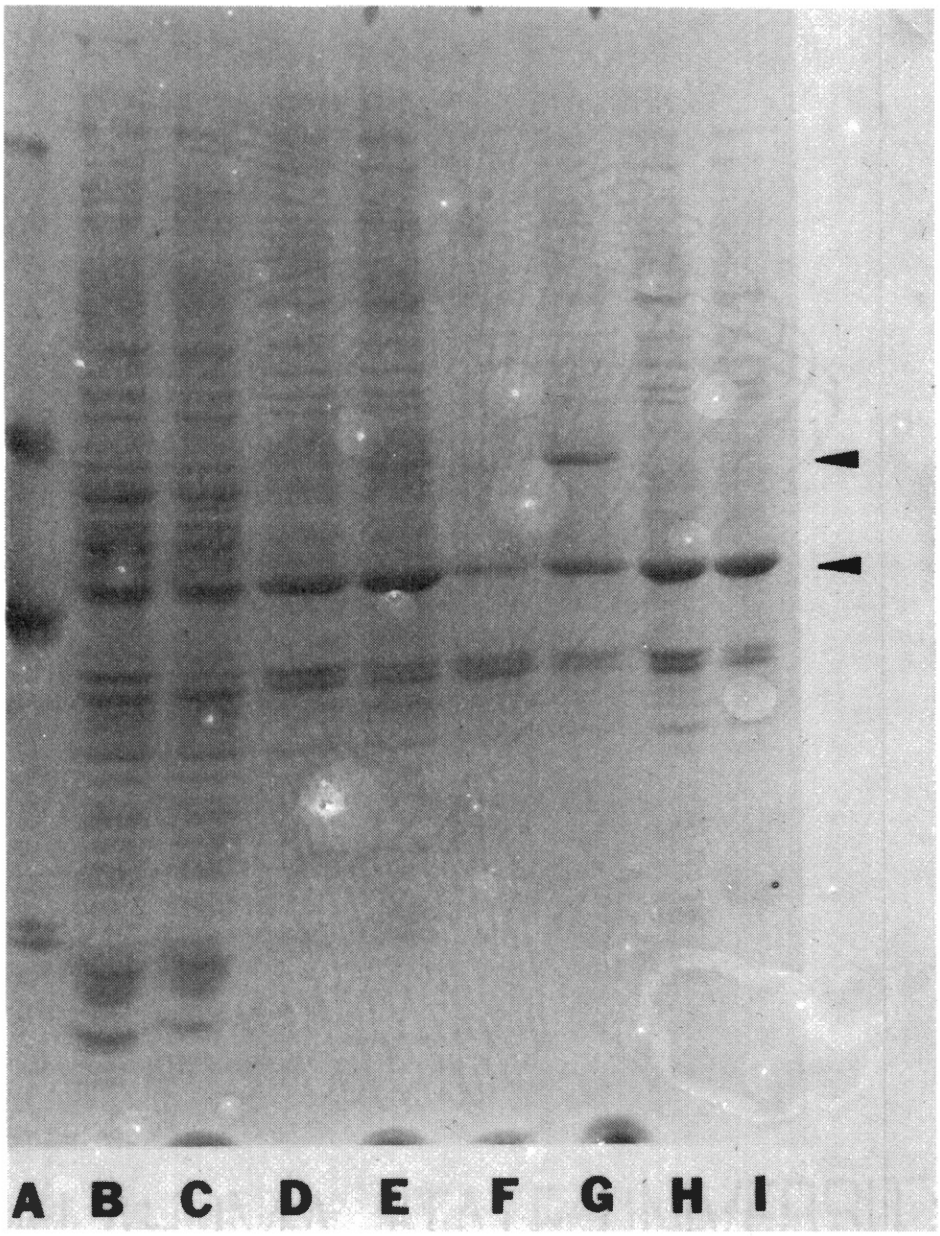

A B C D

Figure 7. SDS Page Gel Stained with Coomassie Blue of Cells, Microvilli and Fractions.

A) Standard containing ovalbumin and bovine serum albumin, B) MAT-B1 cells, C) MAT-C1 cells, D) MAT-B1 microvilli, E) MAT-C1 microvilli, F) MAT-B1 MF1, G) MAT-C1 MF1, H) MAT-B1 Tris residue, I) MAT-C1 Tris residue. Top arrowhead shows position of 58 K. Bottom arrow designates actin.

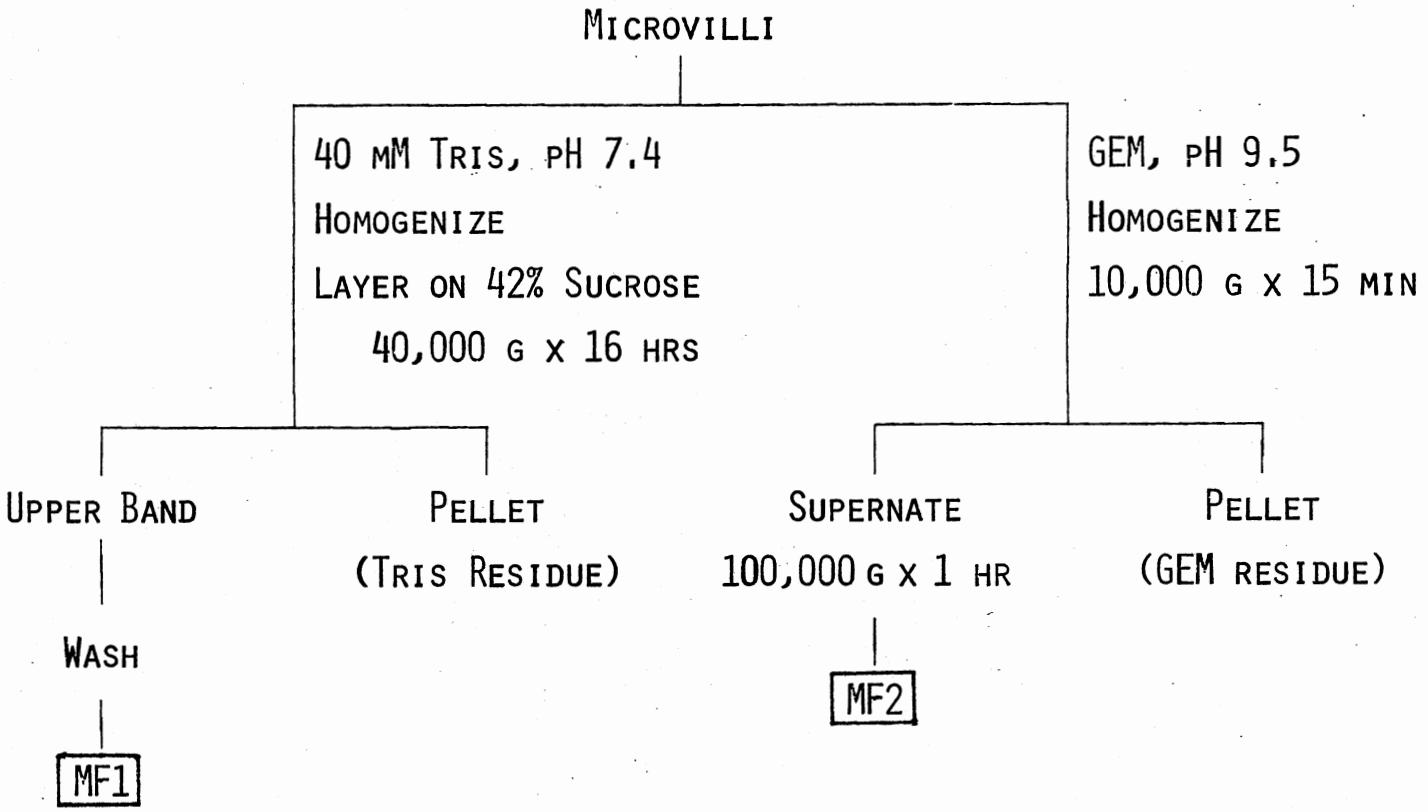

A B C D E F G H I

differences between the microvilli of the two sublines will be present in one or both of these structures. Therefore the microvilli were fractionated into their component parts as outlined in Figure 8.

In the first effort to obtain membranes, microvilli were homogenized in 40 mM Tris. When the homogenate was fractionated on a linear sucrose gradient, two fractions were obtained. The same fractionation was achieved more quickly by sedimenting the homogenate through a 42% sucrose layer to give a pellet (Tris residue), comprising approximately 60% of the homogenate protein, and a retarded fraction (MF1, above sucrose cushion), comprising approximately 40% of the homogenate protein. Electron microscopy showed that MF1 from both sublines was a heterogeneous mixture of membrane vesicles and fragments, fragmented microvilli and densely-stained spherical bodies covered with fibrillar material (Figure 4). Analyses of MF1 for plasma membrane markers showed increased specific activities compared to microvilli for MAT-C1 (Tables II and III), but a decrease for MAT-B1 (Table III). SDS PAGE showed a substantial increase in the amount of 58 K protein to the MAT-C1 MF1 fraction (Figure 7, lane G) relative to the MAT-C1 microvilli and a depletion of 58 K in the Tris residue (Figure 7, lane I). Although MF1 clearly contains cell surface elements from the microvilli, including ASGP-1 (Figure 6), it is not substantially enriched in plasma membrane markers, and the behavior of the MAT-B1 and MAT-C1 fractions are not parallel. Nevertheless, the enrichment for 58 K was intriguing and suggests a specific localization, although it is not possible to specify this localization because of the heterogeneity of MF1.

Since MF1 contained densely stained material bounded by plasma

Figure 8. Scheme for Isolation of Fractions of Microvilli.


MICROVILLI
40 mM TRIS, pH 7.4
HOMOGENIZE
LAYER ON 42% SUCROSE
40,000 G X 16 HRS
GEM, pH 9.5
HOMOGENIZE
10,000 G X 15 MIN
UPPER BAND
PELLET
(TRIS RESIDUE)
SUPERNATE
100,000 G X 1 HR
PELLET
(GEM RESIDUE)
WASH
MF1
MF2

membrane, an extraction-homogenization procedure similar to that which eluted spectrin/actin from erythrocyte ghosts (30) and actin and associated proteins from ascites cell surface envelopes was used (Figure 8). The objective was to decrease the amount of microfilament core material associated with the plasma membrane. After homogenization of microvilli in GEM buffer the suspensions were centrifuged at 10,000 x g to remove large microvilli fragments (GEM residue), and the supernatant was centrifuged at 100,000 x g to sediment a membranous fraction (MF2). Electron microscopy showed that MF2 contained predominantly membrane vesicles and fragments (Figure 4) for both MAT-B1 and MAT-C1 preparations. Moreover, there were significant and parallel increases in specific activities of the plasma membrane markers (Table III) for MF2 from MAT-B1 and MAT-C1 microvilli.

To demonstrate that MF2 is not an atypical cell surface fraction selected by the isolation procedure, cells from both sublines were labeled with [^{125}I] using lactoperoxidase and fractionated. As shown in Figure 9 for MAT-C1 cells and fractions, the labeling patterns for cells, microvilli, and MF2 are very similar. Essentially the same results were obtained for MAT-C1 MF1 fractions, MAT-B1 cells and MAT-B1 fractions, though the distribution of label in the components was quantitatively different from MAT-C1, as previously shown (50).

SDS PAGE showed a great enhancement of the amount of 58 K in the MAT-C1 MF2 fraction compared to MAT-C1 microvilli (Figure 10). As expected, the 58 K band was absent from MAT-B1 MF2. Another noteworthy difference was the decreased amounts of bands of lower molecular weight than actin in MAT-C1 MF2 compared to MAT-B1 MF2.

Figure 9. SDS PAGE and Autoradiography of Cells, Microvilli and Membrane Fractions from MAT-C1 Cells Labeled with ^{125}I and Lactoperoxidase.

A) MAT-C1 cells, B) MAT-C1 microvilli, C) MAT-C1 MF2. Arrowheads indicate positions of 58 K and actin.

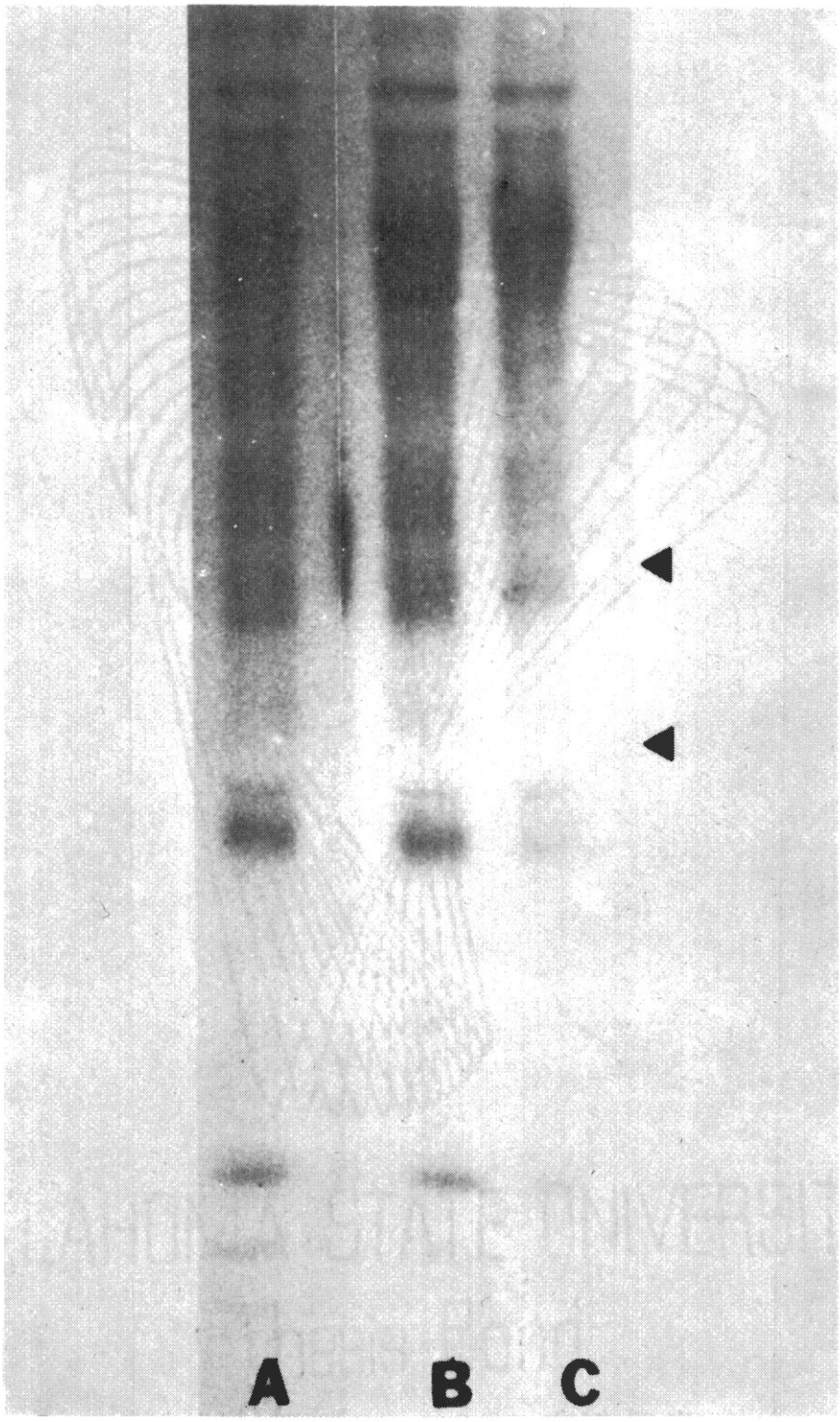
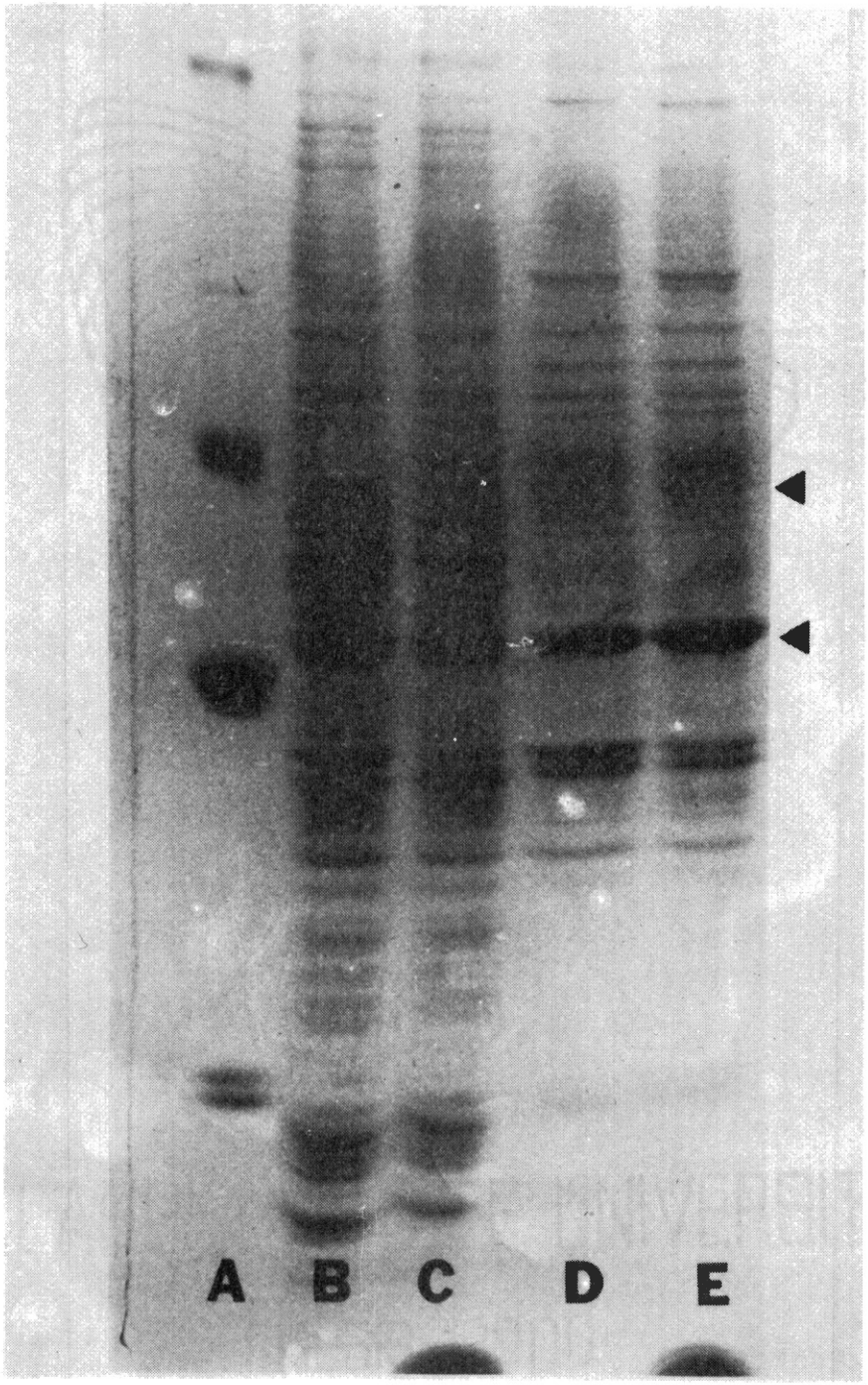

A
B
C

Figure 10. SDS PAGE Stained with Coomassie Blue of A) Standards, B) MAT-B1 Microvilli, C) MAT-C1 Microvilli, D) MAT-B1 MF2, E) MAT-C1 MF2.

Top arrowhead shows position of 58 K. Bottom arrowhead designates actin.


A
B
C
D
E

Association of 58 K with the MF2 Membrane

The previous results suggest that 58 K is associated with the plasma membrane. However, considering the preparation method it is possible that an aggregate of 58 K or 58 K and other proteins co-sediments with the membranes. Therefore the MAT-C1 MF2 fraction was further examined by flotation on a sucrose gradient. Membranes were found in two peaks, a sharp peak at about 45% sucrose, and a broad peak at 50-60% (Figure 11). Fractions from these peaks were examined by SDS PAGE. Each fraction was shown to contain both 58 K and actin (Figure 12).

Solubilization of MF2 with increasing concentrations of DOC in GEM or PBS revealed a gradual decrease of components from MF2 pellet fractions (Figure 13) and a corresponding increase in the supernate fractions (Figure 14), demonstrating almost complete solubilization of MF2 proteins. The soluble material was clarified by centrifugation at 200,000 x g for 1.5 hr and vesicles were reconstituted by two methods to test whether 58 K reconstituted with the lipid fraction.

First, [^{14}C]-PC was added to the clarified DOC soluble fraction followed by extensive dialysis for 24 hr at room temperature against PBS. The dialysate was floated in a sucrose gradient; the [^{14}C]-PC fractionation profile is shown in Figure 15. Most of the PC was located at or near the top of the gradient. However, peaks at about 30% and 40% sucrose indicated populations of vesicles with various densities. The SDS PAGE of fractions (Figure 16) shows 58 K and two minor components floating in the gradient, indicating 58 K has been reconstituted into lipid containing vesicles.

The second method used to reform lipid vesicles was chromatography

Figure 11. Sucrose Gradient Fractionation of MF2.

MF2 samples were mixed with 60% sucrose and overlayered with 55% and 45% sucrose bands and buffer for flotation gradient. Fractions for electrophoresis were combined as shown by bars.

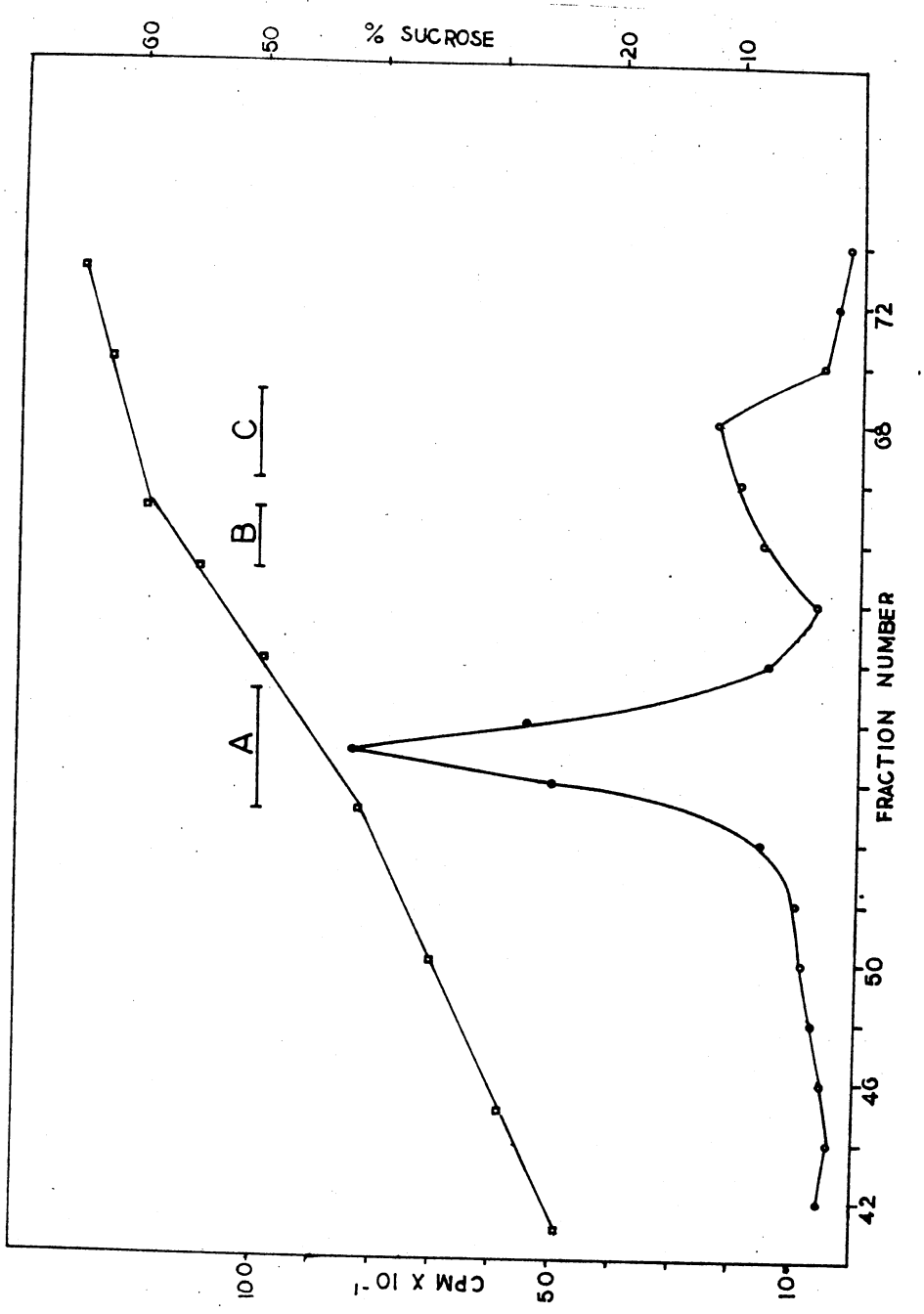

% SUCROSE
60
50
20
10
A
B
C
CPM X 10⁻¹
100
50
10
42
46
50
68
72
FRACTION NUMBER

Figure 12. SDS PAGE of Gradient Fractions from Figure 11.

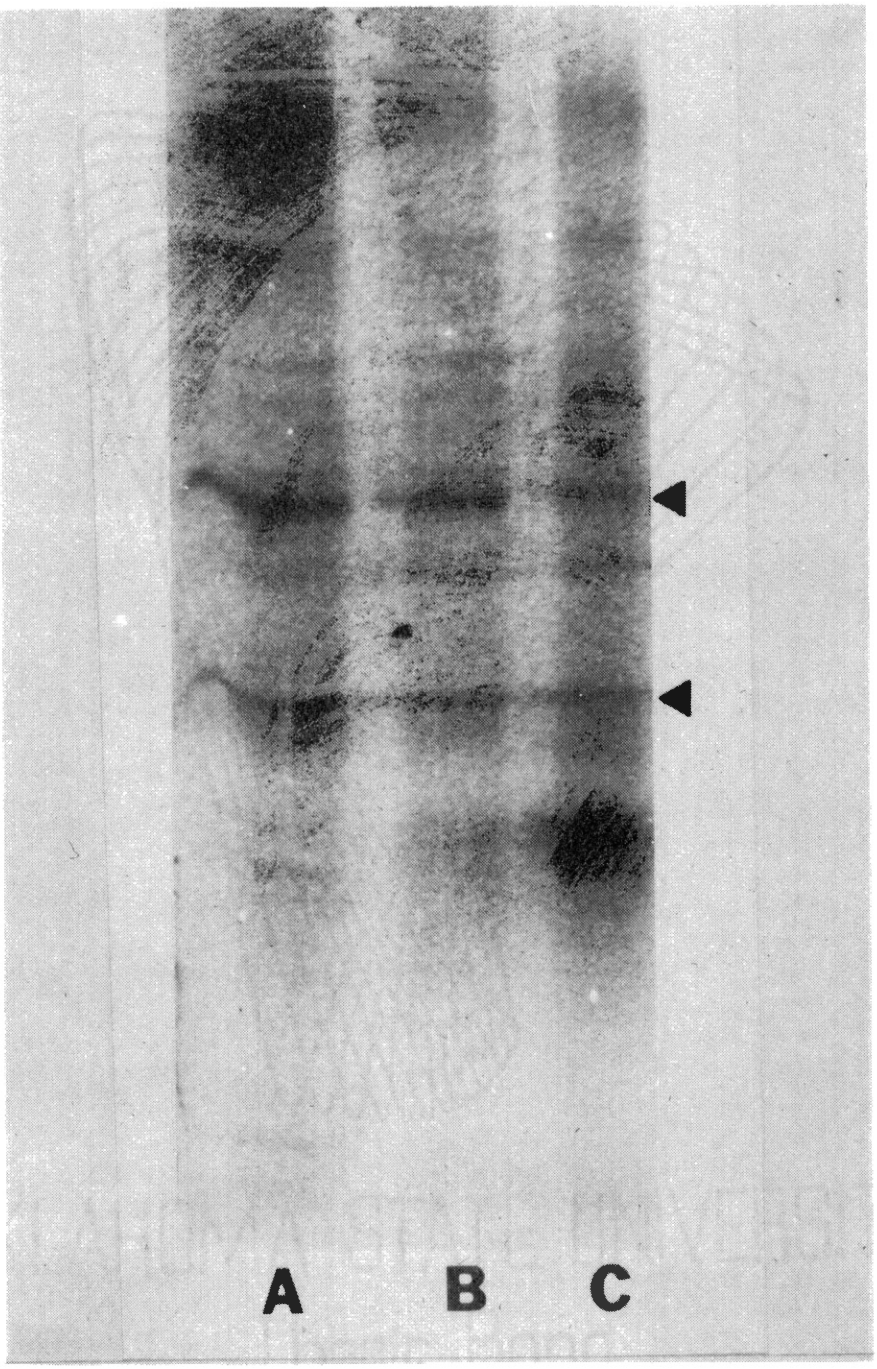

A
B
C

Figure 13. DOC Extract Pellets.

MF2 was extracted for 15 min, room temperature in various concentrations of DOC, centrifuged at 42,000 rpm for 1 hr in SW 50.1 rotor. Pellet fractions were electrophoresed. S) standards, 1) 0.25%, 2) 0.17%, 3) 0.1%, 4) 0.05%, 5) 0.01%, 6) 0.005% DOC.

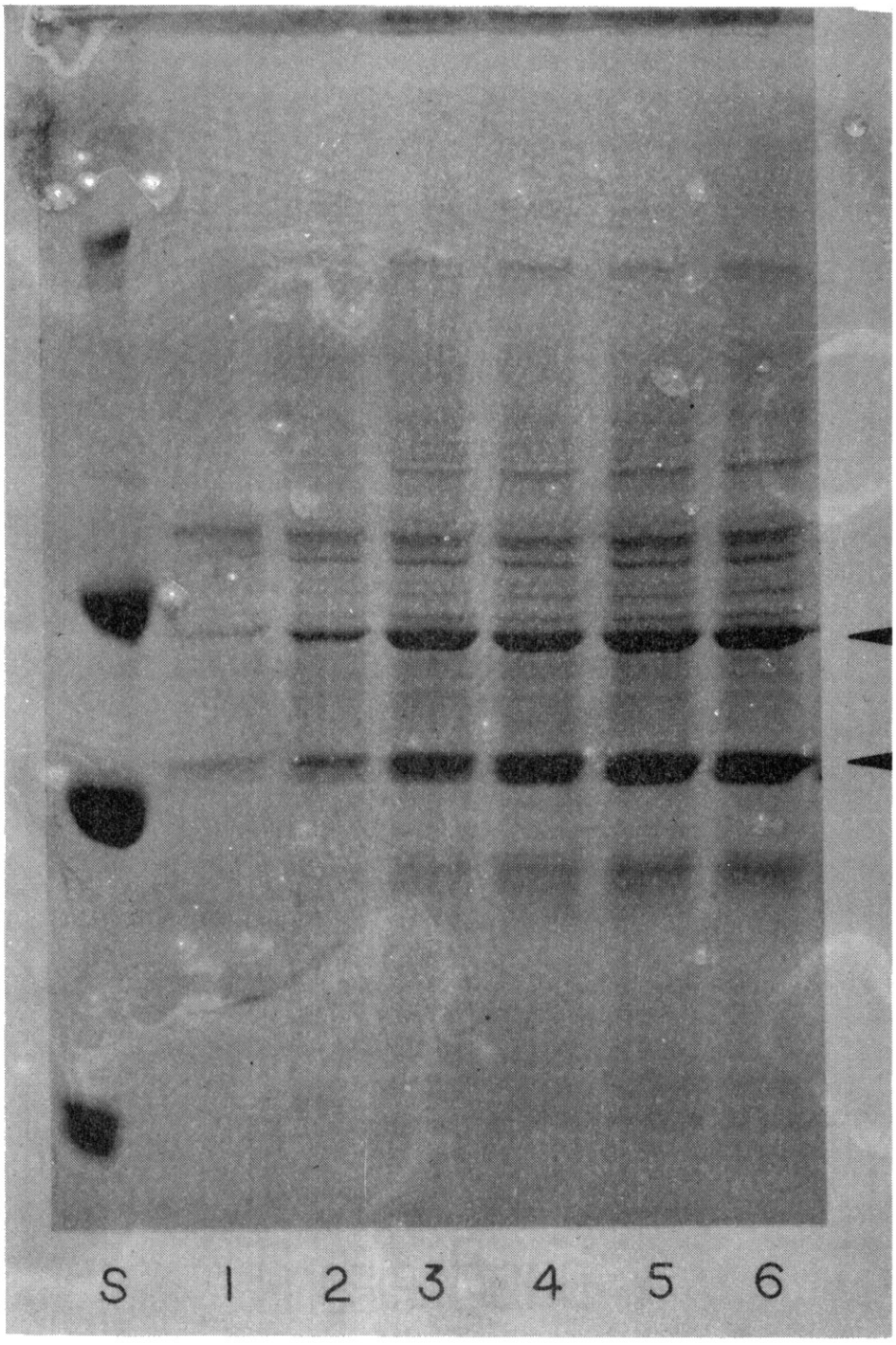

S
1
2
3
4
5
6

Figure 14. DOC Extract Supernates.

Experimental as in Figure 13. S) standards, 1) 0.25%, 2) 0.17%, 3) 0.1%, 4) 0.05%, 5) 0.01%, 6) 0.005% DOC.

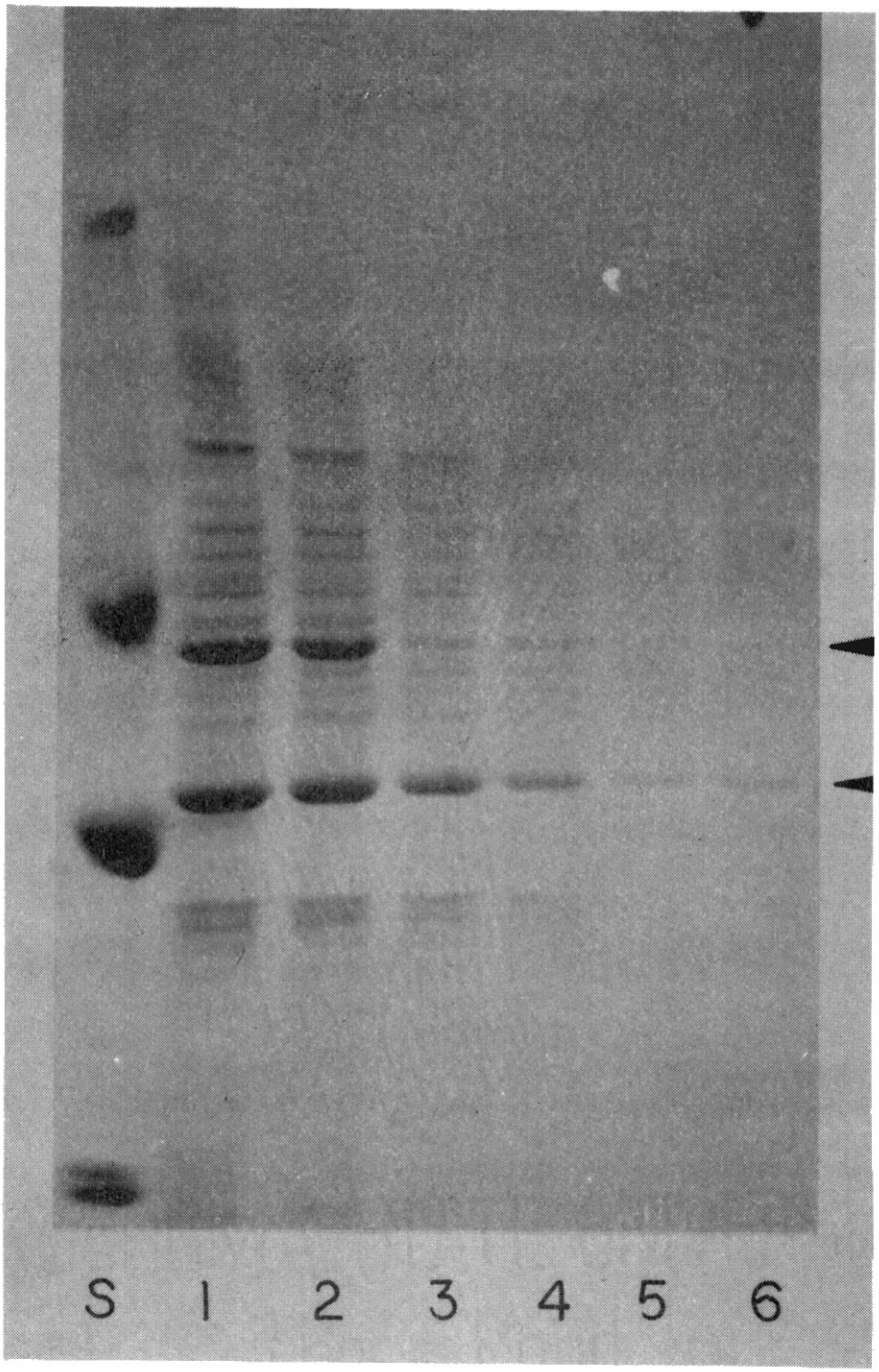

S
1
2
3
4
5
6

Figure 15. Fractionation Profile of Sucrose Flotation Gradient.

MF2 was extracted in 0.5% DOC for 15 min at room temp, centrifuged at 36,000 rpm x 1 hr in SW 50.1 head. 200 µCi [^{14}C]-phosphatidyl choline was added to the soluble fraction and dialysed at room temp for 24 hrs with at least 4 changes against PBS. The dialysate was made up to 60% sucrose and a 20-50% sucrose gradient was layered on top followed by centrifugation at 23,000 rpm x 18 hrs in the SW 27.1 rotor. Numbered bars are fractions pooled, dialysed against 0.2% SDS for electrophoresis. Dashed line indicates sucrose concentration.

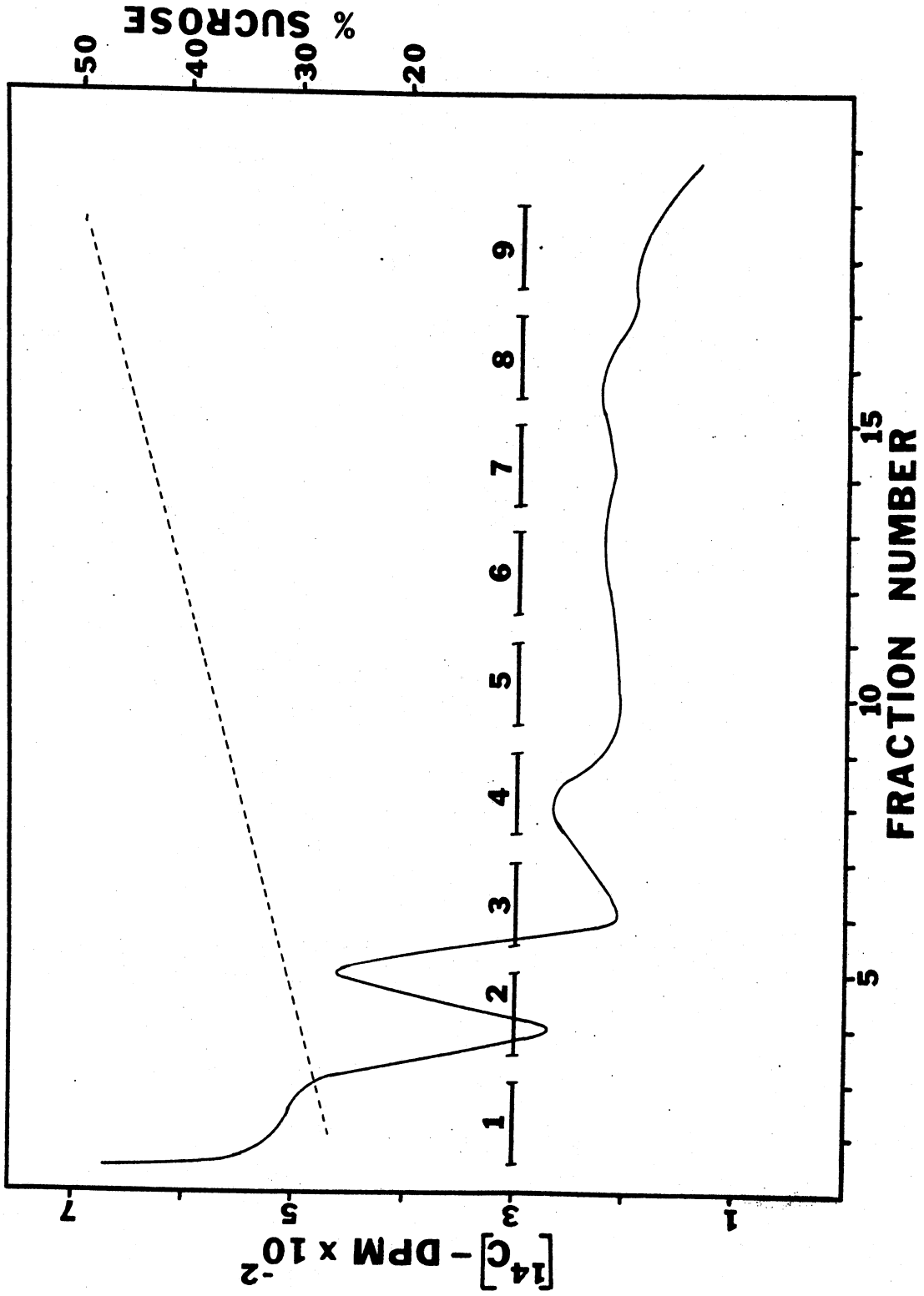
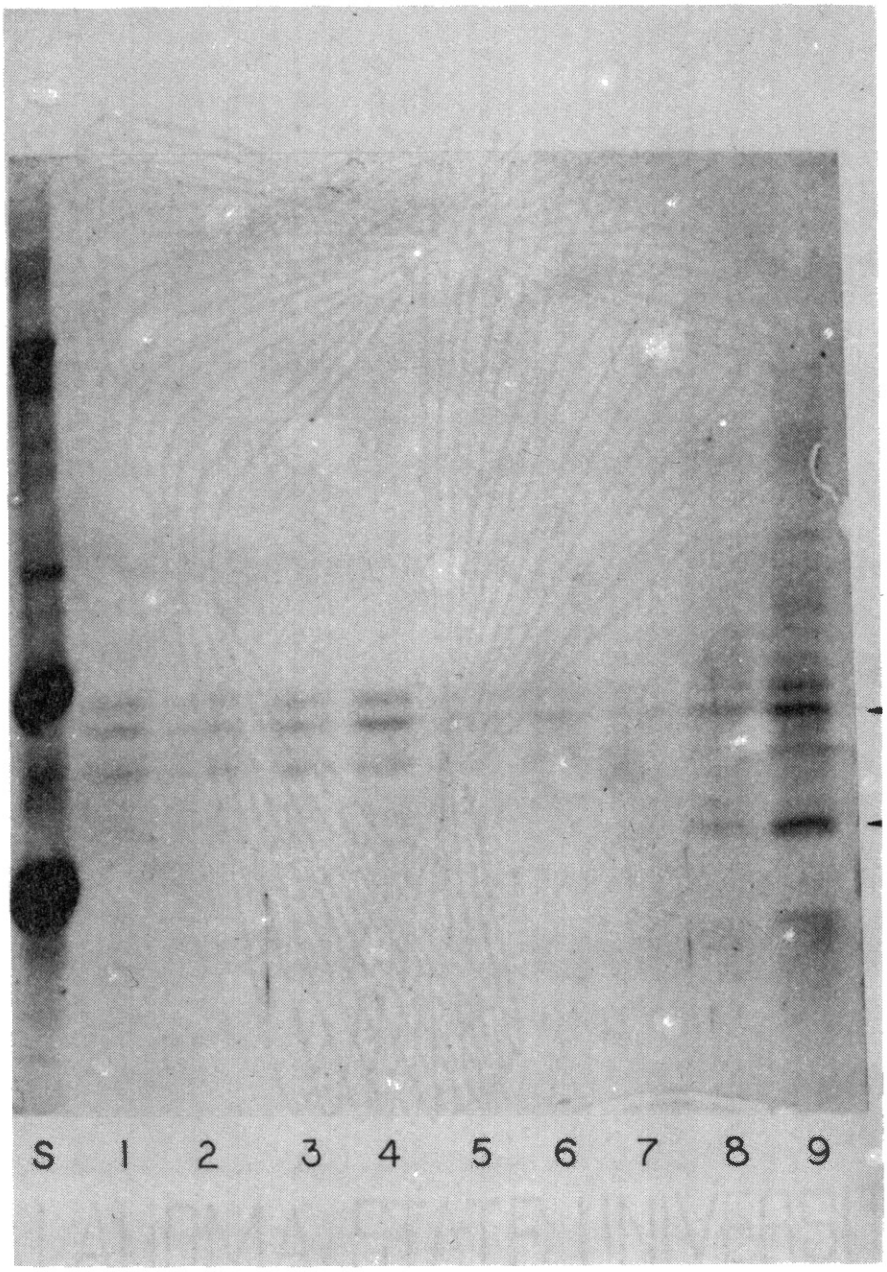

% SUCROSE
50
40
30
20
[14C] – DPM x 10-2
7
5
3
1
1
2
3
4
5
6
7
8
9
5
10
15
FRACTION NUMBER

Figure 16. SDS PAGE of Fractions Indicated in Figure 15.

Upper arrow indicates 58 K, lower arrow actin.


S 1 2 3 4 5 6 7 8 9

over a Sepharose 4B column without DOC of the [^{14}C]-PC added DOC soluble material. Figure 17 is the [^{3}H]-leucine and [^{14}C]-PC elution profile of the Sepharose 4B column. Two populations of vesicles are seen: One near the void volume of the column; the other a heterogenous mixture of smaller vesicles spread throughout the column. Protein is seen in both of these populations. SDS PAGE of the marked fractions appear in Figure 18 and show the 58 K protein eluting near the void volume, again suggesting a reassociation of 58 K with lipid vesicles.

Chromatography of the DOC solubilized MF2 fraction over a Sepharose 6B column in buffer containing DOC is shown in Figures 19 and 20.

The results indicate that 58 K has a smaller size when DOC is present than when DOC is removed. Calibration of the column revealed a molecular weight of approximately Mr 320,000 and a Stoke's radius of 61.25 Å.

Association of 58 K with Actin

Extraction with nonionic detergents has been widely used to remove hydrophobic components of cells (14), ghosts (63), isolated microvilli (38) and membrane envelopes (37), leaving behind a cytoskeletal residue composed of actin and associated proteins. Triton extraction of MAT-B1 or MAT-C1 microvilli removes essentially all of the lipid and the glucosamine-labeled material, leaving a residue composed predominantly of actin for MAT-B1 microvilli and actin and 58 K for MAT-C1 microvilli (C. Carraway, R. F. Cerra, P. B. Bell and K. L. Carraway, submitted for publication). These results suggest an association of 58 K with the cytoskeleton core of the MAT-C1 microvilli. However, it

Figure 17. Elution Profile of Lipid Reconstitution Sepharose 4B Column.

MF2 was extracted for 15 min at room temp in 0.5% DOC, centrifuged at 36,000 rpm x 1 hr in SW 50.1 rotor. 200 µCi [^{14}C]-phosphatidyl choline was added to the soluble fraction and chromatographed over a Sepharose 4B column without DOC. Fractions were collected, counted, and electrophoresed. Solid line indicates [^{3}H]-leucine, dashed line [^{14}C]-PC. Numbered bars indicate fractions pooled and electrophoresed.

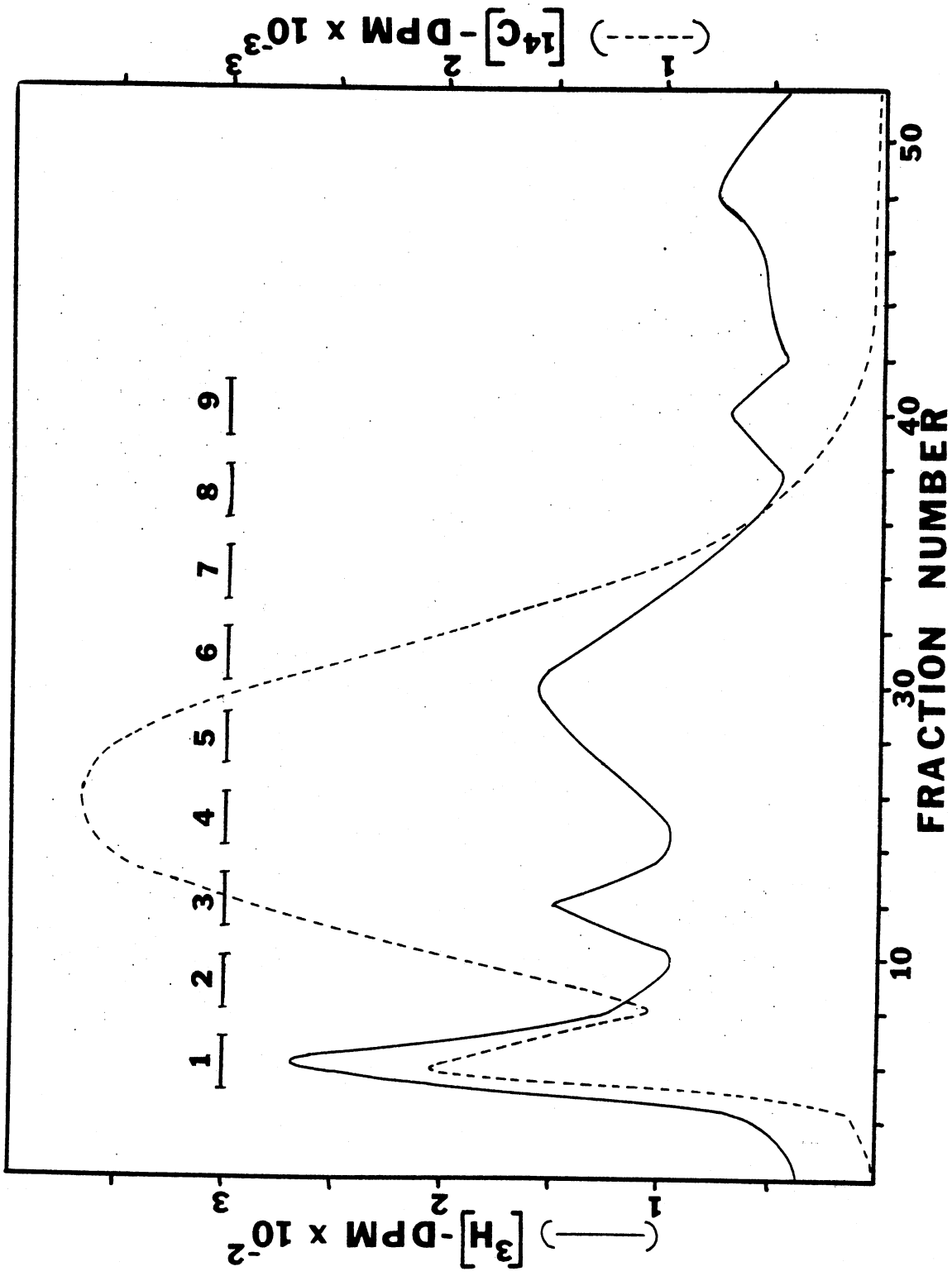

(——) [3H]-DPM x 10-2
(------) [14C]-DPM x 10-3
FRACTION NUMBER
1
2
3
4
5
6
7
8
9
10
30
40
50

Figure 18. SDS PAGE of Fractions Indicated in Figure 17.

Upper arrow denotes 58 K, lower arrow actin.

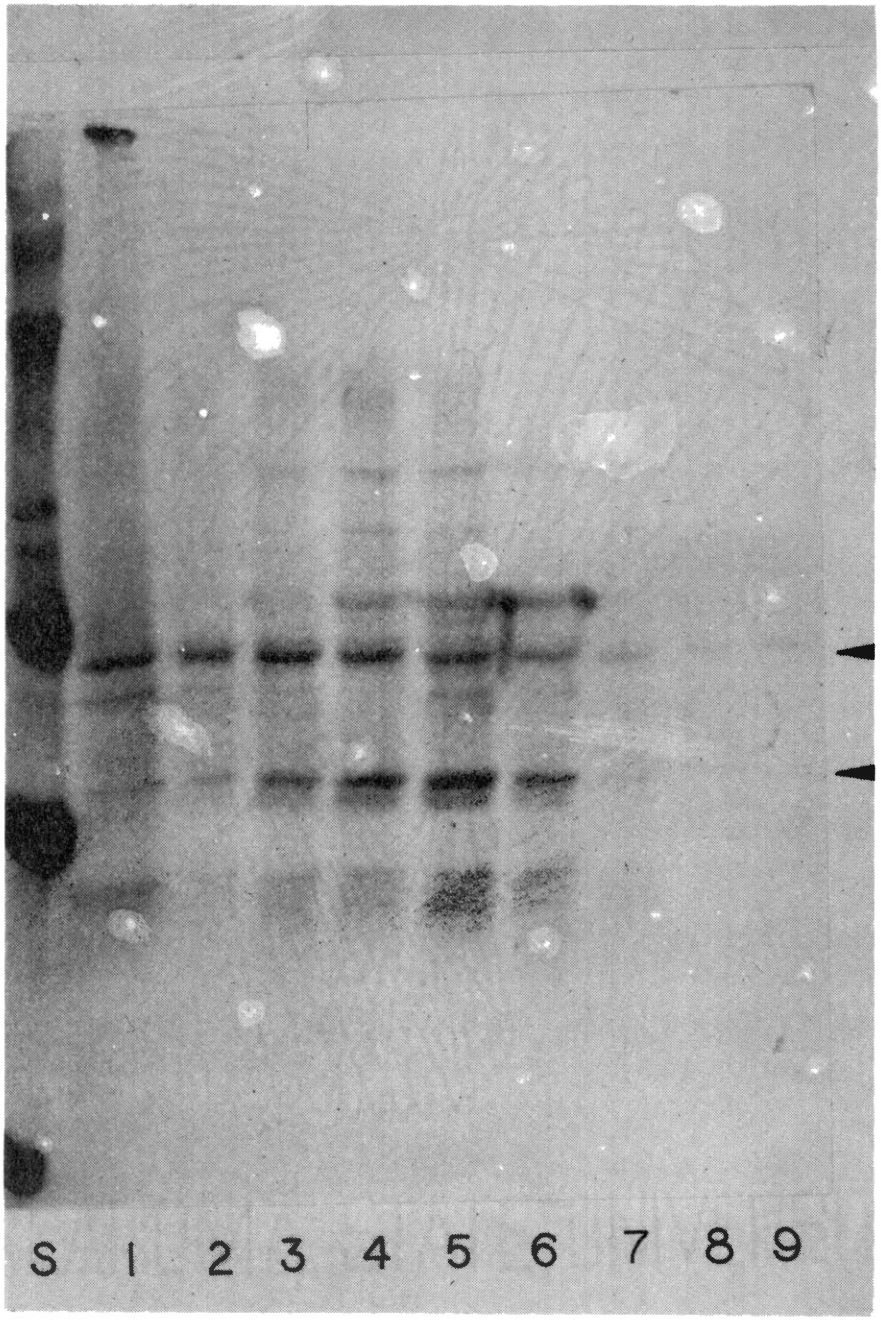

S 1 2 3 4 5 6 7 8 9

Figure 19. Elution Profile of GEM-DOC Sepharose 6B Column.

MF2 was extracted in 0.5% DOC in GEM for 15 min at room temp and chromatographed over a Sepharose 6B column. Fractions were counted. Triangles indicate fractions electrophoresed, solid line [^{3}H]-leucine CPM. V_o) Void volume, T) Thyroglobulin, F) Ferritin, C) Catalase, G) G-Actin, V_r) Retention Volume.

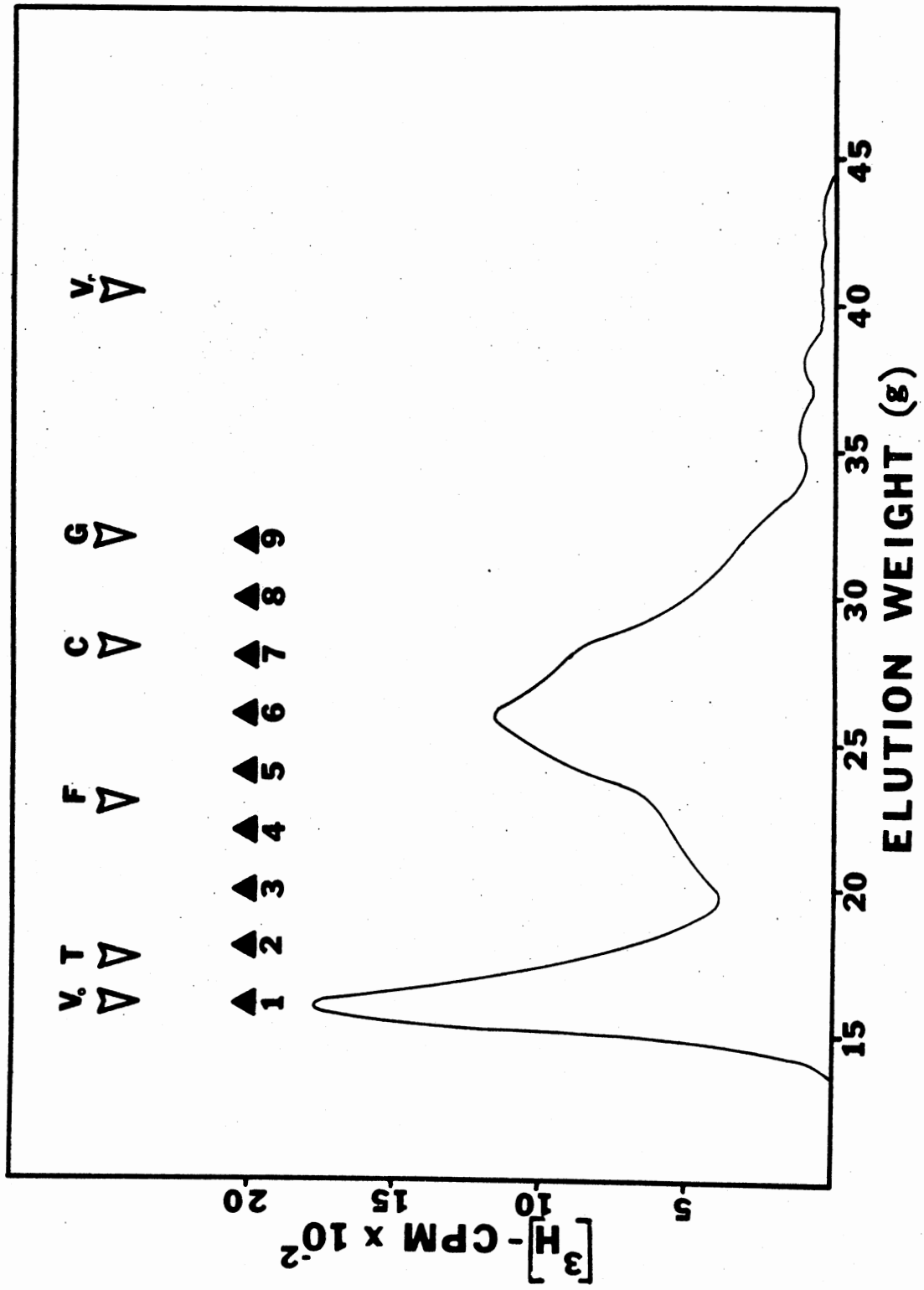
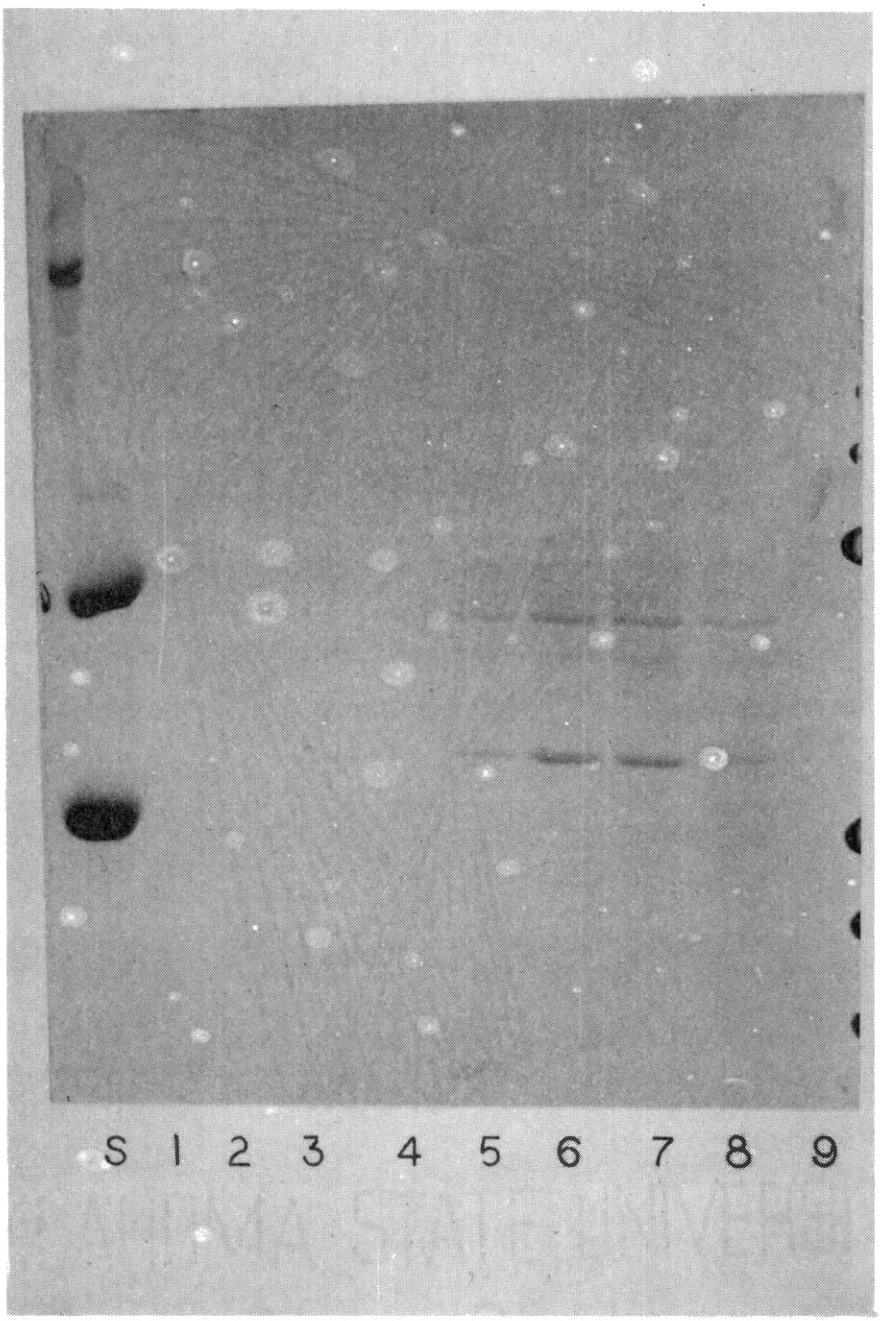

V_o
T
1
2
3
F
4
5
6
C
7
8
G
9
V_t
V_x
5
10
15
20
$[^3H]$ - CPM x 10^2
15
20
25
30
35
40
45
ELUTION WEIGHT (g)

Figure 20. SDS PAGE of Fractions Shown in Figure 19.

Upper arrow indicates 58 K, lower arrow actin.


S 1 2 3 4 5 6 7 8 9

is possible that actin and 58 K aggregates co-sediment after extraction. An attempt was made to separate these components by sequential differential centrifugation using centrifugal forces from 10,000 x g to 200,000 x g. Actin was found by SDS PAGE in all of these sedimented fractions (Figure 21) and the 58 K protein was found at all but the pellets of highest speeds (150,000 g and 200,000 g). Likewise, rate-zonal sucrose density gradient fractionation failed to separate 58 K from actin; the actin was present across the gradient. It appears either that actin is present in different forms or that extraction fragments the core microfilament bundles. Thus the centrifugation results are consistent with the possibility of an association of 58 K with microfilaments in a larger aggregate, but they are not conclusive.

If the 58 K polypeptide is associated with the microfilament core, it should not be exposed on the cell surface. A band corresponding to the 58 K region does not appear in the lactoperoxidase labeling study shown in Figure 9, but the failure to label may have resulted from the absence of accessible tyrosines on the protein or the amount of label incorporated. To resolve these questions MAT-C1 microvilli and MF2 fractions were heavily labeled with [^{125}I] using lactoperoxidase in the presence and absence of deoxycholate. As shown in Figure 22 neither actin nor 58 K is labeled substantially in microvilli or MF2 in the absence of detergent, but both are heavily labeled in its presence. These results are also consistent with an association between 58 K and the microfilaments, and indicate that both microvilli and MF2 are sealed.

To investigate further the interaction between actin and 58 K, MF2 was extracted in an actin de-stabilizing (D) buffer, stabilizing

Figure 21. SDS PAGE of Sedimented Material Obtained After Sequential Differential Centrifugations of Triton X-100 Extracts of MAT-C1 Microvilli.

A) Standard containing ovalbumin, bovine serum albumin and α-actinin, B) intact microvilli, C) 10,000 g x 15 min residue, D) 15,000 g x 15 min residue, E) 20,000 g x 15 min residue, F) 100,000 g x 30 min residue, G) 150,000 g x 30 min residue, H) 200,000 g x 30 min Residue. Upper arrow denotes 58 K, lower arrow actin.

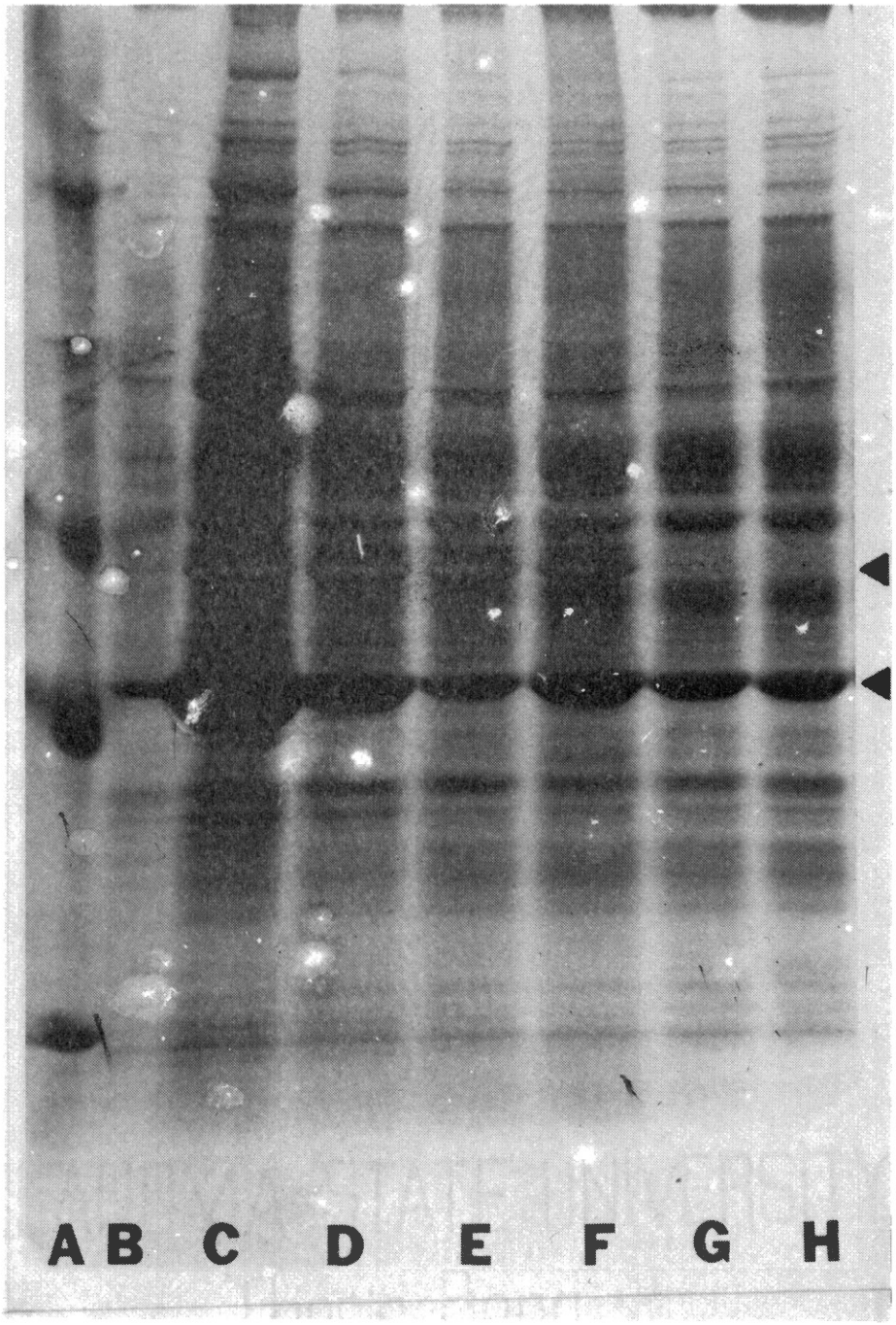
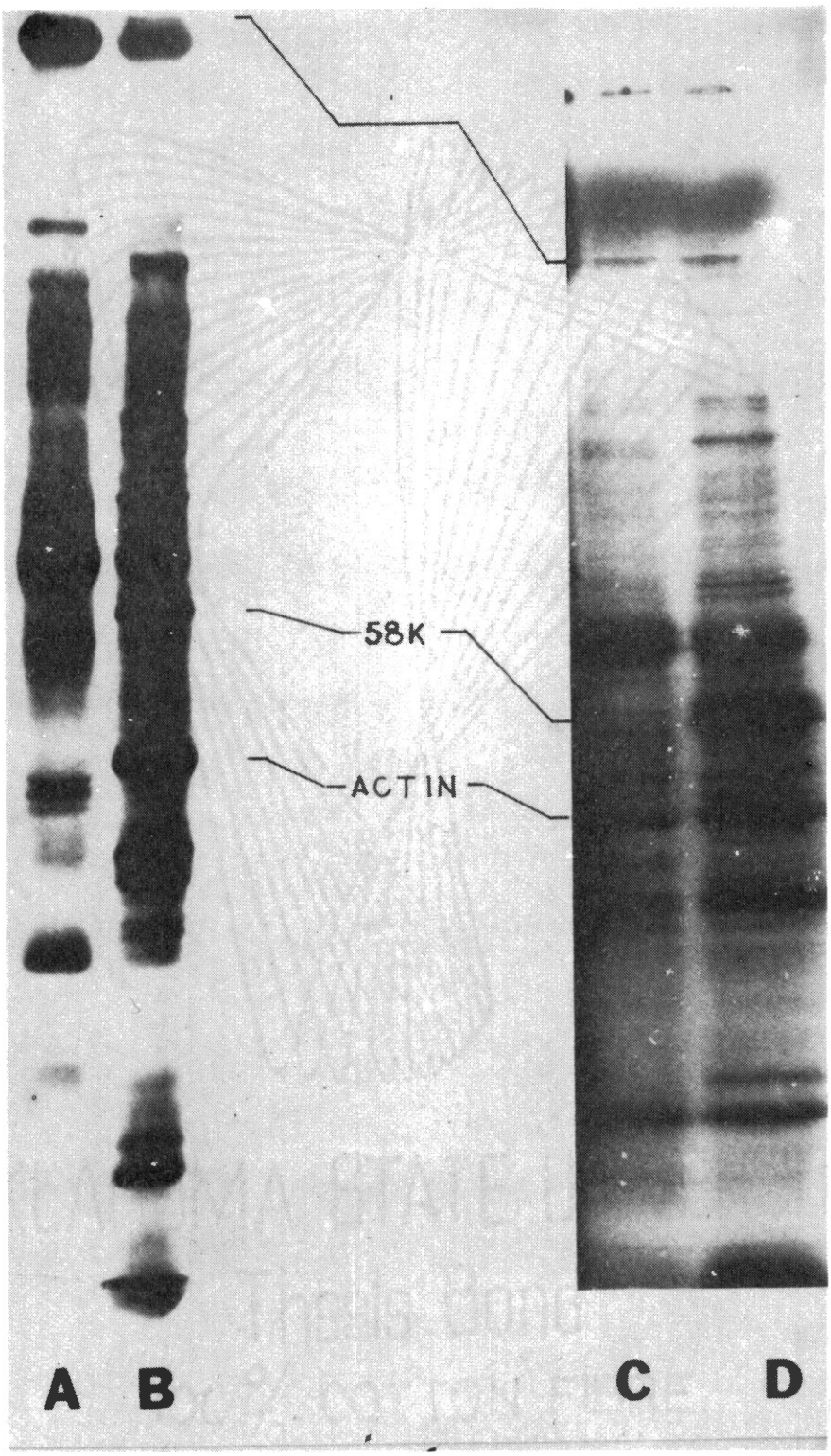

A B C D E F G H

Figure 22. SDS PAGE and Autoradiography of Microvilli (A, C) and MF2 (B, D) Fractions Labeled with Lactoperoxidase and ^{125}I in the Presence (C, D) and Absence (A, B) of Deoxycholate.

The protocol for labeling with and without detergent has been described previously (30).


58K
ACTIN
A
B
C
D

(S) buffer or in a GEM containing 0.2% Triton buffer. The extracts were centrifuged at 42,000 rpm for 1.5 hr in the SW 50.1 rotor and the supernate and pellet fractions were quantitated. Table IV shows that >90% of the protein is released by treatment with the GEM-Triton and D buffers while >78% is released by the S buffer; >75% of the glucosamine labeled moieties are released in all buffers. Densitometric analysis of Figure 23 indicated that >75% of the actin was solubilized in all these buffers (Table V). However, the 58 K protein was only partially soluble in the GEM-Triton buffer, soluble in D, and insoluble in S buffer. The Gem-Triton residue (MF2R) was further extracted in pH 9.0 Tris, 0.6 M KI-GEM and 0.6 M KCl-GEM (Table VI). Actin was only partially (<55%) soluble (non-sedimentable at 200,000 x g for 1 hr) under these conditions. 58 K was nearly completely soluble in high salt and insoluble in a low salt buffer. This solubility is not due to irreversible denaturation of protein since removal of the salt by dialysis allows >98% of 57 K to become insoluble (Table VII). The actin in the pellet fraction may still be in a polymerized form or complexed with the now sedimentable 58 K aggregate.

To show a direct complex between 58 K and actin the myosin affinity technique of Koch and Smith (31) was used. Polymerized actin and components complexed with it bind to myosin and become sedimentable at low g forces. Table VIII shows that of the labeled actin, 37% from MF2 and 60% from MF2P (S buffer residue) binds to myosin. Almost 60% of MF2 and >99% of the MF2P 58 K protein co-sediments with the actin. It is possible that 58 K binds myosin, however, when all the data are considered there is strong evidence for an association between actin and the 58 K protein.

TABLE IV

EXTRACTION OF MF2

	[^{3}H]-leu		[^{14}C]-glucosamine	
	CPM	%	CPM	%
GEM Triton				
Supernate	59,200	90	164,800	89
Pellet	6,400	10	20,400	11
S Buffer				
Supernate	54,400	78	146,400	75
Pellet	16,000	22	50,000	25
D Buffer				
Supernate	44,800	90	147,200	78
Pellet	5,200	10	40,400	21

Figure 23. Extraction of MF2.

MF2 was extracted in Buffer D, S, or GEM containing 0.2% Triton for 15 min at room temp, centrifuged at 42,000 rpm x 1 hr in SW 50.1 rotor. Super and pellet fractions were dialysed against H_2O followed by O/N dialysis against 0.2% SDS then electrophoresed. S) Standard, 1) D supernate, 2) D pellet, 3) S supernate, 4) S pellet, 5) Triton supernate, 6) Triton pellet. Upper arrow indicates 58 K, lower arrow actin.

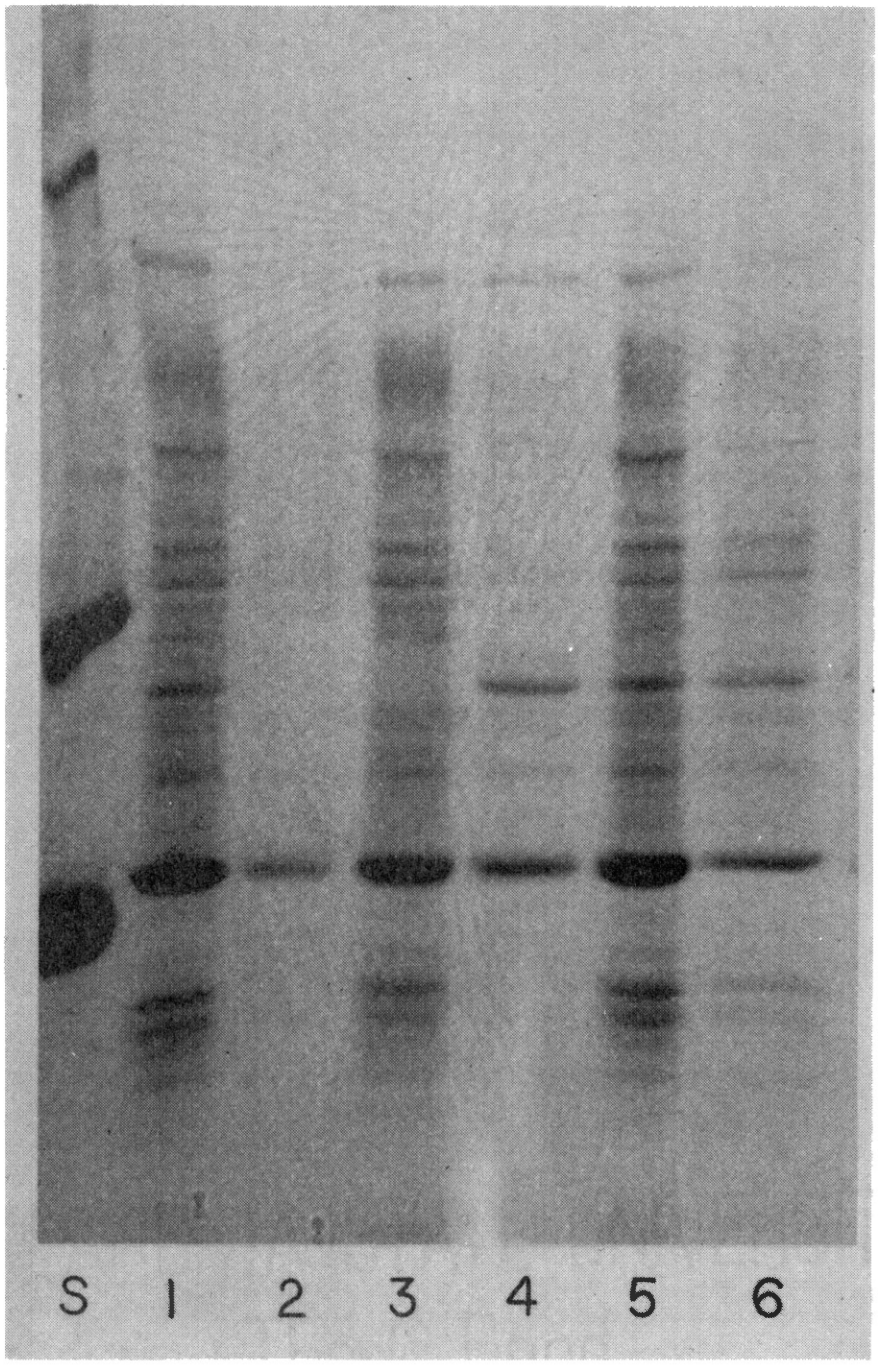

S
1
2
3
4
5
6

TABLE V

EXTRACTION OF ACTIN AND 58 K

	% Extracted					
	D		S		G-T	
	Sup	Pel	Sup	Pel	Sup	Pel
Actin	78.2	21.8	92.7	7.3	77.2	22.8
58 K	>98	<1	<1	>98	50.6	49.4

TABLE VI

EXTRACTION OF MF2R

Component	pH 9	0.6 M KI	0.6 M KCl
		% Extracted	
58 K	<2	91	>98
Actin	50	34	54

TABLE VII

REVERSAL OF 58 K SOLUBILITY

	GEM		PBS	
	Sup	Pellet	Sup	Pellet
58 K	<2%	>98%	<2%	>98%
Actin	40%	60%	30%	70%

TABLE VIII

MYOSIN AFFINITY TECHNIQUE

		Actin			58 K		
	µg Prot	CPM	CPM/µg Prot	% Cont	CPM	CPM/µg Prot	% Cont
MF2	30	370	12.3	37.0	213	7.1	59.2
MF2M	20	91	4.6		83	4.2	
MF2P	30	229	7.6	60.3	181	6.0	>99
MF2PM	12	55	4.5		81	7.0	

State of Actin in the MF2 Fraction

Although actin is a major component of the microvilli, MF1 and MF2, no structures resembling microfilaments are observed in the MF2 preparations (Figure 4). Even if small numbers of microfilaments were present and unobserved, they would be too few to account for the abundance of actin. Moreover, the hypotonic conditions used in the membrane preparation method should effectively release and dilute any G-actin present. These results suggest that the microvillar actin is in a form other than microfilaments.

The DNase inhibition assay of Blickstad et al. (8) was used to determine the state of the actin in MF2 and microvilli (Table IX). However, only 80% of the total actin in MF2 was accounted for by the DNase assay, using rabbit muscle actin as standard. To determine the nature of the actin in the MF2 preparations, the properties of the actin were examined under the conditions of the assay using the S and D buffers.

As already mentioned, electrophoretic analysis on dodecyl sulfate gels indicated that >75% of the actin was also solubilized (not sedimented at 150,000 x g for 1 hr) in both buffers. In contrast a substantial fraction of actin was sedimented from microvilli preparations treated with S buffer (but not D buffer), results consistent with the observation of microfilaments in microvilli but not in MF2.

Extracts of leucine-labeled MF2 in S- and D-buffers were chromatographed on Sepharose 6B and the peak fractions examined for actin by electrophoresis. In the S buffer (Figure 24A, 25) much of the actin eluted at a position intermediate between G-actin and F-actin.

TABLE IX

STATE OF ACTIN

Fraction	Myosin Affinity	DNase Inhibition	
	% Bound	F/G	% Detected
Microvilli	57.2	1.56	78.7
MF2	36.2	1.14	80.7
MF2S	12.3	0.16	41.0
MF2P	63.5	0.75	59.0

Figure 24. Elution Profiles of MF2.

MF2 was extracted for 15 min at room temp in S or D buffer at 0.5 mg protein MF2/ml and chromatographed over a Sepharose 6B column. A) Profile of extraction in S buffer, B) profile of extraction in D buffer. Solid line ^{3}H-leucine DPM, Dashed line % F-Actin, Broken line % G-Actin. % Actin calculated by densitometric analysis. V_o = Void volume, V_r = retention volume, F_e = ferritin standard, A = aldolase standard.

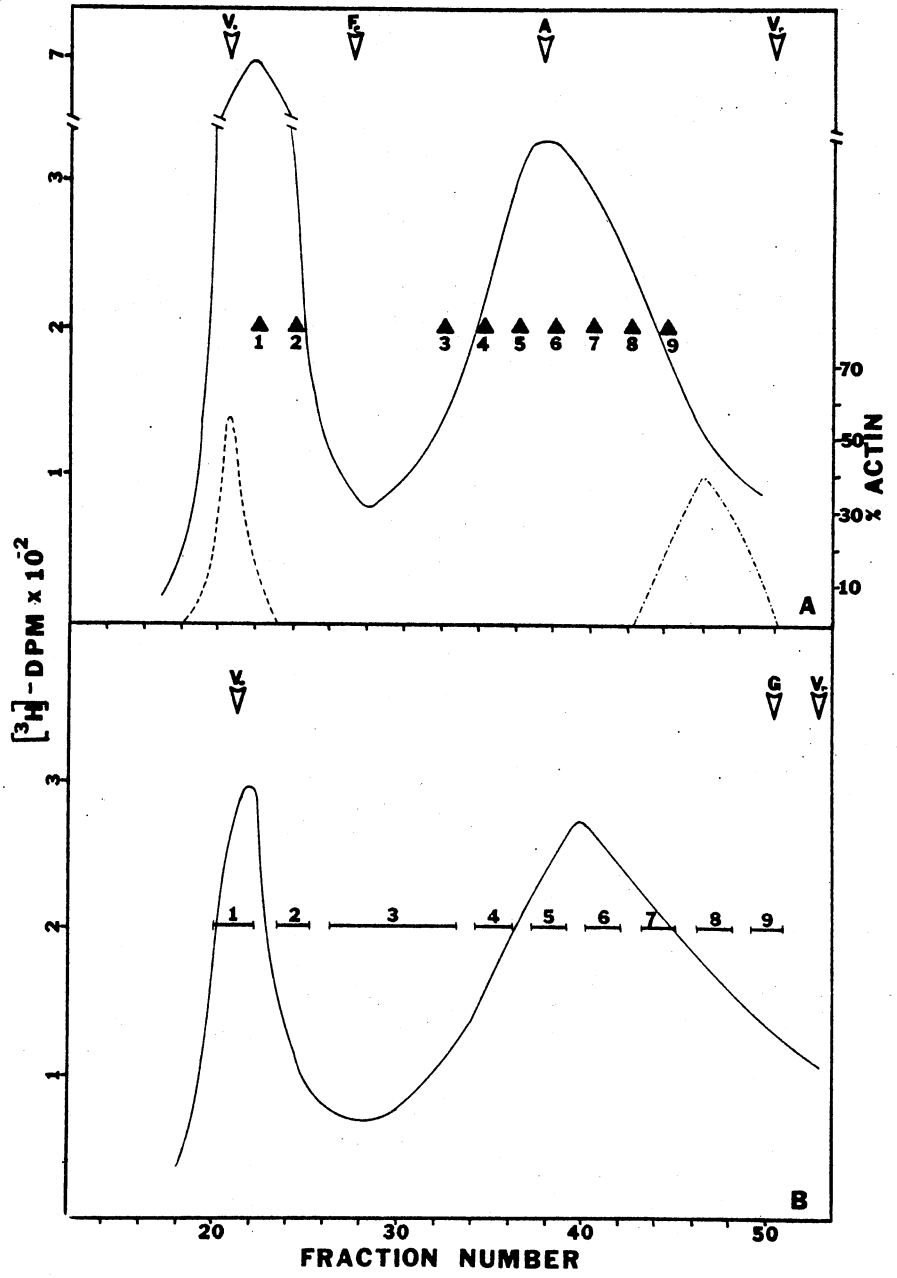
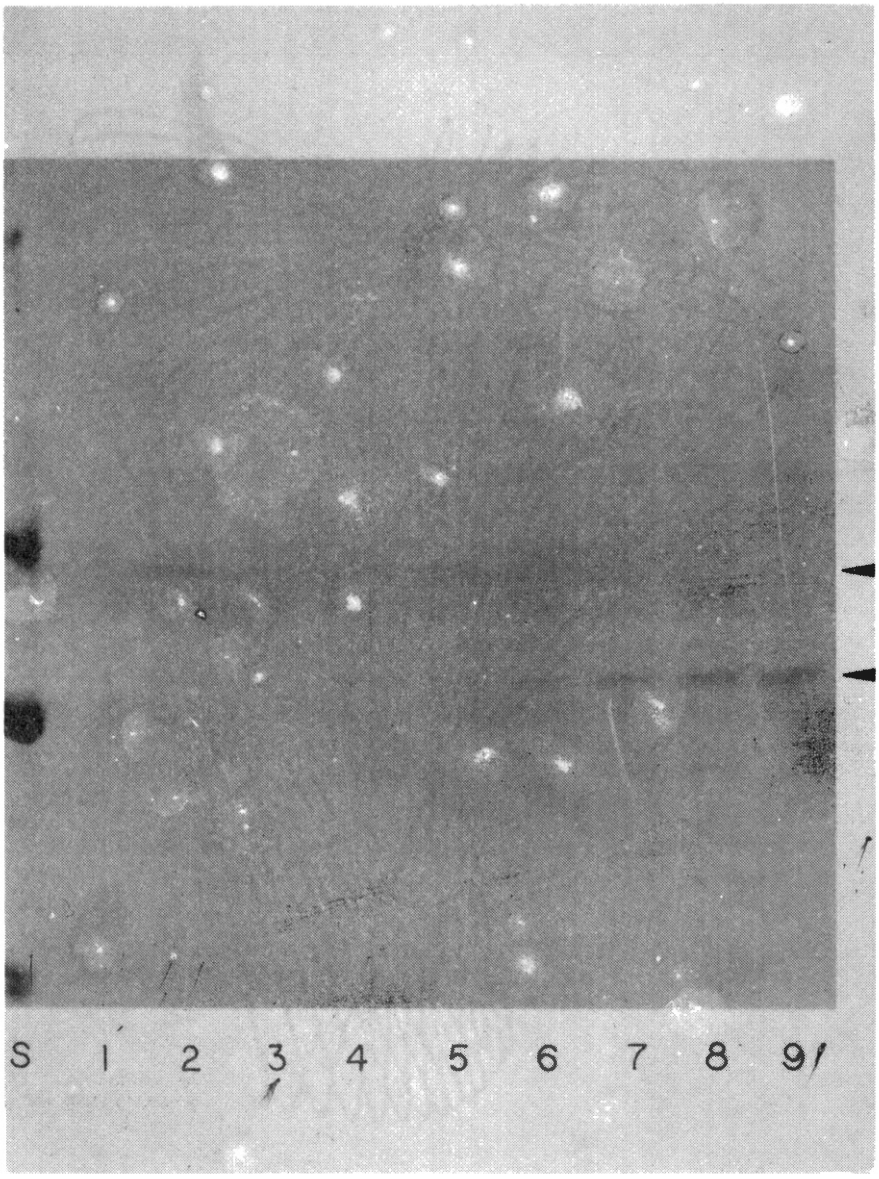

V0
Ferritin
Aldolase
Vt
1
2
3
4
5
6
7
8
9
70
50
30
10
% ACTIN
A
G
B
[3H]-DPM x 10-2
FRACTION NUMBER
20
30
40
50

Figure 25. SDS PAGE of Fractions Marked in Figure 24A.


S 1 2 3 4 5 6 7 8 9

In D buffer (Figure 24B, 26), however, the actin eluted predominantly at a volume corresponding to that of rabbit muscle G-actin, as would be predicted from the previous observations on the depolymerization of actin in this buffer (8). Similar results were obtained when the size of the actin in the extracts was examined by density gradient centrifugation. In S buffer (Figure 27A, 28) the actin in the extracts sedimented at an intermediate position between G- and F-actin, while in D buffer (Figure 27B, 29) it sedimented predominantly with rabbit muscle G-actin.

By both gel filtration and density gradient centrifugation the intermediate form of actin is nearer G-actin than F-actin, suggesting the presence of short oligomers. Additional studies will be necessary to characterize more completely the range of sizes.

To assure that the presence of the intermediate actin did not result from degradation of F-actin during the MF2 preparation, microvilli were subjected to the same procedures. As shown in Figures 30 and 31, F-actin was a substantial component of the microvilli, as expected, along with the intermediate form of actin. These results also show that the intermediate form of actin can be obtained from the microvilli and thus is not an artifact of the microvillar membrane preparation.

A possibility exists that the oligomeric actin units are formed by G-actin polymerization during extraction in the S buffer. To clarify this point, S buffer containing soluble [^{35}S]-methionine labeled G-actin was used to solubilize the MF2 fraction prior to chromatography. If actin was polymerizing, the [^{35}S]-actin would be incorporated into the oligomeric units. Figure 32 shows the elution profile of MF2 + [^{35}S]-

Figure 26. SDS PAGE of Fractions Marked in Figure 24B.

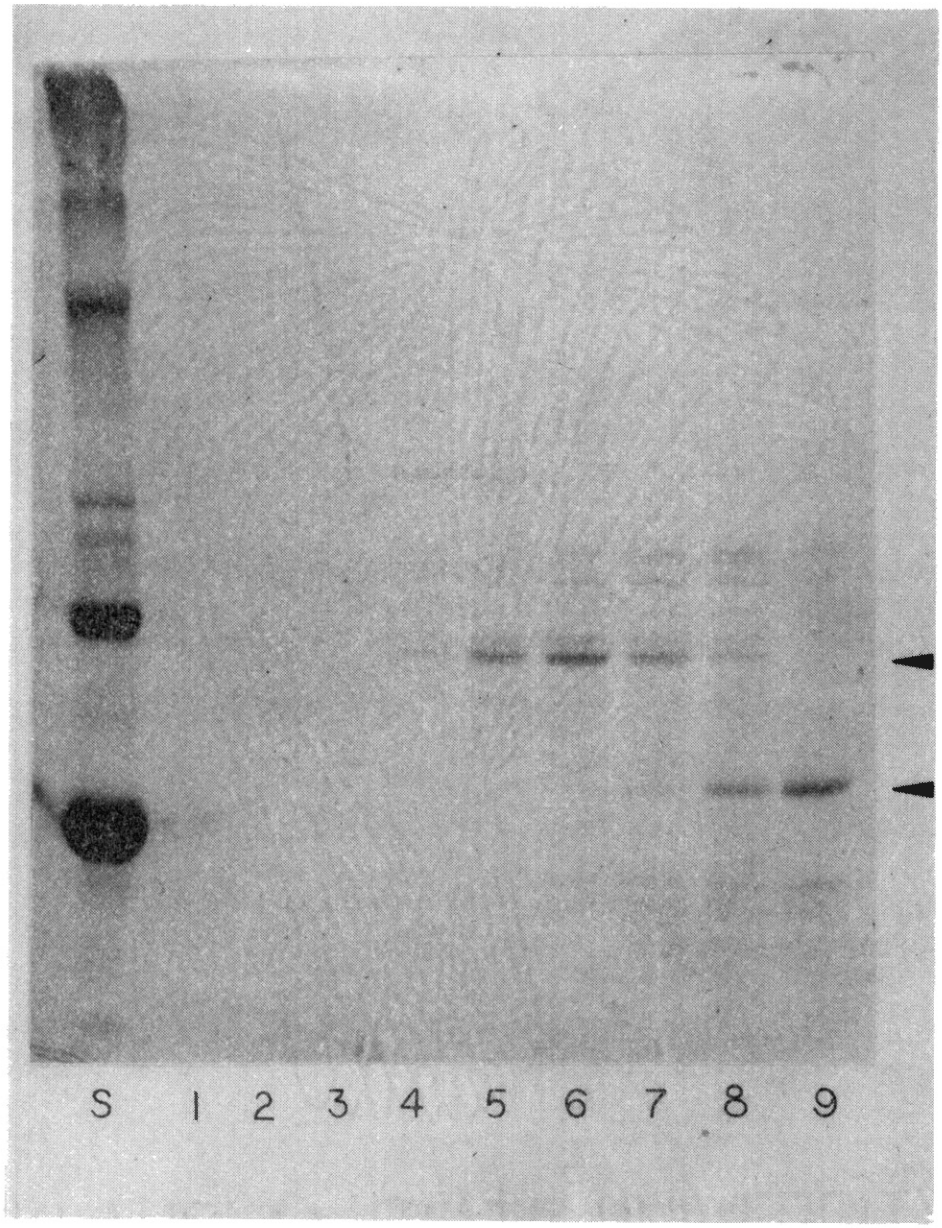

S
1
2
3
4
5
6
7
8
9

Figure 27. Rate-Zonal Fractionation Profile of MF2 on Sucrose Gradient.

MF2 was extracted for 15 min at room temp in S or D buffer and sedimented over a linear 5-20% sucrose gradient at 22,000 rpm x 40 hrs in the SW 27.1 rotor. Fractions were counted, pooled, dialysed and electrophoresed. A) Sucrose density gradient centrifugation profile of [^{3}H]-leucine-labeled MF2 solubilized in S buffer. B) Same as (A) except solubilization in D buffer. Solid line in [^{3}H]-leu DPM, dashed line indicates sucrose concentration, broken line is G-actin standard. G = G-actin, F = F-actin standard.

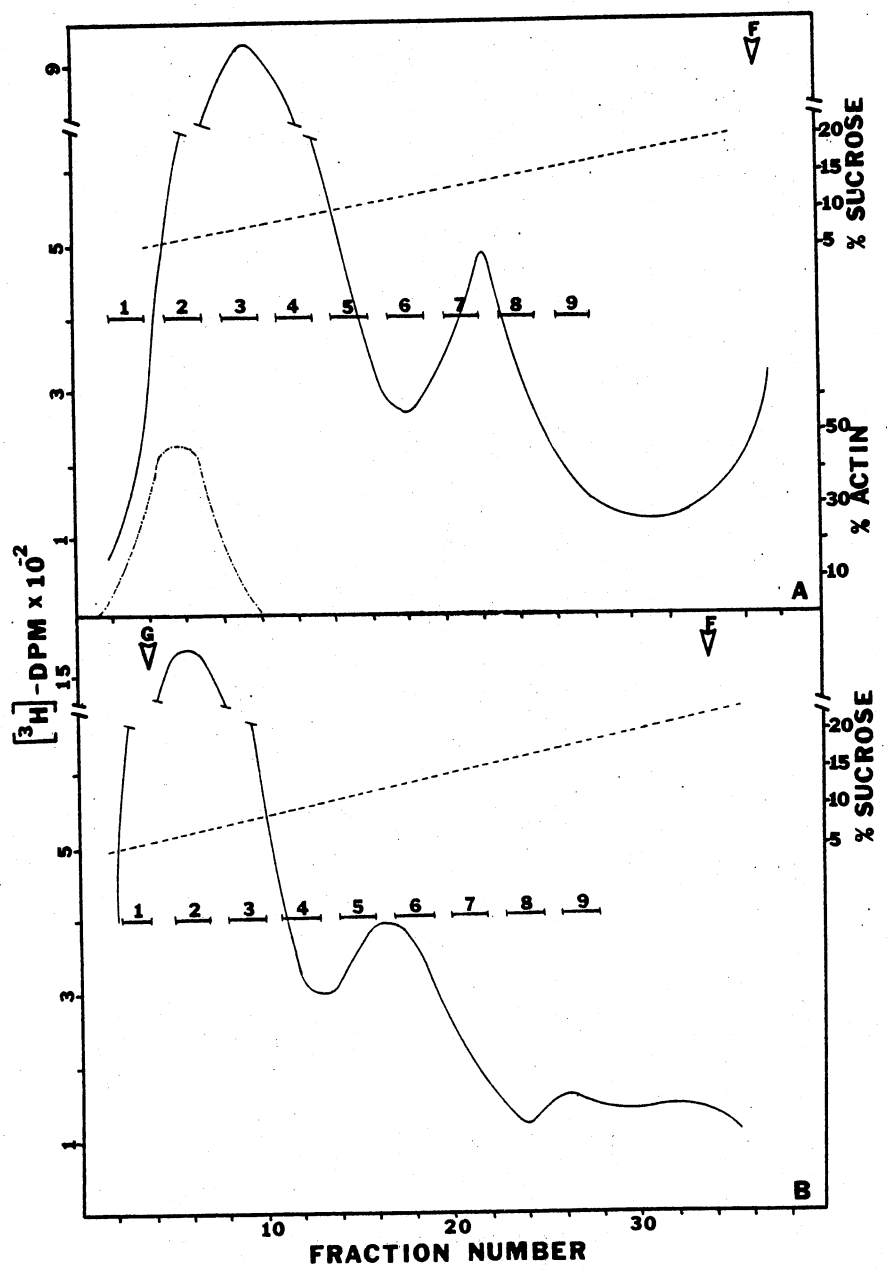

[3H]-DPM x 10-2
% SUCROSE
% ACTIN
% SUCROSE
FRACTION NUMBER
A
B
G
F
F
1
2
3
4
5
6
7
8
9

Figure 28. SDS PAGE of Fractions Marked in Figure 27A.

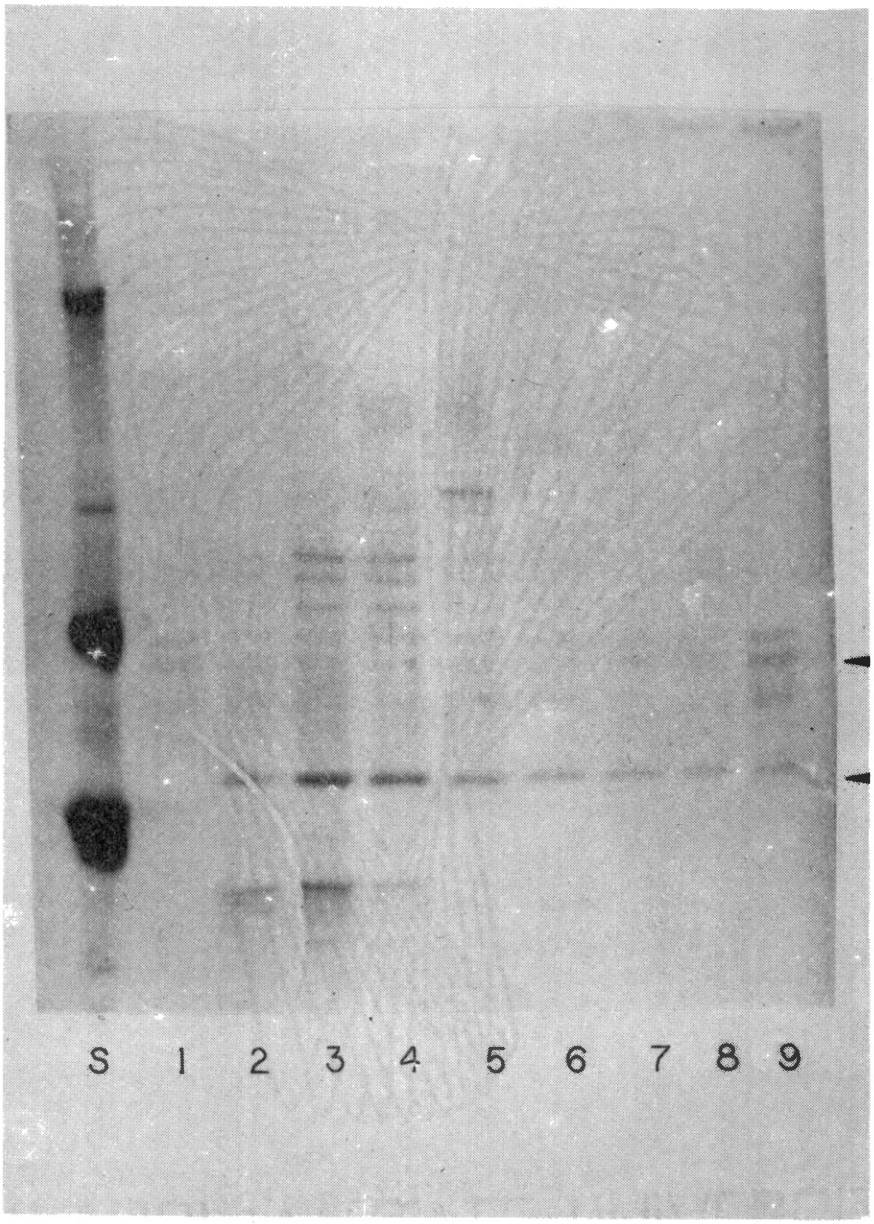

S 1 2 3 4 5 6 7 8 9

Figure 29. SDS PAGE of Fractions Marked in Figure 27B.

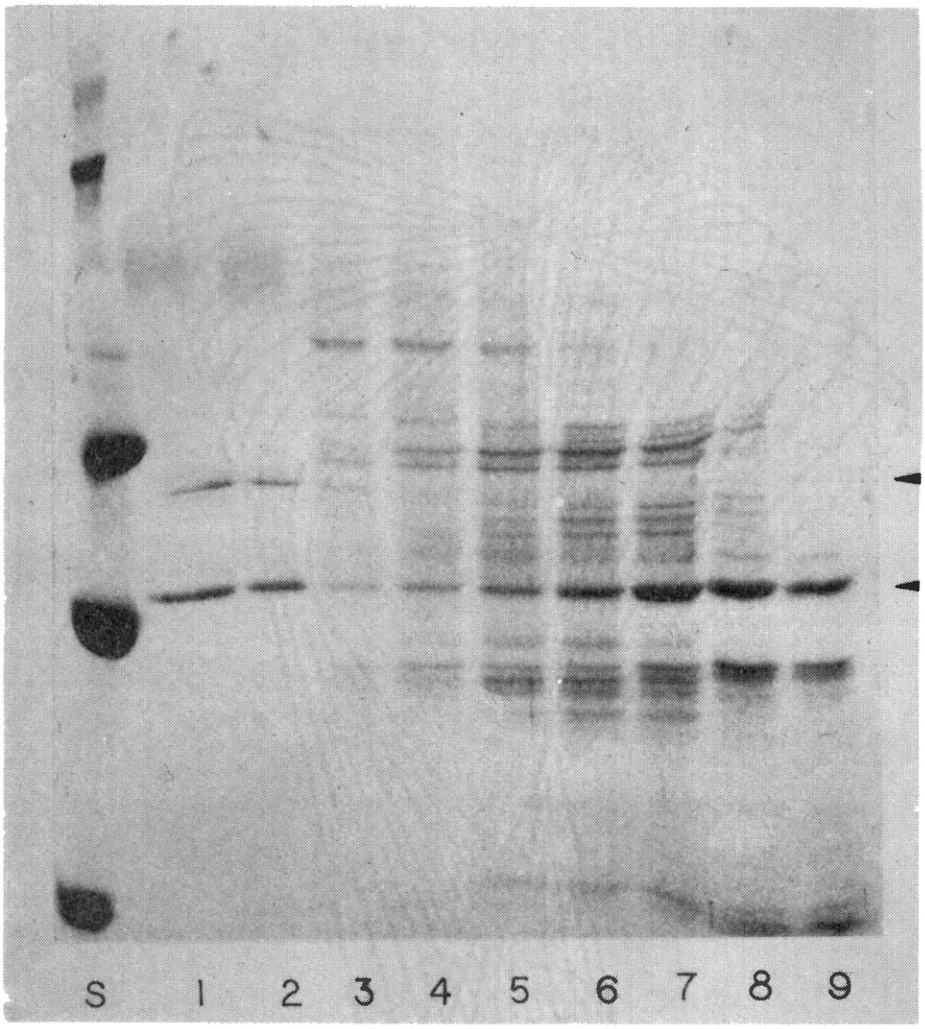

S
1
2
3
4
5
6
7
8
9

Figure 30. Elution Profile of Extracted Microvilli.

Microvilli were extracted in S buffer for 15 min at room temp and chromatographed over a Sepharose 6B column. F = F-actin, G = G-actin standard.

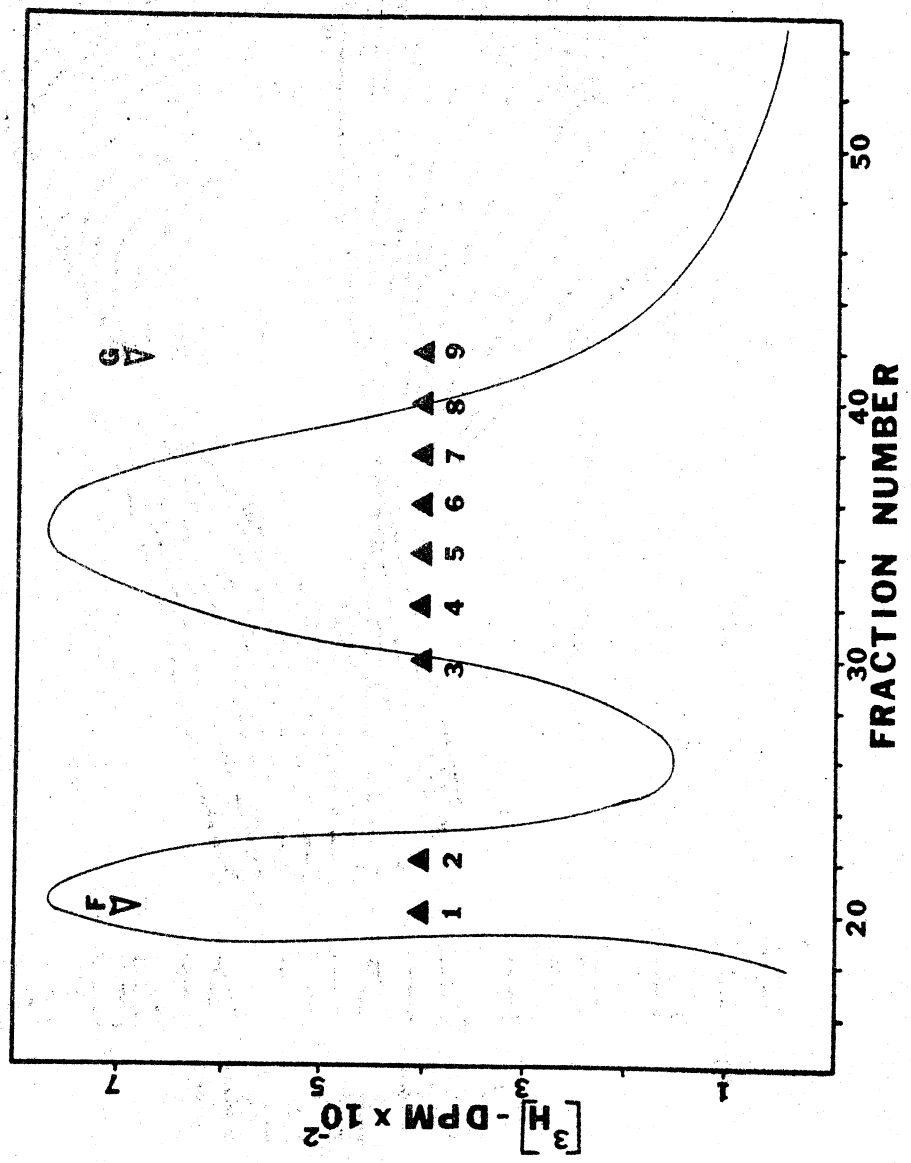

FA
GA
1
2
3
4
5
6
7
8
9
20
30
40
50
FRACTION NUMBER
1
3
5
7
[3H] - DPM x 10-2

Figure 31. SDS PAGE of Fractions Marked in Figure 30.

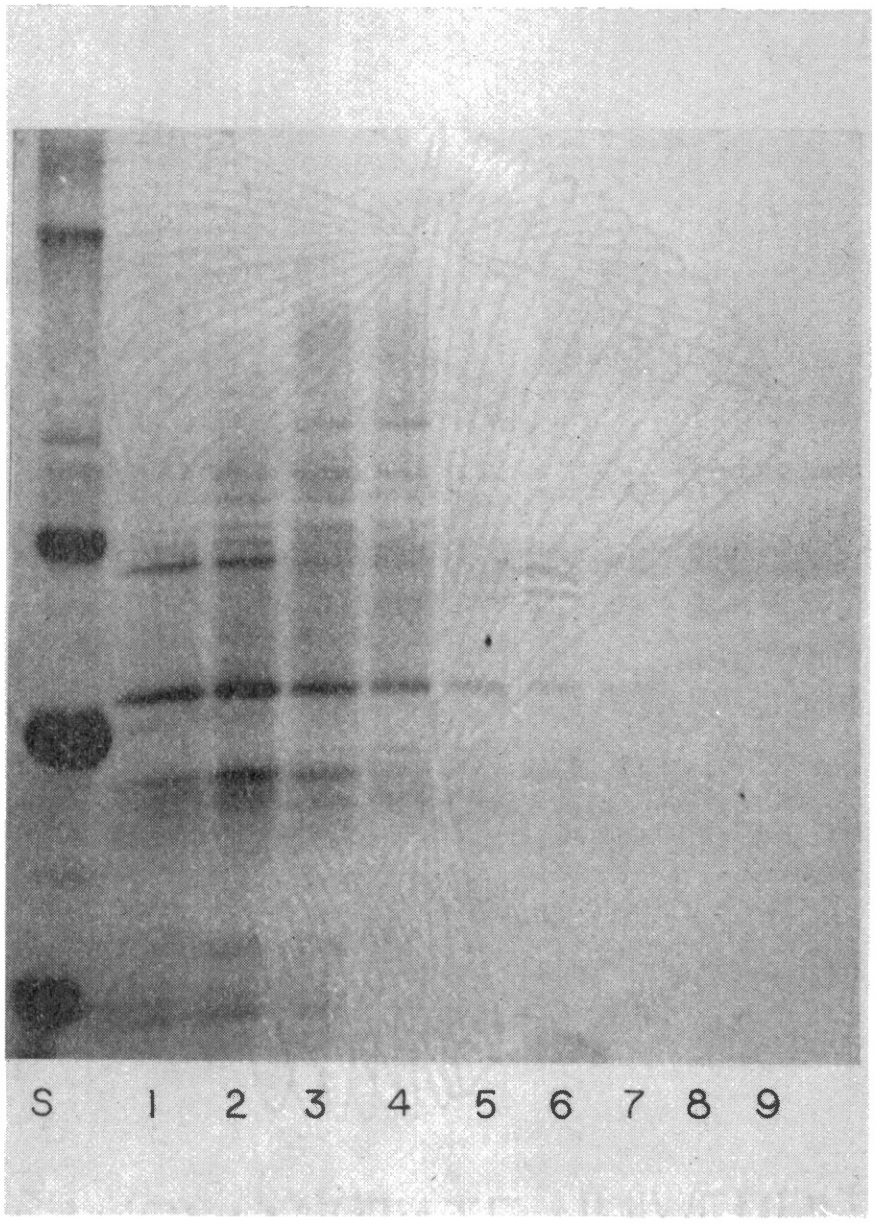
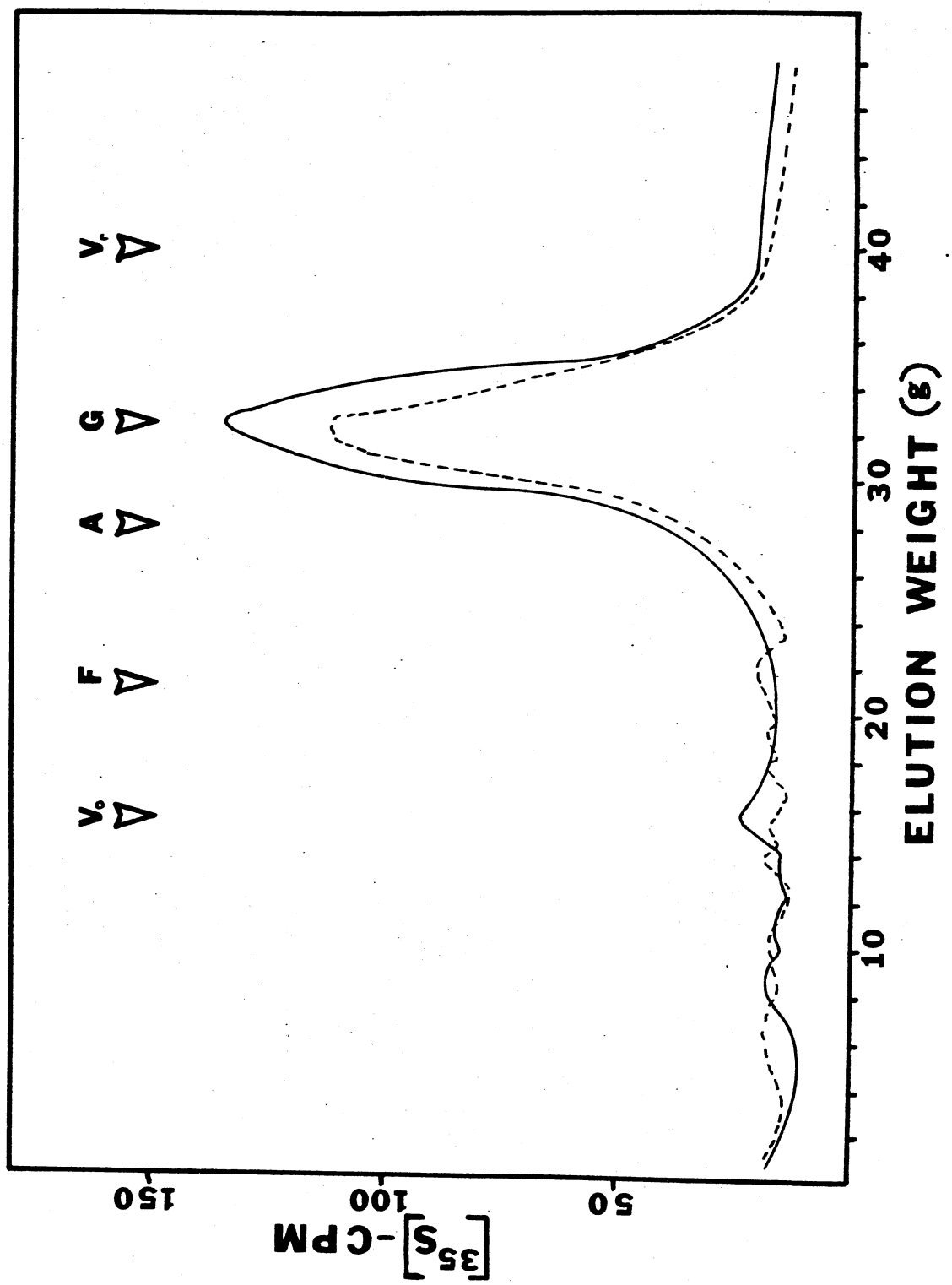

S
1
2
3
4
5
6
7
8
9

Figure 32. Elution Profile of MF2 Incubated with [^{35}S]-actin.

The MF2 pellet was solubilized in S buffer containing [^{35}S]-actin for 15 min at room temp and chromatographed over a Sepharose 6B column. A 250 µl aliquot was taken of each fraction and counted. The [^{35}S]-actin was also chromatographed over the 6B column under the same conditions and had an elution volume the same as G-actin. Standards were: V_o) void volume, F) ferritin, A) adolase, G) G-actin, V_r) retention volume.


V_o
F
A
G
V_t
150
100
50
[35S]-CPM
10
20
30
40
ELUTION WEIGHT (g)

actin and the [^{35}S]-actin alone. There was no evidence of incorporation of the labeled actin into oligomeric units. To show that the isolated [^{35}S] G-actin was not denatured, it was dialysed against a 0.6 M KCl buffer and centrifuged at 42,000 rpm x 1.5 hr in the SW 50.1 rotor. The supernate and pellet fractions were dialysed and electrophoresed showing actin in both fractions; thus indicating the [^{35}S]-G-actin has polymerization capabilities.

CHAPTER IV

DISCUSSION

Branched and unbranched microvilli from MAT-C1 and MAT-B1 cells, respectively, can be isolated using the same procedure. The isolated microvilli can be disrupted further to give plasma-membrane enriched fractions, MF1 and MF2. Comparison studies involving electron microscopy, enzyme analyses, radiochemical labeling and polypeptide analyses indicate the microvilli and fractions are very similar except for the MAT-C1 branched microvilli morphology and in the presence of a 58 K polypeptide in the MAT-C1 microvilli.

The 58 K polypeptide appears to be a membrane associated protein. It co-isolates with membrane enriched preparations of the microvilli and remains with the membrane fraction when floated in a sucrose gradient. In addition, after solubilization of the membrane fraction with DOC, 58 K associates with reconstituted lipid vesicles.

This 58 K protein, however, is not a typical integral membrane protein. It is soluble in DOC, high salt and guanidine but remains insoluble after Triton extraction, suggesting an association with the cytoskeleton. The [^{125}I] labeling studies show that 58 K is not exposed on the cell surface, but is localized on the cytoplasmic side of the MF2 membrane. Sequential differential centrifugation of Triton-extracted MAT-C1 microvilli failed to separate 58 K and actin. Also, the Myosin Affinity Technique detects 58 K as a protein interacting

with actin. These results strongly suggest an association between 58 K and actin.

To understand the nature of the 58 K-actin interaction it is important to know the state of actin, i.e. is it in the F, G or an intermediate form? The results presented strongly suggest the presence of an oligomeric form of actin associated with the microvillar membranes. The DNase assay does not detect all of the actin associated with the membrane fractions. Chromatography and sucrose density centrifugation both demonstrate that the actin in MF2 is in a form intermediate between F-actin and G-actin. This intermediate form is not the result of either degradation of F-actin or polymerization of G-actin during extraction. The oligomeric state of actin is not caused by the association of G-actin with another protein, since actin is by far the predominant component in the intermediate peak fractions. However, this observation does not rule out the possibility of proteins associated at lower stoichiometries with oligomeric actin. Since such proteins might play a role in regulating actin oligomer size, it will be of interest to purify the oligomers and examine them for associated proteins.

What is the nature of oligomeric actin? It may simply be short segments of F-actin. Alternatively, it may be a different chemical or conformational form. Additional biochemical and biophysical characterizations are needed to assess these possibilities and to determine how the oligomeric actin is associated with microfilaments, the plasma membrane and the 58 K protein.

Evidence presented shows 58 K is associated with both the cytoskeleton and with the membrane. If this is so, then it becomes a

candidate for a linking protein, a protein to serve as a bridge between the cytoskeleton and the membrane. As a linking protein, 58 K may serve as a lateral connection between microfilament cores and the membrane or as a cross-linker of short actin units at the membrane.

A component having a linking function might aid in the stabilization of the microvilli and microvillar cell surface. Molecules attached to a stabilized cytoskeleton would not be as able to patch and cap as those attached to an unstabilized structure.

Also, this stabilization could prevent retraction of microvilli into the cell body and breakdown as the cell proceeded through cell division. Thus newly forming microvilli would be pushed out from the cell surface behind the microvilli already present. This process alone could lead to the intricate branching patterns observed with MAT-C1 cells. Since the branched structures provide a barrier to approach by another cell or antibody, they offer a selective advantage for survival of the MAT-C1 subline. It has recently been observed that xenotransplantability of sublines of the 13762 tumor is more closely correlated with the presence of branched microvilli than with any other cell surface feature that has been examined (S. Howard, unpublished observations).

CHAPTER V

SUMMARY

Microvilli and microvilli fractions were isolated from the MAT-B1 and MAT-C1 sublines and were characterized by electron microscopy, plasma membrane marker enzyme analyses, metabolic labeling with glucosamine and polypeptide analysis by dodecyl sulfate polyacrylamide gel electrophoresis. Comparison of the two sublines showed them to be very similar except for the presence of a Mr 58,000 protein (58 K) in MAT-C1 microvilli which was essentially absent in MAT-B1 microvilli.

The 58 K protein was shown to be associated both with the membrane and with the cytoskeleton of the membrane enriched fraction from the microvilli (MF2). Characterization of the actin associated with MF2 revealed that the actin is in an oligomeric state, either as short F-actin filaments or in a different conformation.

It is therefore suggested that 58 K is a linking protein between actin and the membrane. This link may be a lateral connection between the microfilaments and the membrane in the microvillus. Alternatively, 58 K may serve to cross-link the oligomeric actin units with one another and the membrane. Either type of association would permit stabilization of the microvillus structure. This stabilization could then induce branching of the microvillus or immobility of cell surface molecules.

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