CYTOSKELETAL-MEMBRANE INTERACTIONS IN ASCITES TUMOR CELLS

By

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Dedication

To my father, the memory of my beloved mother and Sri Paramhansa Yoganandaji for their love, council and gentle discipline.

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LIST OF ABBREVIATIONS

Å	Angstrom, 10^{-8} cm
ABP	Actin Binding Protein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
°C	Degrees centigrade
Cyto B	Cytochalasin B
CBB	Coomassie Brilliant Blue R250 stain
Cyto D	Cytochalasin D
cm	Centimeter
cpm	Counts per minute
DEAE	Diethylaminoethyl cellulose
dl	Deciliter
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dpm	Disintegrations per minute
$E^{1\%}_{280}$	Extinction coefficient
EDTA	Ethylenediamine tetraacetate
f/f_0	Frictional coefficient
FITC-Con A	Fluorocein isothiocyanate conjugated Concanavalin A
g	Gravity
GEM	5 mM glycine-1 mM EDTA-5 mM β-mercaptoethanol buffer

gm	Gram
GM	5 mM glycine-5 mM β-mercaptoethanol buffer
HBS	HEPES buffered saline
HMM	Heavy meromyosin
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium dye
IAA	Iodoacetamide
LDH	Lactate dehydrogenase
LIS	Lithium diiodosalicylate
M	Molar
β-EtSH	β-mercaptoethanol
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mw	Molecular weight
NAD^+	Oxidized nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PRP	Platelet rich plasma
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Sedimentation coefficient
SEM	Scanning electron microscopy

SDH	Succinate dehydrogenase
SDS	Sodium lauryl sulfate
TEM	Transmission electron microscopy
Tris	Tris-(hydroxy methyl)-aminomethane
μ	Micron, 10^{-5} cm
μCi	Microcurie
μg	Microgram
μl	Microliter
UV	Ultraviolet light
$\bar{v}$	Partial specific volume

CHAPTER I

INTRODUCTION

Membrane Mobility

The early models for plasma membranes were based on lipid films (1) and expanded to assume a bilayer configuration placing the more hydrophilic moieties in contact with the liquid milieu on both sides and the hydrophobic regions in the interior.

By electron spin resonance (2), X-ray diffraction (3), and nuclear magnetic resonance (4) the bulk of the lipid appears to be in a fluid state at 37°C.

Membrane proteins are of two types: 1) peripheral proteins which are dissociated using hydrophilic agents and 2) integral proteins which are solubilized with hydrophobic agents and may co-isolate with phospholipid. These latter proteins may be highly asymmetric having regions interacting strongly with the internal lipid and the outer aqueous media (5).

Vertical mobility across the plasma membrane is thermodynamically difficult and "flip-flop" of molecules occurs at a very slow rate (6), on the order of minutes or greater. Lateral mobility, however, occurs at much greater rates. Edidin and Fambrough estimated diffusion of antigens in muscle fiber membranes to be in the range of 10^{-9} cm^2 per second (7).

Chemical labelling and hydrolytic methods (8, 9) have demonstrated that there is asymmetry in the membrane itself, i.e. lipid segregation, so that phosphatidyl ethanolamine and phosphatidyl serine had sphingomyelin predominate in the cytoplasmic half while phosphatidyl choline predominates in the outer half of the bilayers. This differential organization of lipids could serve to control fluidity in the two halves. Also the higher anionic grouping in the inner half could sequester cations or facilitate ionic interactions with proteins on the cytoplasmic side.

The Fluid Mosaic Model

The fluid mosaic model (10) was proposed to account for these observations. However, it is necessary to put restraints on this model to account for observations on membrane-bound proteins in bacteria (11) and mammalian cells (12), in which molecular motions such as those induced via lectins or specific antigens (13) are dependent on factors other than lipid interactions. Such processes as patching and capping require restrictions on movements of surface receptors. In cases where capping is allowed to proceed, it usually induces either endocytosis of that part of the membrane containing the immobilized receptors (14) or shedding into the media (15).

Membrane Associated Control Elements

It was noted that reduced temperature, metabolic inhibitors, or drugs could inhibit either capping or patching. Hence patching, but not capping, is independent of cellular metabolism. Drugs known to interact <u>in vitro</u> with either microtubules (16) or microfilaments

(17) could alter the patching and capping phenomena. Such results led to the postulation that membrane receptors are transmembrane linked to one or both of these cytoplasmic systems which can then exert control over the distribution of the surface constituents.

Evidence for association of cytoskeletal elements with plasma membranes has been shown by studies such as those by McNutt et al. (19) for microtubules and Clarke et al. (20) for microfilaments.

Perhaps the best example of possible cytoskeletal control over cell surface components is that from erythrocytes. The work of Ji and Nicolson (21, 22) showed only minor crosslinking of erythrocyte membrane components with dimethylmalonimidate (distance between functional end groups is approximately 5 Å), and this appears to be limited to lipid and glycoprotein components. When lectins were added to the system under conditions where cell surface glycoproteins are aggregated, spectrin became susceptible to cross-linking under the same conditions. Blocking or dissociating the lectin before cross-linking or lowering the temperature to prevent receptor movement allowed no spectrin cross-linking.

This transmembrane coupling could be demonstrated alternatively by reacting with anti-spectrin, fixing with glutaraldehyde and observing sialic acid at the membrane surface via electron microscopy (23). Clustering was seen with prior anti-spectrin treatment.

Contractile Components of the Cytoskeleton

Both actin and myosin are constituents in every animal cell type analyzed. Since actin-myosin interactions are well established for generating force in muscle tissue, these proteins were postulated

to likewise be responsible for motility, cytoplasmic streaming, cleavage and phagocytosis in non-muscle cells.

Identification of actin can be achieved by electron microscopy using decoration of actin filaments by the trypsin cleavage product of myosin, heavy meromyosin, or the papain cleavage product, subfragment 1. Other common means for identification are SDS PAGE gel mobility and polymerization-depolymerization behavior in alternating high and low salt. Additional biochemical means utilize amino acid analysis, peptide mapping of enzymatic or cyanogen bromide cleavage products, hydrolysis of ATP and viscosity measurements.

Actin is present in amounts as high as 10 percent of the total cellular protein. It is present in both globular and filamentous forms although stress fibers are seen only in spread cells. Recently, it has been shown that DNase I can bind and be inhibited by actin (24) and this has become an additional test for identity.

Myosin is present, but at a concentration an order of magnitude less than is normal for skeletal muscle. Usually electron microscopic visualization does not show characteristic thick filaments in any great numbers. Negative staining of platelet actomyosin does not reveal the sarcomeric arrangement typical of muscle. Instead, the myosin molecules form short, thin, bipolar filaments which bridge parallel actin filaments (25). As yet, no evidence has been described that a similar organization exists in other non-muscle cell types.

Other muscle proteins (α-actinin, tropomyosin, and the troponin Ca^{++} regulation complex) have been found in nonmuscle cells. Most of this work is based on immunofluorescence data (26). Unfortunately, this technique does not allow quantitation since the fluorescence is

usually amplified by a sandwich staining method or by the optic system used for visualization of the labelled molecules. It is also difficult to discern true fibrillar structures from binding at sufficiently close intervals that overlap of fluorescence can occur. The methods used to allow access to the cells may themselves induce artifacts (Carraway, unpublished observations). Dr. Robert Goldman is currently seeking to overcome massive destruction of membrane permeability barriers by short (3 second) exposure to low levels of non-ionic detergents prior to flooding the cells with specific antibody.

Changes in Organization with the Cell Cycle and Transformation

Sanger (28, 29) has used the immunofluorescence technique to localize bulk actin in fibroblasts during the cell cycle. Resting cells showed a cable appearance (stress fibers) but as the cell rounded for mitosis, actin staining became diffuse. It subsequently became localized in the mitotic apparatus region and finally at the developing cleavage furrow.

Heaysman et al. (30) have observed that a thick array of actin filaments is associated with the plasma membrane at the site of contact when two fibroblast cells meet each other. There is an associated cessation of membrane ruffling in this same area and it was suggested that the filament formation may induce localized rigidity of the membrane and hence inhibition of movement.

Brinkley et al. (31) have shown that SV40 infected 3T3 cells exhibit very few cytoplasmic microtubules by immunofluorescence.

Since this phenomenon may correlate with cell rounding in general, it is not clear that this is a specific consequence of transformation. Additional evidence from Fine and Taylor (32) suggest that the total tubulin content in SV403T3 cells is decreased. A similar decrease in tubulin synthesis accounted entirely for the former. The half-life of the protein determined by pulse-chase experiments showed no change between transformed and normal cells growing in log phase.

Normal epithelial cells of rat kidney contain actin localized at the cell periphery. When tumors were induced by intraperitoneal injection of dimethylnitrosamine, the actin became diffused throughout the cytoplasm (33).

Likewise skin fibroblasts cultured from patients with autosomal dominant colon and rectal cancer had a diffuse matrix of actin localization in the cytoplasm. Cables as seen in normal cells were not apparent (34).

Actin Binding Protein

Kane in 1975 (35) demonstrated that extracts of sea urchin eggs formed a gel when allowed to warm to room temperature.

This observation was subsequently confirmed by Hartwig and Stossel (36) using macrophages and Weihing (37) with HeLa cell extracts. When gelled extracts were centrifuged, the pelleted material contained large amounts of actin and a 250,000 dalton protein named actin-binding-protein (ABP) by Hartwig and Stossel. These authors also partially characterized this protein. Their data has been listed along with that published by Wang (38) and Shizuta et al. (39) for filamin from gizzard tissue in Tables I and II. Both are

TABLE I

PROPERTIES OF ABP FROM HARTWIG AND STOSSEL*

1.	Co-precipitates with actin in high speed extracts of cells made 50-100 mM in KCl.
2.	Completely solubilizes in 0.6 M KCl.
3.	Soluble in both 2 mM EDTA at pH 9 and in 80% EtOH.
4.	Precipitates in 5 mM $CaCl_2$ and 50-100 mM KCl. Spectrin precipitates at 75 mM KCl.
5.	Contains no K^+-EDTA or Ca^{++}-Mg^{++} ATPase activity.
6.	Elutes in both excluded and included forms on 4% agarose columns.
7.	Insoluble between 50 and 100 mM KCl.
8.	Viscosity = 15.6 dl/gm in 100 mM KCl, pH 7.
9.	Negative staining shows beaded aggregates but no filaments.

*Data taken from reference 36.

TABLE II

PROPERTIES OF FILAMIN FROM WANG[1] AND SHIZUTA[2]

1.	Can be cross-linked with dimethyl adipimate at high ionic strength.
2.	Native protein contains no intra-molecular disulfides but oxidation reagents can cause inter-disulfide bonds.
3.	Soluble from 2 mM to 800 mM KCl, pH 6.2 to 10.0, 15 mM Ca^{++} or 15 mM Mg^{++}.
4.	Contains no detectable K^{+}-EDTA ATPase activity.
5.	Sedimentation coefficient = 9.4 ± 0.2 S in high salt.
6.	$\bar{v}$ = 0.734 ml/gm.
7.	f/fo = 2.2 to 2.3.
8.	Stokes radius = 120 ± 5 Å in 0.6 M KCl on 4% agarose columns.
9.	$E_{280}^{1\%}$ = 7.4 in 0.25 M K_2HPO_4, 280/260 = 1.95.
10.	Contains less than 2 µg hexose and pentose per 0.5 mg.
11.	Contains a blocked N-terminus.
12.	Dimensions in EM = 175 X 100 Å.
13.	Filamin is part of the intracellular filamentous cell structure by indirect immunofluorescence.
14.	No bipolar filaments are observed in aggregated solutions.
15.	The protein is soluble but aggregated if stored at physiological salt concentration for more than a few days at 4°C.
16.	Filamin is antigenically distinct from myosin and MAP proteins.

[1]Data taken from reference 38.

[2]Data taken from reference 39.

dimeric proteins and have the same native monomeric molecular weight. ABP does not appear to form filaments but rather undergoes aggregation. Filamin also aggregates easily.

The criteria normally used to show binding of ABP to actin is sedimentation with actin under conditions where actin is insoluble (i.e. 0.1 M KCl). It is likely that ABP itself is also at least partially insoluble under these conditions and therefore may not be truely "bound" to the F-actin filaments.

Kane (40), using sea urchin egg extracts reported that the protein when added to actin in 0.1 M KCl caused the lateral association of actin filaments into a gel comprised of ordered arrays if he used partially purified 43,000 and 250,000 MW components and allowed them to interact for several hours before observation. If observed immediately after mixing, an unordered interaction was seen suggesting a re-orientation of conformational preferences is required for the former.

Gelation and Cellular Motility

The cytoskeletal components just enumerated are believed to function in a manner similar to muscle contraction in eukaryotic cells to effect cell motility. This includes the events of cytokinesis, cleavage furrowing, phagocytosis, pseudopod elongation and membrane ruffling.

In lower organisms, such as various species of amoebae, a sol-gel transformation occurs in the cytoplasm (41). Micro-injections of Ca^{++} (7×10^{-7} M) cause contraction and a streaming of the cytoplasm (42). The extent of gel formation and contraction are dependent on

the pH of the extracts. Pollard and Ito (43) showed that cytoplasmic extracts from a species of amoeba increased in viscosity prior to movement and suggested this was due to filament formation.

Pollard has also shown that actin purified from the gelled extracts can undergo a temperature dependent polymerization (44) in the presence of KCl, ATP and $MgCl_2$. Addition of fragments of actin filaments derived from muscle accelerated the polymerization step.

Other major components of the gel include ABP and myosin. The concentration of myosin seems to be dependent on the length of time allowed before centrifugation of the gel; longer times cause larger amounts of myosin to be associated with the pelleted material. Additionally, at longer times, syneresis of the gel may occur. This step is dependent on the presence of ATP and Ca^{++} (approximately 10^{-6} M) and has been assumed to be the consequence of an actomyosin contractile event.

Conflicting reports concerning the nature of the interaction of actin-binding protein have been generated in several laboratories. While the original work suggested its requirement for gel formation, Pollard has shown actin isolated from Acanthamoeba will form a gel without addition of any other protein (44). Recent evidence from Maruta and Korn (45) also suggests actin binding protein may not be the "gelation factor". They have isolated four low molecular weight proteins from Acanthamoeba extracts which cause 97% of the gelation activity of the unfractionated extract. More recent evidence, however, suggests ABP is degraded to several low molecular weight polypeptides if EDTA is not added to the mixture (46). An interesting possibility in this regard concerns a Ca^{++} activated protease studied in platelets

which cleaves the high molecular weight proteins of greater than 200,000 daltson (47).

Effects of Perturbants on Cytoskeletal Proteins

Ca^{++} and diamide, a sulfhydryl oxidizing agent, inhibit tubulin polymerization. Addition of dithioerythritol can reverse the inhibition of diamide. The calcium effect has been postulated to occur via formation of disulfides (48).

The cytochalasins are fungal metabolites which alter a number of cellular functions dependent on cell motility. Cytochalasin B has been used more extensively than the others in this series. The perturbant inhibits gel formation in crude cell extracts and in purified actin-ABP solutions (49).

Lin and Spudich have shown that cytochalasin B binds to two sites in red cell membranes (50). Most of the high affinity site binding can be blocked by hexoses and therefore is probably involved in sugar transport. The low affinity sites are not affected by sugars and may involve cytoskeletal proteins.

Lens epithelial cells are prevented from differentiation in the presence of levels of cytochalasin B and D which disrupt microfilament bundles (51). These authors suggest the action is through stabilization of the non-fibrillar actin organization in rounded cells as seen via immunofluorescence.

Other evidence suggests the action of cytochalasin D is on actomyosin itself. The drug induces a shortening of muscle fibrils while preventing dissociation so that a sustained, extensive contraction results (52).

Scope of the Research

The current work was carried out as part of an extensive study of cell cytoskeletal proteins and their function and role in cell surface events at the biochemical level.

Major cytoskeletal proteins of the membranes of SA-180 cells were analyzed and identified. They were isolated in order to characterize them as contractile proteins. Concurrent with this, electron microscopy studies confirmed the postulated association with and extractability from the cell plasmalemma.

The roles of ABP in stabilization of the membrane and in gelation phenomena have been investigated. A proposed model for the association of the various cytoskeletal proteins with the plasma membrane is presented.

CHAPTER II

METHODS

Growth and Isolation of Ascites Cells

Sarcoma 180 ascites tumor cells were transferred by weekly injections into the peritoneal cavity of naive mice (strain COBS CD-1 ICR BR from Charles River Breeding Laboratories, Inc.) of approximately 4×10^5 cells per 0.1 ml of Hepes balanced salt solution (53). Cells were allowed to reach near plateau growth (54) before recovery by aspiration; approximately 3×10^8 cells were recovered per mouse. Recovered cells were washed with cold HBS by centrifugation at 750 rpm (210 g min) for three minutes in a Sorvall refrigerated centrifuge using an SS-34 rotor (53).

Mammary adenocarcinoma cells of the B1 and C1 types were grown identically in Fischer strain 344 rats by weekly intraperitoneal injection. These ascites cells were likewise recovered via aspiration and washed several times with HBS before use (56).

Plasma Membrane Isolation

Membranes were isolated from the ascites form of Sarcoma 180 cells. The washed cells were suspended in 40 mM Tris, pH 7.4 and allowed to stand at 4°C for a maximum of four minutes. The cells were then centrifuged at 2000 rpm for 3 minutes (1445 g min) and

resuspended a second time in the same buffer. Upon the second cell recovery, the Tris wash was repeated if the supernatant showed contaminating red blood cells or membranes and/or substantial quantities of hemoglobin. This procedure serves a dual function: 1) it lyses erythrocytes to allow removal by differential centrifugation and 2) it pre-swells the tumor cells before "fixing" by the subsequent procedure. It is necessary to swell the cells sufficiently so they are easily sheared.

Two volumes of 1 mM $ZnCl_2$ (pH 6.8) in water were added and the cells allowed to remain at ice temperature for twenty minutes with occasional swirling. Cells were then disrupted using the tight pestle of a Dounce homogenizer with the minimal number of gentle strokes as required, by phase contrast microscopy monitoring, to release the plasma membranes as large envelopes while retaining the nuclei intact. Generally 85-90% of the cells were disrupted in 8-11 strokes.

The homogenate was diluted with an equal volume of 40 mM Tris, pH 7.4, and centrifuged for 10 minutes at 2000 rpm. Resuspension in 40 mM Tris and centrifugation at 1000 rpm for 3 minutes sedimented whole cells and the majority of the nuclei while the supernatent was enriched in plasma membrane envelopes. This step was repeated until only a small percentage of the membranes were not recovered into the supernatent. The crude membranes were then recentrifuged at 2000 rpm for 12 minutes and the sedimented material layered onto discontinuous sucrose gradients in 40 mM Tris. The gradients were also made 40 mM in Tris and were composed of ten mls 55% sucrose, twelve mls 46%, twelve mls 35% and three mls of crude membrane. Centrifugation was

on a Model L5 65 Beckman ultracentrifuge in an SW-27 rotor at 16,000 rpm for 50 minutes at 4°C. Material at the 35-46% interface was essentially free of nuclei, whole cells, lipid vesicles, and other particulate matter. These purified plasma membranes were then washed with 40 mM Tris or isotonic HBS or PBS in a Sorvall centrifuge with an SS-34 rotor for 15 minutes at 2000 rpm to free them of sucrose.

A few initial experiments were done using the two-phase purification method of Brunette and Till (57). In this method, the homogenized cells were centrifuged at 2000 rpm for ten minutes and the pellets layered over a gradient composed of Dextran T500 and polyethylene glycol 6000 containing Tris and $ZnCl_2$. Membranes are trapped at the interface upon centrifugation at 10,000 rpm for 15 minutes. Frequent nuclear contamination occured and a sucrose gradient centrifugation was added as a second step to try to remove the contaminating nuclei. Variability in removal of contaminents together with electron microscopy data caused abandonment of this modification for membrane preparations.

Membrane Perturbations

Proteolysis was performed using Pronase, papain, trypsin or chymotrypsin. Membranes were suspended to a final concentration of one mg per ml in HBS, pH 7.4, and appropriate amounts of enzyme added to give a concentration of 5-20 µg enzyme per mg protein. Proteolysis of solubilized proteins was performed at a protease concentration of 5 µg per mg protein. The reaction was terminated by adding hot SDS to 0.4% and heating in a boiling water bath for five minutes. Samples were then dialyzed and lyophilized for subsequent PAGE.

Membranes at one mg protein per ml were extracted in appropriate buffers at 4 or 25°C, with gentle shaking on a rotating shaker bath for periods ranging from 15 minutes to 8 hours and centrifuged at 31,000 x g for 40 minutes. Supernatants were filtered through 0.45 μ Millipore filters and concentrated via lyophilization for SDS PAGE. The media tested for their ability to fragment envelopes and release cytoskeletal proteins are listed in Table III. These media contained a wide range of pH and salt concentrations.

Cross-linking of membrane proteins was performed at a final concentration of one mg protein per ml in PBS at pH 8.0. Several cross-linking reagents were used at concentrations ranging from 0.1 to 3.0 mg per mg protein as listed in Table IV. After 20 to 30 minutes at room temperature the reaction was terminated by the addition of appropriate blocking agents and SDS. Samples were dialyzed against a low ionic strength buffer at room temperature to decrease the salt concentration and remove unbound SDS prior to PAGE. Samples cross-linked by cleavable cross-linkers were solubilized in β-mercaptoethanol deficient sample buffer at a maximum of 50°C by use of a constant temperature dry-block.

Isolation of Smooth Muscle Contractile Proteins

The procedure reported by Bhan and Malhotra (58) for isolation of myosin from cardiac muscle was modified to incorporate the isolation of filamin (38) and α-actinin (59) from chicken gizzard tissue.

Isolation of Filamin

Approximately 100 gms (wet weight) of gizzard tissue was finely

TABLE III

EFFECTS OF VARIOUS EXTRACTING MEDIA ON SARCOMA ENVELOPE FRAGMENTATION AND EXTRACTION

Treatment	Fragmentation	Extraction
GEM, pH 9.5	+	+
GEM, pH 8.5	+	+
GM, pH 9.5	+	+
GM, pH 8.5	+	+
HBS, pH 7.4	-	-
HBS, pH 7.4, 1 mM EDTA	-	-
HBS, pH 7.4, Mg or Ca ATP	-	-
40 mM Tris, pH 7.4	-	-
PBS, pH 8.0	-	-
0.4-0.6 M KCl, pH 7.0	-	-
0.4-0.6 M KCl, pH 7.0, Mg or Ca ATP	-	-
Distilled HOH, pH 7.4	-	-
Distilled HOH, pH 7.4, followed by PBS, pH 9.5	+	+
PBS, pH 8.0, followed by PBS, pH 9.5	-	-
DMSO in HBS (1%)	-	-
Cytochalasin B in HBS	-	-
Actin Depolymerizing Buffer	-	-
Homogenization Buffer for Myosin from Cultured Cells	-	-
Lis in PBS, pH 8.0	-	-

TABLE IV

CROSS-LINKING REAGENTS FOR PRODUCING HIGH MOLECULAR WEIGHT COMPLEXES OF CYTOSKELETAL PROTEINS

Reagent	Cross-Linking Ability
Copper phenanthroline (3:1)	Most proteins
1,5-Difluoro-2,4-dinitrobenzene	Essentially all
Dimethyl malonimidate dihydrochloride	Relatively few
Dimethyl-3,3'-dithiobispropionimidate dihydrochloride	Most proteins
Dimethyl adipimate dihydrochloride	Mostly myosin
Dimethyl suberimidate dehydrochliride	Mostly myosin

minced in blending buffer (containing 50 mM KCl, 10 mM KH_2PO_4 and 1 mM EDTA, pH 6.8) and blended in a Waring blender for three intervals of 30 seconds each. Sixty second cooling periods were allowed between bursts. The residue was re-blended and supernatants pooled. Triton X-100 was added to the blending buffer to 0.25% and the residue re-extracted for one hour at 40°C. This supernatant was combined with those obtained previously and the total brought to 30% saturation with ammonium sulfate to precipitate filamin. The residue from this step was carefully resuspended in 0.6 M KCl buffer containing 20 mM sodium thiosulfate, 10 mM Tris-HCl, 5 mM β-EtSH, 2 mM $MgCl_2$ and 0.05% NaN_3, pH 7.2. A glass rod was used to break up the gummy precipitate. The solution was dialyzed vs. a low ionic strength buffer containing 50 mM KCl, 5 mM $MgCl_2$, 10 mM imidazole, 0.4 mM β-EtSH, 1 mM EDTA and 0.05% NaN_3, pH 6.8 at 4°C for 24 hours with two changes of this latter buffer. Any precipitate was removed by centrifugation at 100,000 x g for two hours. The clarified supernatant was then concentrated with XM-50 (50,000 m.w. cut-off) ultrafiltration filter in an Amicon pressure flow apparatus and passed over a 4% agarose column in the 0.6 M KCl buffer to yield purified filamin.

Isolation of α-Actinin

The residue from the initial extractions above was brought to pH 8.5 in 1 mM $NaHCO_3$ and extracted at 4°C for several hours. Re-extraction was repeated for an additional hour. The supernatants were brought to 30% saturation with ammonium sulfate and the precipitated material recovered by centrifugation. This material was resuspended in the above buffer and dialyzed against several changes

of the same buffer. Centrifugation at 100,000 x g for two hours gave presumptive α-actinin. The concentrated solution was then applied to a DEAE column in 20 mM Tris buffer and eluted with a linear KCl gradient. Fractions showing A_{280} in the correct salt concentration range were pooled and concentrated. The material by SDS PAGE did contain polypeptides other than α-actinin but further purification was not attempted. Material for antibody induction was isolated via gel electrophoresis.

Isolation of Myosin

The residue from the α-actinin step was re-adjusted to pH 7.0 and the salt concentration returned to that of the high salt buffer given above. Extraction proceeded for 4-6 hours at 4°C. The supernatant was collected and diluted 10:1 with cold distilled water and stirred slowly overnight at 4°C. Precipitated material was collected and resuspended in the 0.6 M KCl buffer and centrifuged at 100,000 x g for 2 hours. The clarified solution was again precipitated by dialysis against the 50 mM KCl buffer, collected, resolubilized and recentrifuged to clarify it. The material was then applied to a 4% agarose column in this same buffer after concentration using an Amicon pressure-flow apparatus with an XM-50 membrane. Fractions were pooled, concentrated and subjected to SDS PAGE to determine protein purity.

Isolation of ABP

The method of Hartwig and Stossel (36) for isolation of ABP from macrophages was followed with only slight modification. The enriched

gelation pellet (see below) was resuspended in the high salt buffer cited above and dialyzed against the 50 mM KCl solution over 24 hours and two changes of buffer. Precipitated material was removed and the clarified supernatant concentrated and applied to a 4% agarose column in the 0.6 M KCl buffer. Material eluting after the void volume was pooled and concentrated.

Gelation

The methods of Stossel and Hartwig (60) and Weihing (61) were used for obtaining high speed supernatants of the sarcoma cells. The cells were recovered as previously stated and washed with hypotonic buffer to remove contaminating erythrocytes. In some experiments the cells were washed with isotonic ammonium chloride since it seemed to produce less swelling of the cells. After washing, the cells were resuspended with one volume of 40 mM Tris, pH 7.4 containing EDTA and β-mercaptoethanol to final concentrations of 1 mM each. One to five mM ATP was added prior to or after homogenization. Fifty to sixty strokes were usually needed to completely break all cells. The homogenate was poured into ultracentrifuge tubes taking care not to dilute it and was spun for one hour at 100,000 x g in an SW-27 rotor on a Beckman Model L5 65 ultracentrifuge. In some experiments, the supernatant from this step was respun an additional three to six hours at the same speed. Floating material was removed using a 45° bent needle with syringe aspirator device. The remaining supernatant was decanted and used in the various gelation experiments.

Electron Microscopy

Sarcoma 180 Ascites Cells

Samples were prepared for electron microscopy by fixing in two percent glutaraldehyde in 0.1 M cacodylate buffer at a pH of 7.4 on ice. After washing in the same buffer containing sucrose, the cells were fixed in one percent osmium tetroxide in the cacodylate buffer, dehydrated in a graded series of ethanol and embedded in Epon-Araldite. Silver sections were stained with uranyl acetate and lead citrate and observed with a Phillips EM200 electron microscope.

Samples for scanning electron microscopy were fixed in two percent glutaraldehyde in 0.1 M cacodylate-0.1 M sucrose, placed on either serum-coated glass coverslips or Nucleopore filters, dried on a Polaron critical point dryer from liquid CO_2, coated with gold/paladium (100 Å) and observed in a JEOL JSM-35 scanning electron microscope at 15-20 KV at 0° tilt.

Platelets

Platelet preparations were fixed by the method of Mattson et al. (62) by suspension into a ten-fold excess of freshly prepared 0.1% glutaraldehyde in 0.1 M cacodylate buffer. Fixation was for 30 minutes at room temperature. An equal volume of 6% glutaraldehyde in 0.1 M cacodylate buffer was added and fixation continued for at least 24 hours at room temperature. The platelets were washed free of fixative by repeated centrifugation from distilled water and dehydrated in a graded series of alcohol and placed on polylysine coated coverslips and prepared as above.

Platelets

Collection of 500 ml of bovine blood was performed with the aid of a veterinary student at the University dairy facilities. Blood was drawn by free-flow from a jugular puncture into 13 ml acid citrate dextrose solution per 100 ml blood. The blood was centrifuged in a Sorvall at 20°C using an HB-4 rotor at 3500 rpm for 90 seconds. The straw colored supernatant was drawn off using a rubber tubing fitted with a straight-cut needle and connected to a 50 ml syringe. Barber-Jamieson (63) buffer was added to approximately the same volume of drawn fluid, mixed, and centrifuged. The supernatants were pooled and re-centrifuged at 1350 rpm for 20 minutes to pellet erythrocytes. Generally, one spin cleared 90% or better of the red blood cells. The platelets were then collected by centrifugation at 1750 rpm for 25 minutes. The pellet was washed by swirling gently in Barber-Jamieson buffer and centrifuged again. Resuspension in the same buffer gave the working platelet preparation. All manipulations were done in plastic containers and with plastic instruments.

FITC-Con A Labelling of Cells and Membranes

Sarcoma 180 ascites cells were labelled with fluoroscein-conjugated Concanavalin A (FITC-Con A) at various stages during the preparation utilized for membrane isolation. The cells were incubated at 37°C with 50 μg FITC-Con A per five mls of media containing approximately 2×10^7 cells. At the end of the incubation period of one hour the cells were collected by centrifugation and washed several times with fresh buffer. They were then fixed by adding five times

a minimal volume (one ml) of a freshly prepared solution of formaldehyde in phosphate buffer for one hour, centrifuged, washed and observed using UV optics with appropriate barrier filters.

Membranes prepared from sarcoma cells were suspended in buffer at approximately five mg protein per ml and labelled under the identical protocol as above.

Vesicles prepared by EDTA extraction of membrane envelopes were treated in a similar manner.

Control samples of each of the above were prepared by pre-fixing in formaldehyde solution for one hour, washing, and post labelling with FITC-Con A solution.

FITC-IgG Labelling of Membranes

Plasma membranes of the sarcoma cells were labelled by incubating a membrane solution (two mg protein per ml in PBS) with antibodies produced in albino New Zealand rabbits. A 0.1 volume of antibody was added to the membrane suspension and incubated at room temperature for 30 minutes. The membranes were collected by centrifugation and washed with fresh buffer several times. They were then resuspended to the original volume used and 0.01 volumes of FITC conjugated anti-rabbit antibodies was added. Incubation was at room temperature for 20 minutes. Several washes with fresh buffer were done to remove unbound reagent before suspension in minimal volume for observation using UV optics in a light microscope.

Antibody Preparation

Purified contractile proteins (myosin, α-actinin, filamin, and ABP) were separated by SDS PAGE on 5% gels. After staining with CBB to visualize, the appropriate bands were sliced from the gels and soaked at least 3 hours in distilled water to remove acetic acid. The bands were re-gelled into short glass tubes of increased diameter. These were fitted closely at the bottom end with visking tubing and placed in an electrophoresis chamber and electrophoresed for 36 hours. The dialysis sacs were removed and dialyzed vs. 5 mM KH_2PO_4 buffer pH 7.0 overnight and then lyophilized. The material thus obtained was resuspended in distilled water (one ml) and an equal volume of Freund's adjuvant added and the solution emulsified by repeated passage through a small bore needle. The first two injections were subcutaneous above the shoulder blades on the rabbit's back. Subsequent injections were done either via this same route or intramuscularly into the thigh tissue of the hind legs. Generally, two to five bleedings from the marginal ear vein were done prior to beginning the injection program to obtain pre-immune serum. Approximately five injections at weekly intervals were required before observance of good titres were obtained.

Amino Acid Analysis

Partially purified proteins were further separated on SDS PAGE after column chromatography. The protein bands were stained with Coomassie brilliant blue for localization and identification and then excised for hydrolysis. The gel slices were soaked in distilled

water and hydrolyzed in a final concentration of 6 N HCl containing 0.2% phenol and 0.1% thioglycolic acid for 22 hours at 110°C. A sample of acrylamide gel containing no protein was hydrolyzed as a blank. The amino acid composition was obtained on a microanalyzer designed and built by Dr. T.-H. Liao (64).

Analytical Procedures

SDS PAGE

Electrophoresis in the presence of the detergent, sodium lauryl sulfate, followed that described by Weber and Osborn (65). Membranes were solubilized by boiling for five minutes in buffer containing 0.2% SDS, 4% β-mercaptoethanol, 20% glycerin and 0.19 M phosphate final concentrations. Cells were solubilized by SDS treatment and nucleic acid sheared by passing the solution quickly several times through a small bore needle or by sonication. Aliquots were then treated with sample buffer as above. Electrophoresis was carried out in a Bio Rad unit at 6 mA per gel for tube gels and 50 mA total amperage for slab gels.

Non-SDS PAGE

In several experiments it was desirable to determine migration without the presence of detergent to allow for charge effects and to prevent denaturation of the proteins. Some of these experiments utilized the phosphate system while others were done using the system of Laemmli (66). The membranes tended to be difficultly soluble at pH 6.8 so that a stacker gel was used at the same pH as the running

gel. This procedure was sometimes coupled to SDS PAGE as a second dimension for identification of the protein bands.

Protein Determination

The method of Lowry et al. (67) was routinely used for determination of protein concentration. Standard curves were based on color development for bovine serum albumin. Membranes were solubilized effectively by treatment with the Lowry reagents themselves and appeared to require no special procedures to obtain accurate and consistent results.

$Na^{125}I$ Labelling of Sarcoma Cells and Membranes

Incorporation of radioactive iodide into membranes and cells was accomplished via the enzyme lactoperoxidase (55). 10 µM cold KI, 100 µCi $Na^{125}I$, and 0.5 µM enzyme were used per five ml volumes of approximately 10^8 cells. H_2O_2 was added as a dilute solution in four aliquots during the reaction time of ten minutes. Membranes (3-5 mg protein) were suspended in two mls of buffer and 2/5 the amounts of the reagents listed above were added. The reaction was terminated by addition of a large excess of cold KI and centrifuging. Cells and membranes were washed several times before solubilization and counting. Counting was done on a gamma counter with window settings appropriate for ^{125}I counting.

$^{65}ZnCl_2$ Labelling of Membranes

Sarcoma cells were treated with 1 X 10^7 dpm $^{65}ZnCl_2$ at a final concentration of 1 mM $ZnCl_2$ in the stabilization step. Membranes were

isolated as usual and radioactivity determined on a gamma counter.

Cholesterol Assay

Analysis was according to the procedure of Glick et al. (68). Membranes were extracted with ethanol for ten minutes and the soluble supernatant collected by centrifugation. Aliquots of the supernatant were assayed for cholesterol content at A_{558} and quantitated by correlation with a standard curve determined using pure cholesterol.

Lipid Phosphate Assay

The method of Folch et al. (69) was used for the isolation of lipids from sarcoma membranes and release of phosphate by acid hydrolysis. The hydrolyzates were treated with oxidant (H_2O_2) to ensure complete oxidation of products. Excess oxidant was removed by addition of water to the samples while at 180°C and boiling until the water had been dissipated. Released phosphate was determined by the micro-method of Taussky and Shorr (70). Membrane samples of one mg or greater were used for formation of sufficient phosphate.

DNase I Inhibition Assay

This assay is based on the hyperchromicity at 260 nm when DNA is depolymerized. Buffer containing appropriate ions and DNA was dispensed and warmed to room temperature. A separate solution of enzyme or enzyme-protein mixture was added quickly to the DNA solution and the change in A_{260} was followed on a recording chart connected to a Gilford Model 240 spectrophotometer (71).

Succinate Dehydrogenase Assay

The procedure as described in Methods of Enzymology vol. 22 was followed using 1% INT to follow the reduction-oxidation catalyzed by the enzyme. The substrate was sodium succinate. The dye was extracted with ethyl acetate and absorbance determined at A_{490} (72).

Lactate Dehydrogenase Assay

The method used was that outlined in the Worthington Enzyme Manual, 1972 (73). Substrate was pyruvate and coenzyme was NADH. The conversion to NAD^+ was followed by A_{340} decrease.

RNA Assay

RNA was determined by the orcinol method (74). Color development was determined by absorbance at 660 nm.

Immunodiffusion

The method used most generally for ascertaining if antibodies were being produced was immunodiffusion. The center well was used for holding antigen while the outer wells contained decreasing concentrations of antibody preparation which had been heat inactivated for 30 minutes at 56°C. This latter procedure is probably not necessary for diffusion of purified proteins but was routinely done so that antiserum could be used against cells at some future time. Diffusion was allowed to proceed at room temperature for 24 hours. The plate was rinsed over several hours with several changes (or overnight) with buffer to remove all unreacted protein. The plate

was then stained with Coomassie brilliant blue for two to three hours and destained in 7% acetic acid to visualize the precipitation bands.

^{3}H-Serotonin Release

An alternate method (75) for determining titres of the antibody preparations was via platelet release of serotonin upon stimulation by antibody-antigen reaction. The platelets were incubated with ^{3}H-serotonin for 30 minutes at room temperature to allow uptake. They were then washed several times to remove excess radioactive material. Addition of various concentrations of antibody and antigen to the loaded platelets and centrifugation allowed determination of the minimal concentration necessary for release of the serotonin and the titre of the preparation.

Determination of Label and Protein in PAGE Gels

PAGE gels were routinely stained with Coomassie brilliant blue for localization of protein and periodic-acid Shiff reagent for carbohydrate. Scanning records were obtained by scanning the gels in a linear transport fitted to a Model 240 Gilford spectrophotometer at 565 nm for the former and 490 nm for the latter. Radioactivity associated with these bands was determined by slicing the scanned gels into one mm slices and counting in a liquid scintillation or gamma counter. Estimation of protein was by weighing peak areas from the scans and expressing as a percentage of the total gel.

Myosin and Actomyosin ATPase Activity

ATPase activity associated with myosin was assayed under conditions appropriate for the protein itself and when complexed to actin according to the procedures of Bárány et al. (76). Enzyme activities were expressed as μg of phosphate released per mg of protein per minute of hydrolysis. Since trichloroacetic acid was used to terminate the reaction, controls were necessary to determine the amount of hydrolysis due to the organic acid itself. The micro phosphate assay of Taussky and Shorr was adopted since color development required no more than two minutes time and readings could be initiated more quickly to minimize non-myosin hydrolysis of ATP.

CHAPTER III

CYTOSKELETAL-MEMBRANE INTERACTIONS

Results

Protein Identification

Sarcoma 180 ascites cells were grown and recovered from the peritoneal cavity by procedures outlined in Chapter II. Membranes were routinely prepared by one of the alternate methods also described.

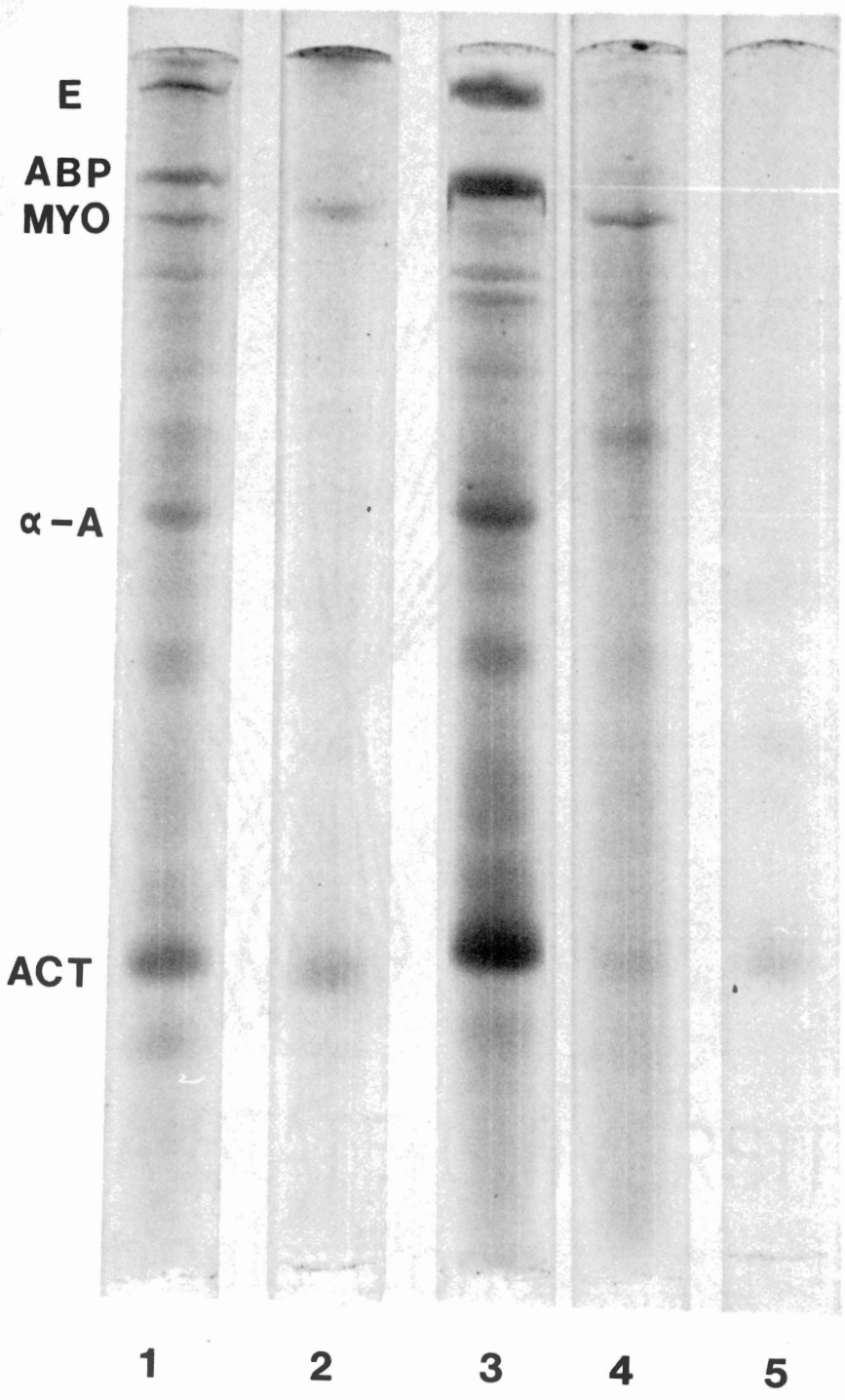
SDS PAGE gels of isolated membranes showed them to be enriched in five major protein bands designated E through A in order of decreasing mobility. These have since been designated E, ABP, myosin, α-actinin and actin respectively (see Figure 1).

Extraction of these membranes with media composed of 5 mM glycine-1 mM EDTA-5 mM β-mercaptoethanol at pH 9.5 (GEM, 9.5) released four of these proteins. The fifth protein remained with the sedimentable residue fraction. Proteins extracted under these conditions are most likely to be peripherally associated with the membranes. PAS staining of SDS gels of whole membranes also showed no substantial quantities of carbohydrate apparently associated with any of these proteins. The inability of the protein designated C and migrating at approximately 210,000 to be solubilized under these conditions is consistent with its later assignment as myosin.

Separation of the solubilized proteins was achieved on a Sepharose

Figure 1. SDS PAGE Profiles of Sarcoma 180 Proteins.

1) Whole membranes, 2) Chicken gizzard actomyosin, 3) GEM pH 9.5 extract, 4) Membrane residue from extraction, 5) Rabbit muscle actin.


E
ABP
MYO
α-A
ACT
1
2
3
4
5

4B column, 1 X 90 cm in the buffer used for extraction. The pH of the column buffer was lowered to pH 8.5 to maintain stability of the agarose matrix. Retention on the column seemed to be improved by the addition of 50 mM Tris-HCl to the GEM media and was routinely incorporated into the elution buffer. Separation of polypeptides E and D, polypeptide B and polypeptide A were achieved (Figures 2 and 3) as three separate fractions.

Recent studies have identified a 43,000 dalton protein as actin in a variety of plant and animal cells (77-79). It has been suggested to be a major protein in bacterial cells (80). Mobility of the protein designated as A in Figure 1 suggested it could be non-muscle actin. Therefore, actin was prepared from rabbit back muscle and chicken gizzard smooth muscle to serve as standards for comparative analyses. These were found to have the same electrophoretic mobility as the membrane protein under both denaturing and non-denaturing conditions.

Amino acid analyses of both the sarcoma and rabbit muscle proteins were performed. The stained gel slices were excised and hydrolyzed. The rabbit actin compared favorably with other rabbit actin values and the sarcoma actin had a mole percent composition analogous to platelet actin (Table V).

As further corroboration of the identity of the protein, isolated membranes were extracted with glycine-EDTA-β-mercaptoethanol and the extract passed over a Sepharose 4B column. The peak area containing the actin protein was concentrated and tested for DNase I inhibiting ability. Figure 4 shows the inhibition curve for actin on DNase I. The maximum inhibition achieved was 75 percent for any actin preparation. The failure to achieve more substantial inhibition is most

Figure 2. Sepharose 4B Profile of GEM, 9.5 Extract from SA 180 Membranes.

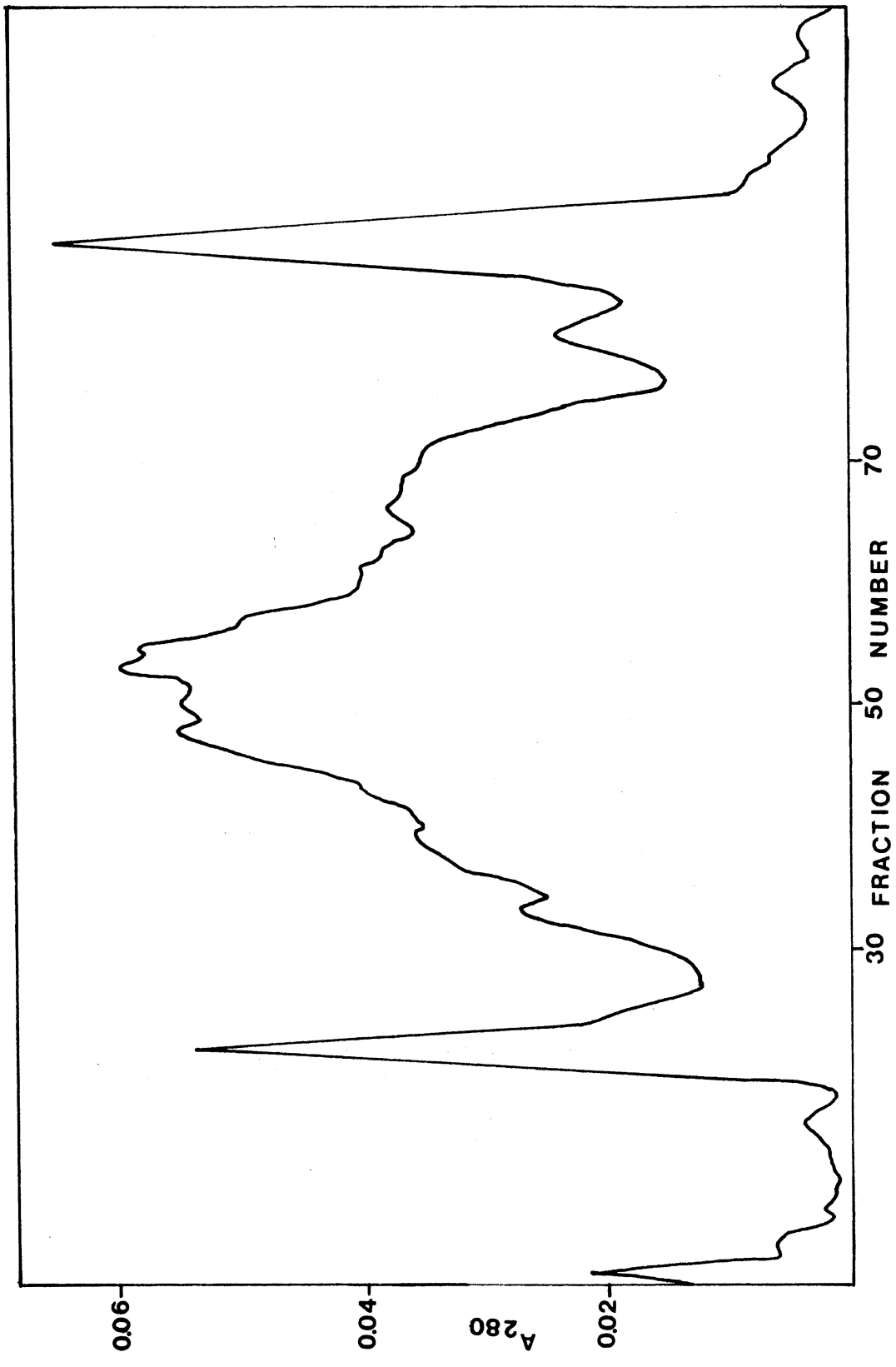

0.06
0.04
0.02
A_{280}
30
50
70
FRACTION NUMBER

Figure 3. SDS PAGE Profiles of Sarcoma 180 Proteins Extractable from Isolated Membranes and Chromatographed on Sepharose 4B (Figure 2).

1) Whole membranes, 2) Chicken gizzard actomyosin, 2) Fraction 39, 4) Fraction 47, 5) Fraction 53.

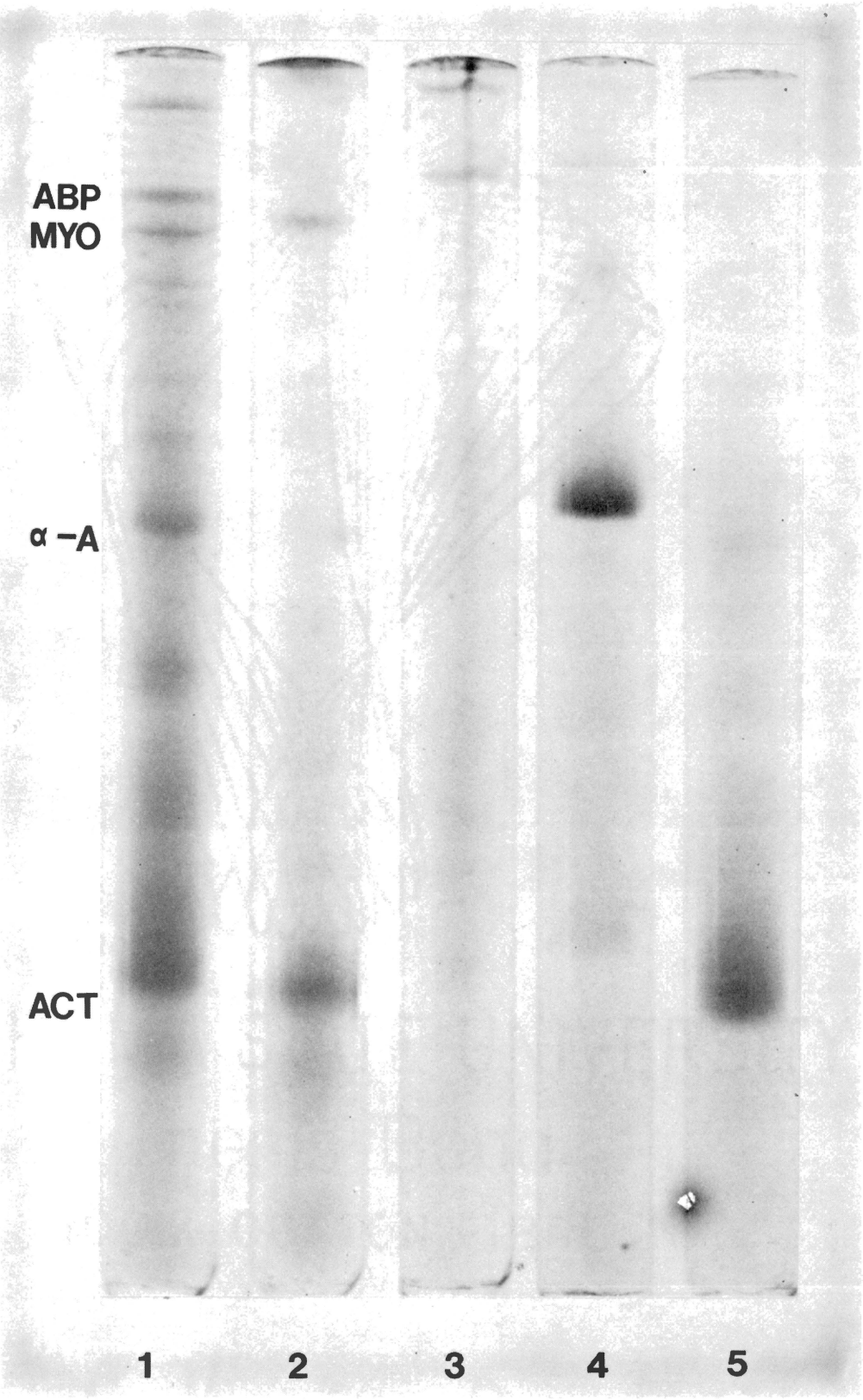

ABP
MYO
α -A
ACT
1
2
3
4
5

TABLE V

COMPARISON OF AMINO ACID COMPOSITIONS

Amino Acid	Band A	Platelet[a] Actin	Band B	Porcine[b] α-Actinin	Band C	Chicken[d] Gizzard Myosin	Band D	ABP[e]
Asp	10.8	9.6	12.3	12.3	12.2	10.2	11.1	8.7
Thr	6.5	7.4	5.5	6.0	5.2	5.8	6.2	6.2
Ser	7.0	7.2	5.9	5.6	6.2	4.8	7.3	6.8
Glu	13.9	13.3	15.6	13.9	18.0	23.1	12.1	11.4
Pro	3.4	nd[c]	3.1	5.3	0.1	0.2	5.4	7.1
Gly	9.7	9.3	7.4	7.0	5.3	2.0	9.6	11.8
Ala	8.8	9.4	8.6	8.8	7.0	6.9	8.1	7.4
Val	4.0	4.7	2.7	6.0	6.6	5.5	8.0	8.5
Met	2.6	2.8	2.3	nd	2.1	2.7	2.8	1.3
Ile	4.6	6.6	5.4	6.5	3.9	4.1	4.5	4.4
Leu	9.2	8.7	10.4	9.6	13.6	12.3	7.9	6.2
Tyr	2.3	3.4	2.1	3.4	3.0	1.7	2.3	3.1
Phe	3.7	3.9	3.8	3.7	3.0	2.5	2.9	3.2
His	2.4	2.6	2.7	2.5	1.4	1.2	2.2	2.2
Lys	7.1	5.4	7.3	4.8	7.5	11.4	5.6	6.0
Arg	5.2	5.1	6.1	5.7	4.0	6.1	4.2	4.1

TABLE V (Continued)

[a]Data of Booyse, Hoveke, and Rafelson

[b]Data of Goll, Mommaerts, Reedy and Serayarian

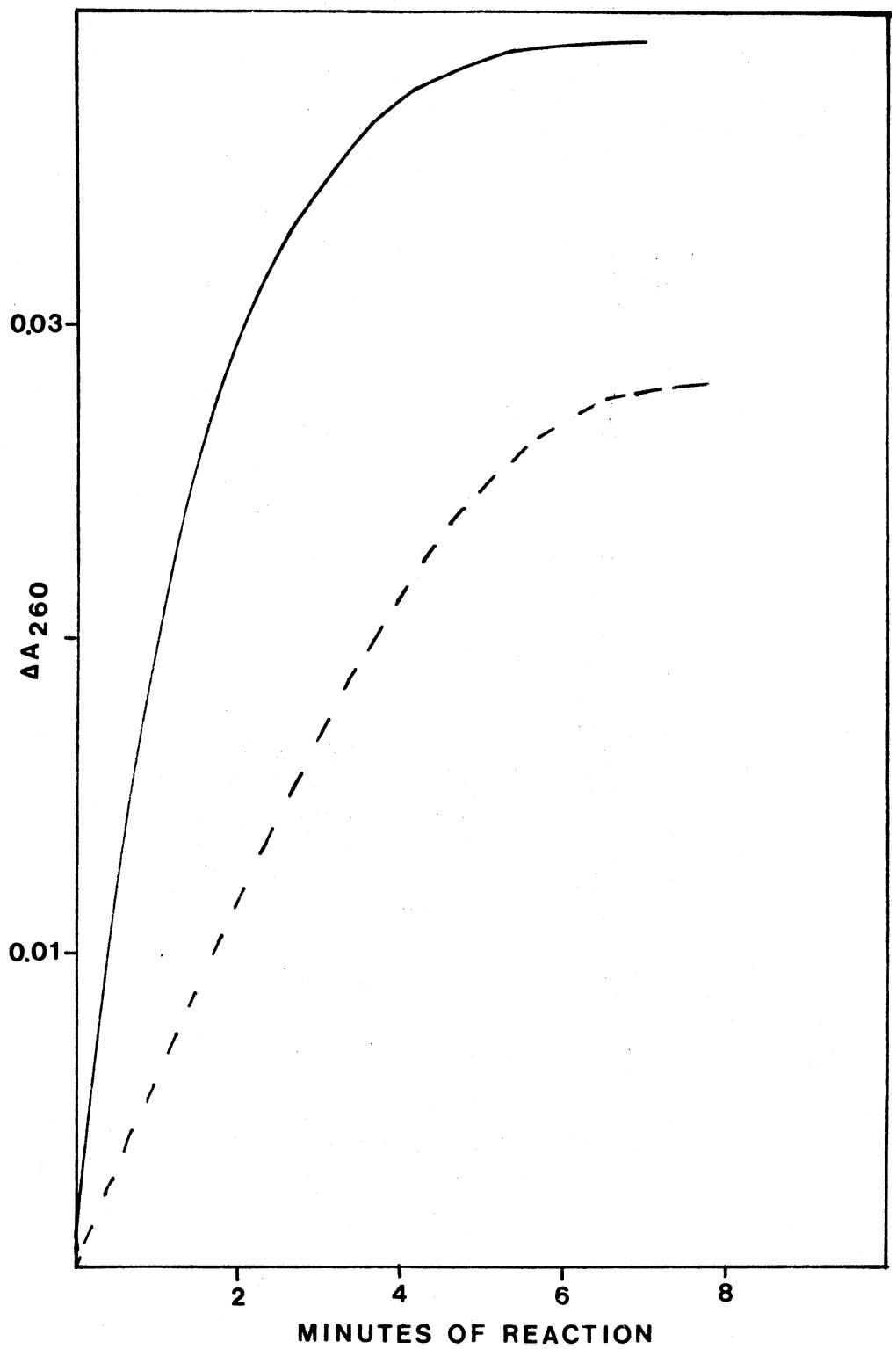
[c]Not determined

[d]Heavy chains only

[e]Data of Stossel and Hartwig

Figure 4. Changes in Hyperchromicity of DNA by DNase I with Time Before and After Treatment of DNase I with Actin Derived from Sarcoma Membranes.

Solid line is no actin treatment. Dotted line is with pre-treatment with actin.


0.03
0.01
ΔA 260
2
4
6
8
MINUTES OF REACTION

likely denaturation of the actin by EDTA in the extraction media. SDS PAGE gels showed no large amounts of contaminants although several minor bands were occasionally present in these column fractions.

The large polypeptide with apparent molecular weight of 210,000 daltons was postulated to be non-muscle myosin. Rabbit muscle actomyosin was prepared and used as standard for conventional ATPase assays for myosin and actomyosin. Intact sarcoma membranes were tested on both assays for activity. Glucose-6-phosphate and β-glycerol phosphate were tested in the system since myosin should be ATP specific. Typical results are shown for the myosin-like activity in Table VI. It can be seen in the table that ATP was the preferred substrate.

Densitometry of SDS PAGE gels (Figure 5) showed that the myosin-like protein also had a mobility identical to purified chicken gizzard myosin but not to the spectrin doublet of erythrocytes.

Direct extraction of envelopes with high salt does not elute myosin. However, if membranes were extracted with GEM, pH 9.5, recovered and re-extracted with 0.6 M KCl for myosin (see below), the polypeptide of 210,000 molecular weight extracts along with several other proteins. Dilution with ten volumes distilled water, collection of the precipitate, and resolubilization in 0.6 M KCl gave a preparation containing predominately the 210,000 dalton protein along with some minor proteins of lower molecular weight (Figure 6). When tested as above, this material had ATPase activity.

An alternate procedure was to pass the concentrated extract through a Bio Gel A15m column in 0.6 M KCl buffer (Figure 7). That

TABLE VI

MYOSIN ATPase ASSOCIATED WITH SA 180 PLASMA MEMBRANES AND SOLUBILIZED PROTEINS

Sample	Substrate	µg/mg/min
Membranes	Glucose-6-phosphate	0.70
	β-Glycerolphosphate	0.70
	ATP Mg^{++}	4.21
	ATP Ca^{++}	5.71
0.6 M KCl Column		
Fraction I	Glucose-6-phosphate	8.82
	β-Glycerolphosphate	3.92
	ATP Mg^{++}	26.40
	ATP Ca^{++}	29.40
Fraction II	Glucose-6-phosphate	4.04
	β-Glycerolphosphate	2.02
	ATP Mg^{++}	23.20
	ATP Ca^{++}	26.20

Figure 5. Correlation of SA 180 Gel CBB Stainable Proteins with Standard Gels of Actomyosin and RBC Ghost Membranes.

Panel A. SA 180 membranes.

Panel B. Chicken gizzard actomyosin.

Panel C. RBC ghost membranes.

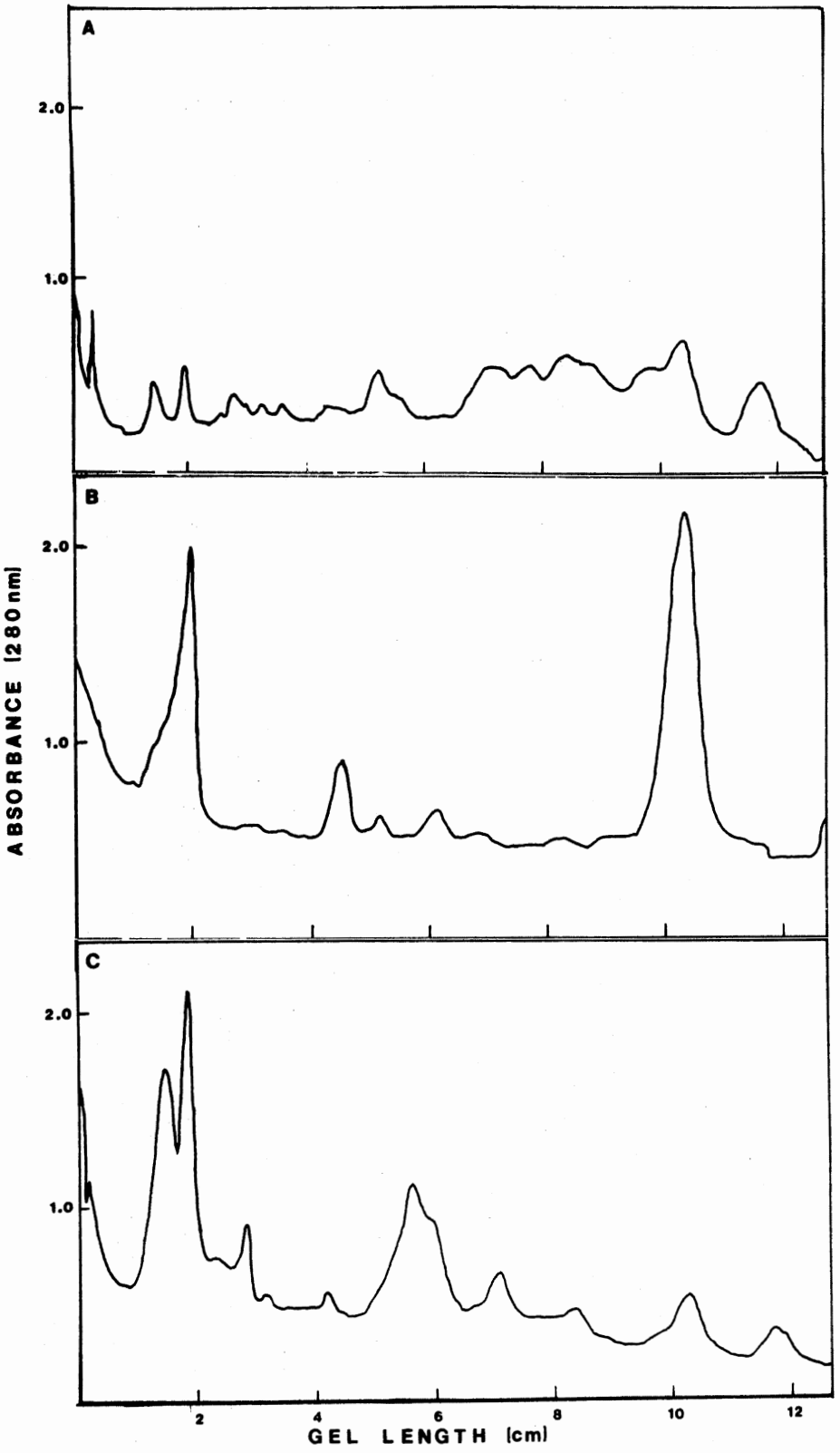

A
B
C
2.0
1.0
2.0
1.0
2.0
1.0
ABSORBANCE (280 nm)
2
4
6
8
10
12
GEL LENGTH (cm)

Figure 6. SDS PAGE of 0.6 M KCl Extractable Material from SA 180 GEM Extracted Membranes.

1) Chicken gizzard actomyosin.

2) 0.6 M KCl soluble, 0.05 M KCl insoluble material.

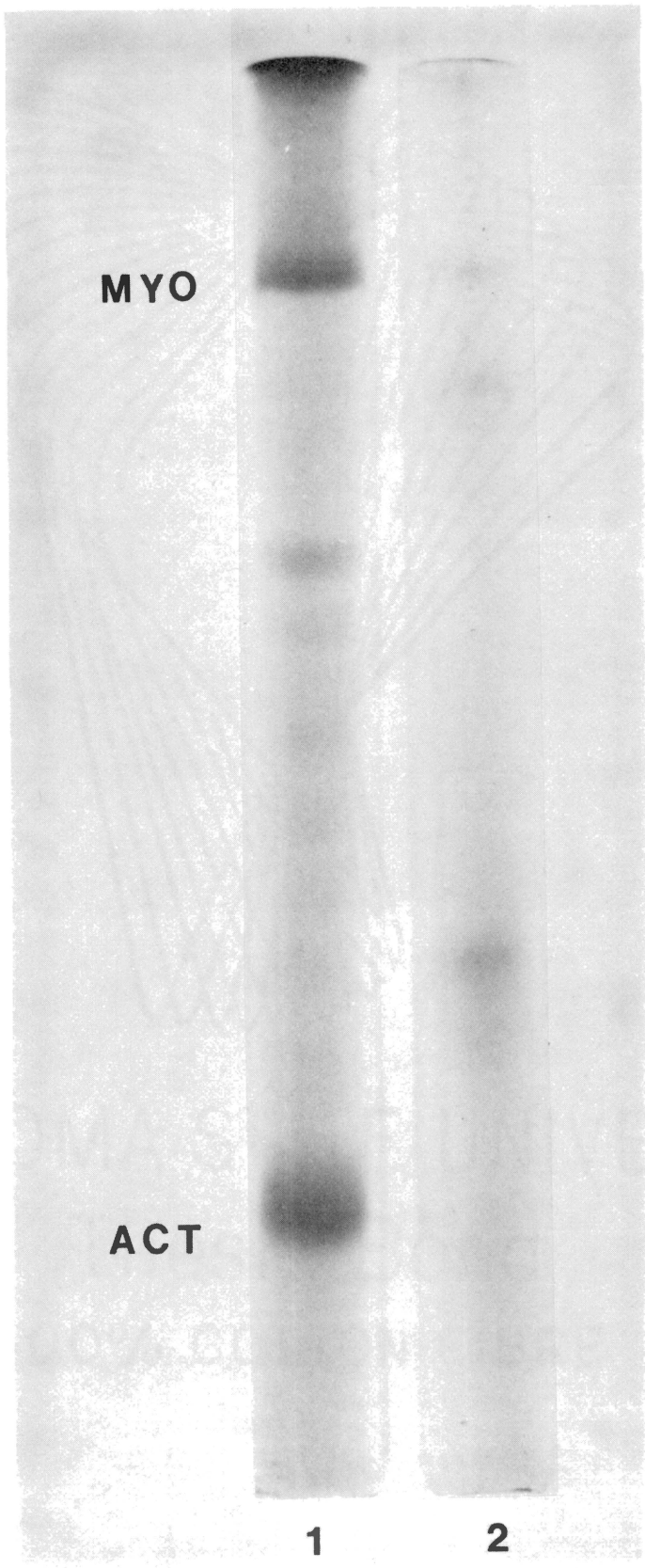
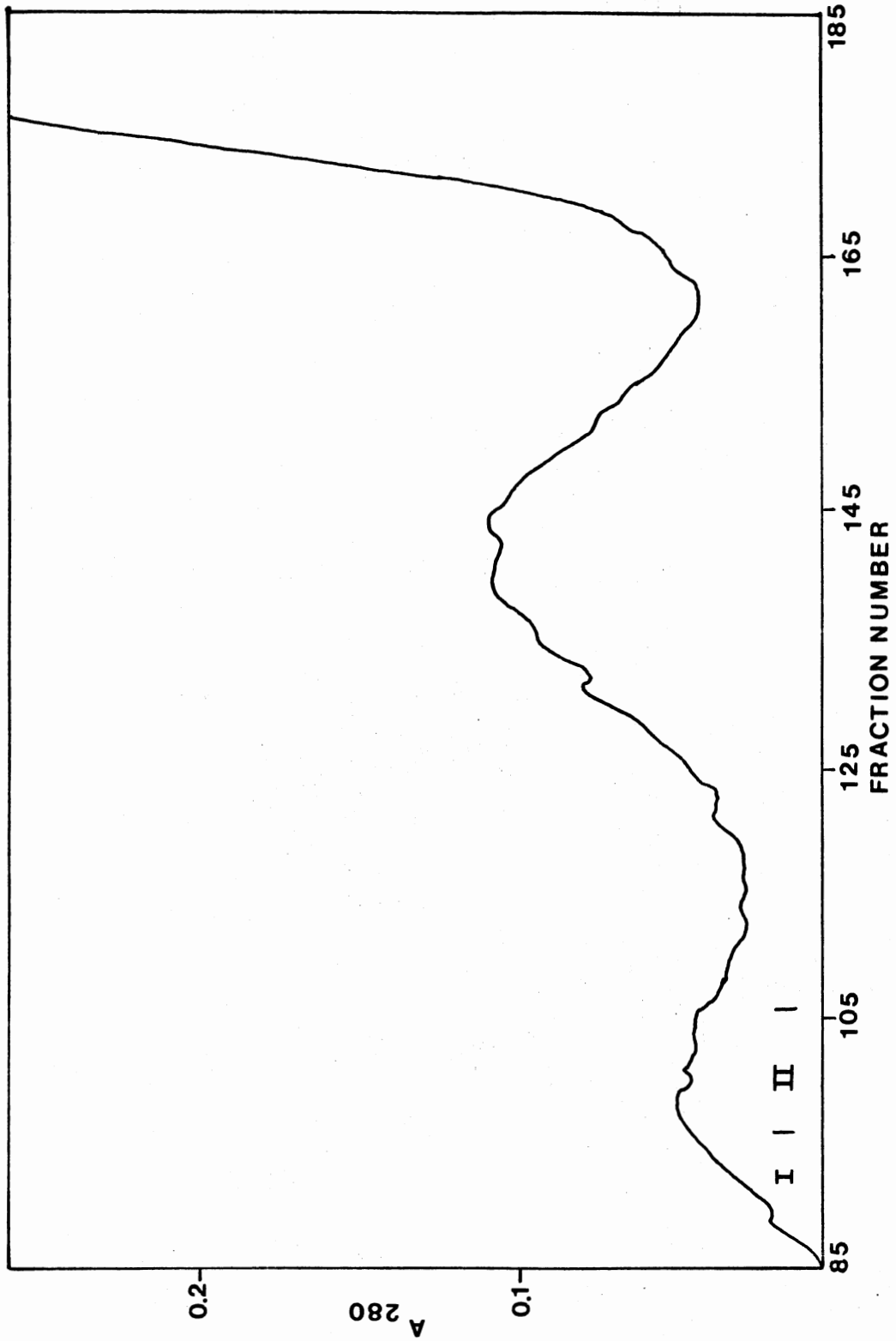

MYO
ACT
1
2

Figure 7. Profile of Absorbance at 280 nm of Fractions from Bio-Gel A15M Chromatography of Material Extracted in 0.6 M KCl from Membranes Previously Extracted with GEM, pH 9.5.

The myosin containing fractions were pooled and divided into two fractions (I and II) for ATPase determination (Table VI).


0.2
0.1
A 280
85
105
125
145
165
185
FRACTION NUMBER
I
II

peak (labelled I and II) showing a polypeptide of 210,000 also contained ATPase activity. One preparation assayed under conditions for myosin activity is also shown in Table VI. Again two alternate substrates were tested. ATP was the preferred substrate, however. Since the peak tested was again not highly purified, it is not surprising that some residual activity due to an apparent co-purifying non-specific phosphatase was present. Many myosin preparations from skeletal sources apparently co-purify with both a phosphorylase and a phosphatase. These enzymes apparently add and remove phosphate from one of the light chains of the myosin molecule which seems to be a regulatory mechanism governing the cation sensitivity of myosin-actin binding (81). While this has not been established to be true in non-muscle systems generally, phosphorylation by a kinase has been shown to be required for actin activation of platelet derived myosin (82).

Also as can be seen in Table VI, the expected increase in Ca^{++} activation over that observed with Mg^{++} did not occur. Dr. A. Martonosi suggested the most likely reason for this result was oxidation of myosin sulfhydryl groups during the isolation procedure. When this occurs an increase in the Mg^{++} and Ca^{++} ATPase levels both occur but to a greater extent with Mg^{++}. Hence, the ATPase values obtained were both higher than expected and loss of the Mg^{++}-Ca^{++} activation difference was observed.

Published work from Kane (35) in sea urchin eggs and Hartwig and Stossel (36) in macrophages, suggested the protein of approximately 250,000 daltons could be related to the actin-binding-protein (ABP) described by these workers. Mobility on SDS PAGE and amino acid

analysis data (Table V) were consistent with this hypothesis.

Isolation of this protein from ascites cells was achieved using the methodology of Hartwig and Stossel. Whole cell homogenates were sedimented at 100,000 x g for one hour and the supernatant allowed to warm to room temperature. The extract became "stiff" or of a Jello consistency. Centrifugation sedimented material rich in ABP and actin. When this material was treated with 0.6 M KCl buffer the ABP was resolubilized whereas the actin remained polymerized and could be removed by centrifugation. Further purification of the ABP was accomplished by dialysis against the 50 mM KCl buffer. This step precipitated contaminating myosin present in the gel. Centrifugation and filtration through a 0.45 μ Millipore filter removed this precipitate. The supernatant was then concentrated and passed over a Bio Gel A15m column in the 0.6 M KCl buffer. SDS PAGE analysis was used for deciding which fraction to pool and concentrate for further studies. Figure 8 shows SDS PAGE gels of purification of ABP during different steps of the isolation.

A sedimentation coefficient was determined for the purified ABP in 0.6 M KCl buffer. Values obtained in several trials are listed in Table VII. Trial 2 was a re-sedimentation of ABP from Trial 1 in which the ABP had been dialyzed against 50 mM KCl buffer. The protein was apparently aggregated by this procedure. KCl was added to bring the concentration back to approximately 0.5 M and the protein re-sedimented in Trial 3. However, the ABP most likely had denatured since it did not disaggregate with the salt increase. Therefore, these values were disregarded for S_{20} estimates.

The fourth protein was postulated to possibly be α-actinin,

Figure 8. Purification of ABP.

1) High speed supernatant.

2) ABP before 4% agarose chromatography.

3) ABP peak eluted from 4% agarose column.

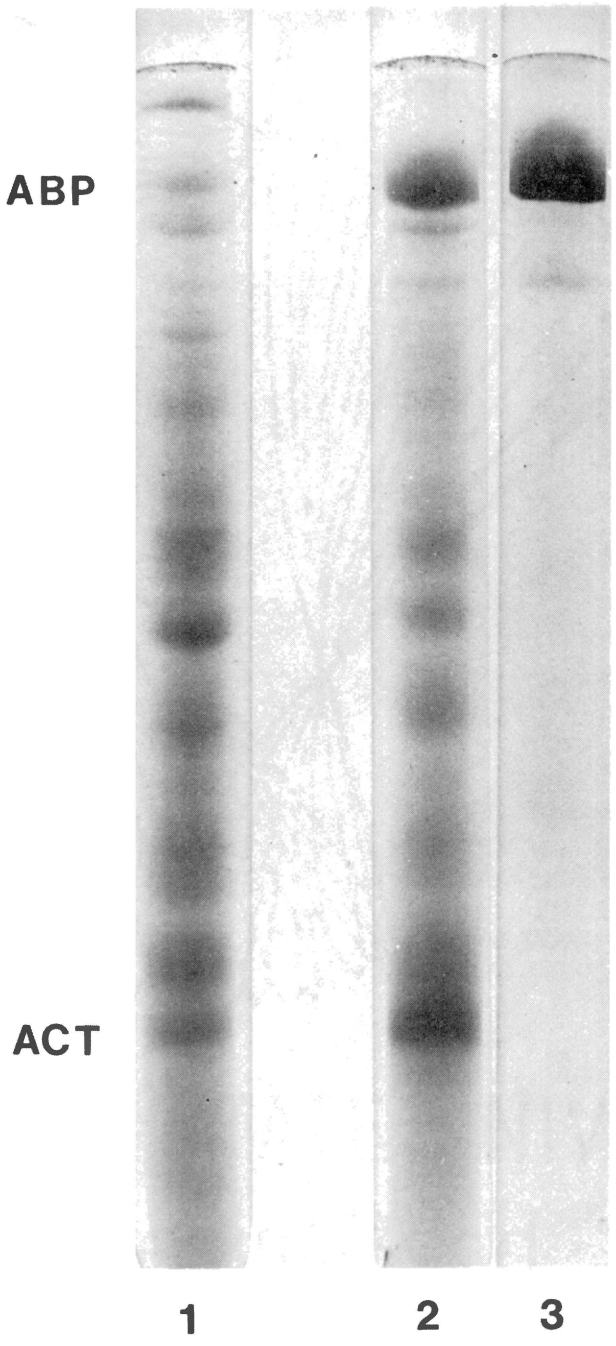

ABP
ACT
1
2
3

TABLE VII

SEDIMENTATION OF ABP*

Trial No.	Temperature	Rotor Speed rpm	S_{app}	S
1	18.1	44,770	8.2	8.6
4	9.2	39,460	5.3	7.2
5	15.0	39,460	10.4	11.9
6	6.0	39,460	6.7	9.8
7	14.4	39,460	7.0	8.1

*Data of A. Sherblom.

which Lazarides (83) had recently found to be present in cultured cells and Mooseker (84) had shown was associated with the tips of the intestinal brush border microvilli.

The protein was purified by column chromatography and SDS PAGE for amino acid analysis. The results of this analysis are shown in Table V. The data were correlated with those from the protein derived from porcine muscle and subjected to analysis also. The method published by Marchalonis and Weltman for determining relatedness (85) was used. A value of 28 was obtained for this protein. (Actin, which is known to be highly conserved throughout evolution gave a value of 18.) Any value less than 50 is considered to indicate closely related proteins.

Mobility on 5% SDS gels (Figure 9) gave a molecular weight of approximately 110,000 daltons (Figure 10). The porcine muscle protein is about 106,000 daltons. Both apparent molecular weight and amino acid analysis suggest this protein may be less well conserved during evolution. Antibodies raised to the porcine muscle protein, however, do cross-react with the non-muscle cell protein (86).

Membrane Perturbations

Proteolysis. Pronase, even at concentrations as low as two µg per ml per mg membrane protein, effectively cleaved most of the proteins as discerned by SDS PAGE. However, the more selective enzymes, trypsin, chymotrypsin, and papain, generally showed specific cleavage of the high molecular weight proteins. Figure 11 shows results of cleavage of SA 180 isolated membranes with five µg papain per mg of protein. Figure 12 is an identical experiment using trypsin.

Figure 9. SDS PAGE of Porcine α-Actinin with SA 180 Membranes.

1) DEAE purified α-actinin.

2) SA 180 membranes.

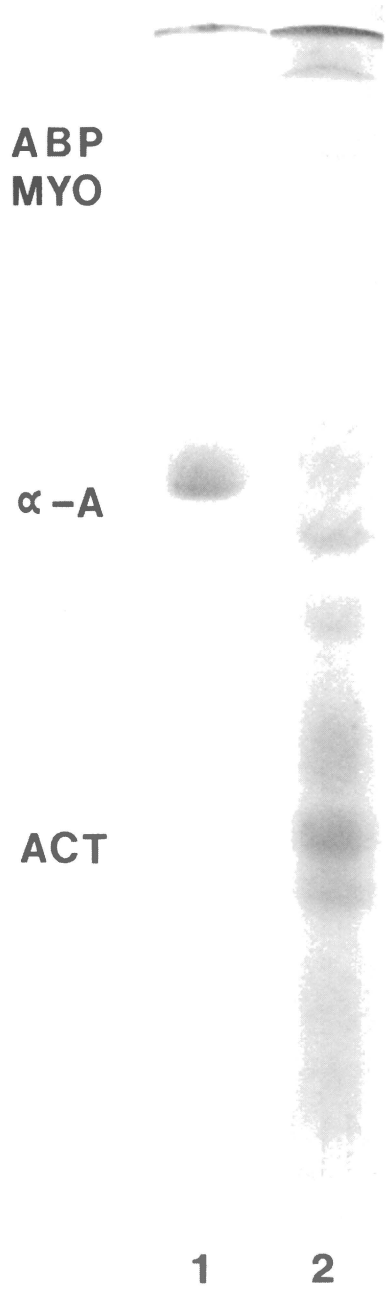

ABP
MYO
α-A
ACT
1
2

Figure 10. Log Molecular Weight vs. R_f-Standard Curve.

Two separate sets of data are shown. Actin and ovalbumin points are coincident.

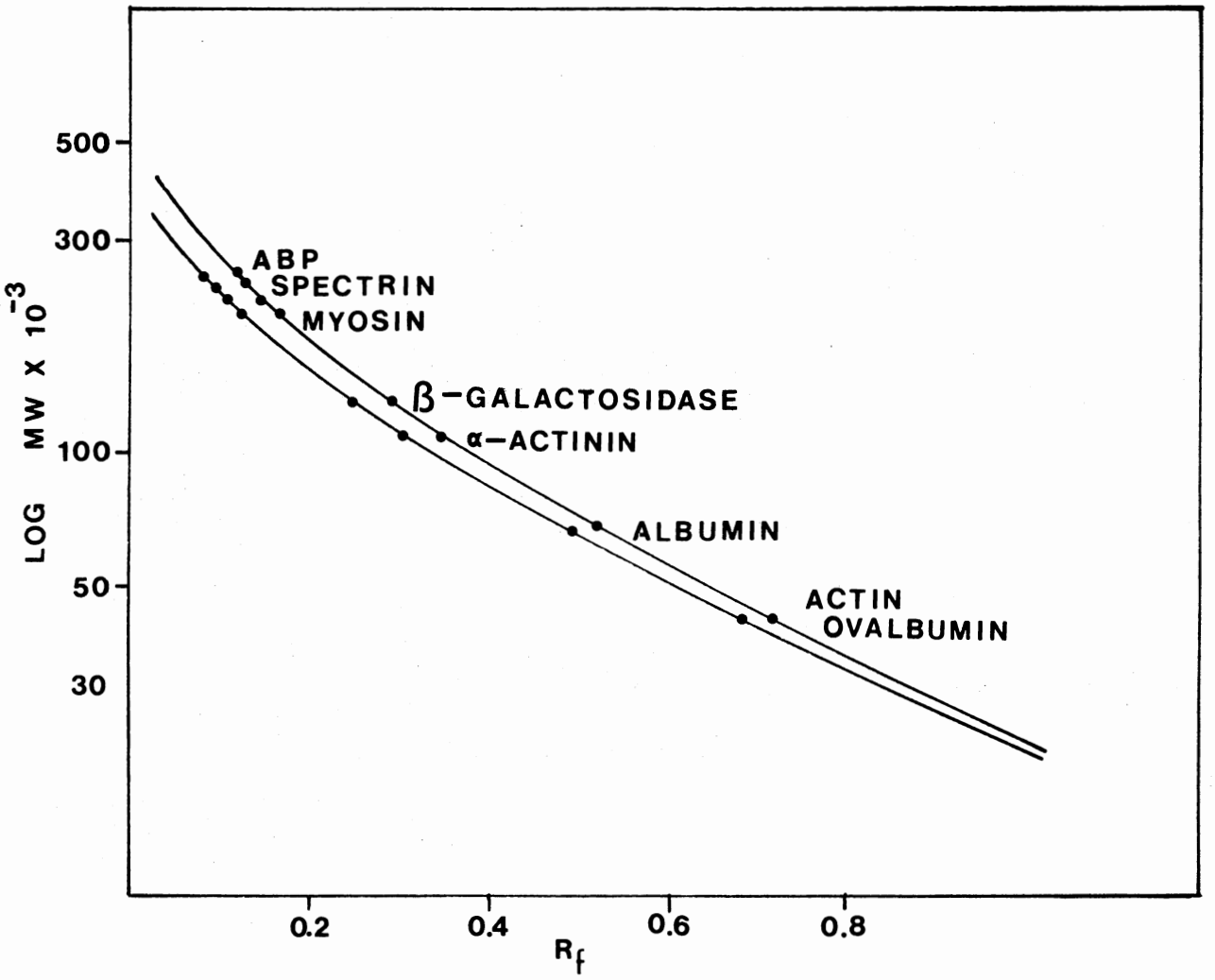

500
300
100
50
30
LOG MW X 10^{-3}
ABP
SPECTRIN
MYOSIN
β-GALACTOSIDASE
α-ACTININ
ALBUMIN
ACTIN
OVALBUMIN
0.2
0.4
0.6
0.8
R_f

Figure 11. Proteolysis of Isolated Membranes with Papain.

A) Whole membranes, B) Proteolyzed membranes, 1) Membranes incubated without enzyme, 2) Membranes treated with papain for 2 min., 3) Membranes treated for 5 min., 4) Membranes treated for 10 min., 5) Membranes treated for 20 min.

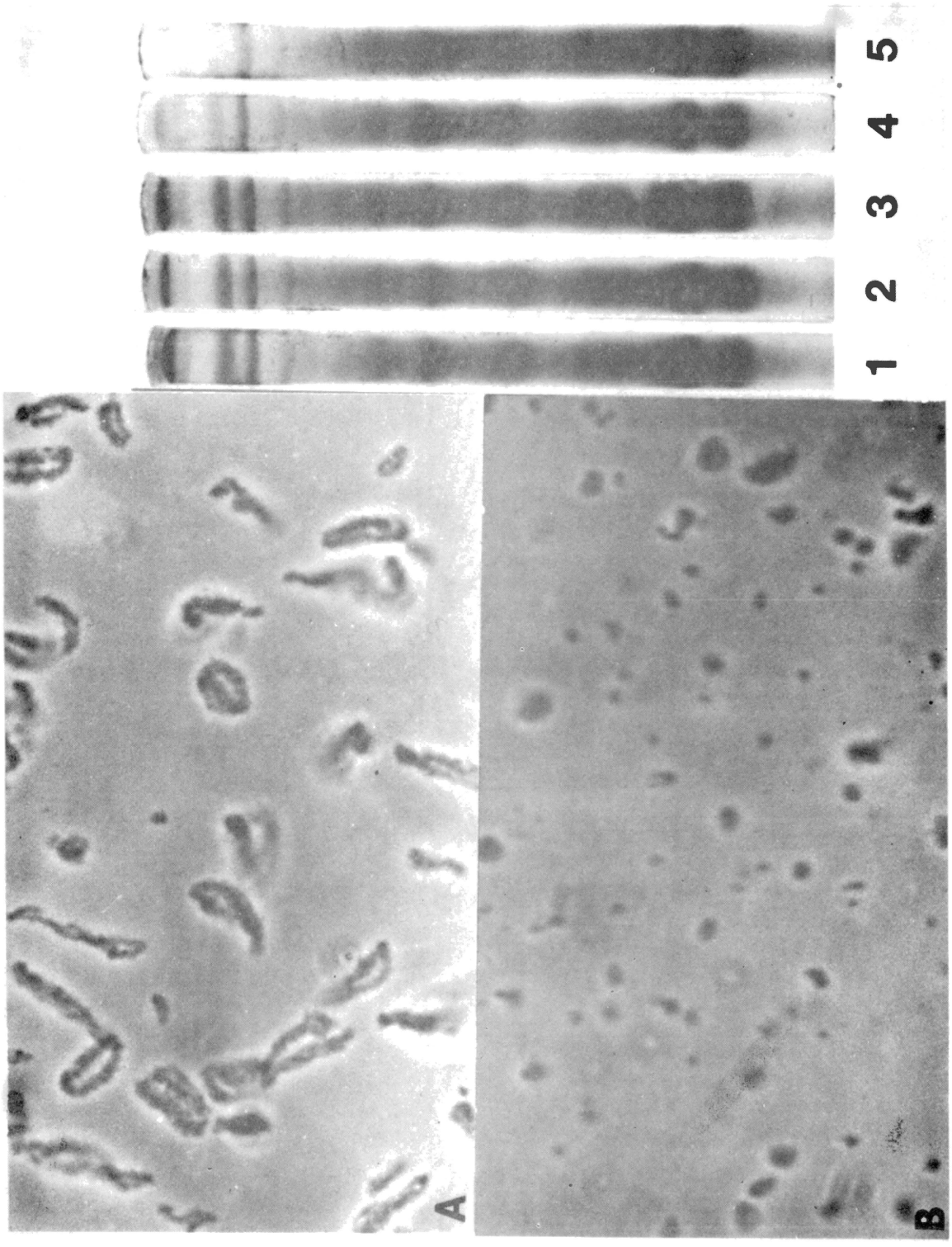
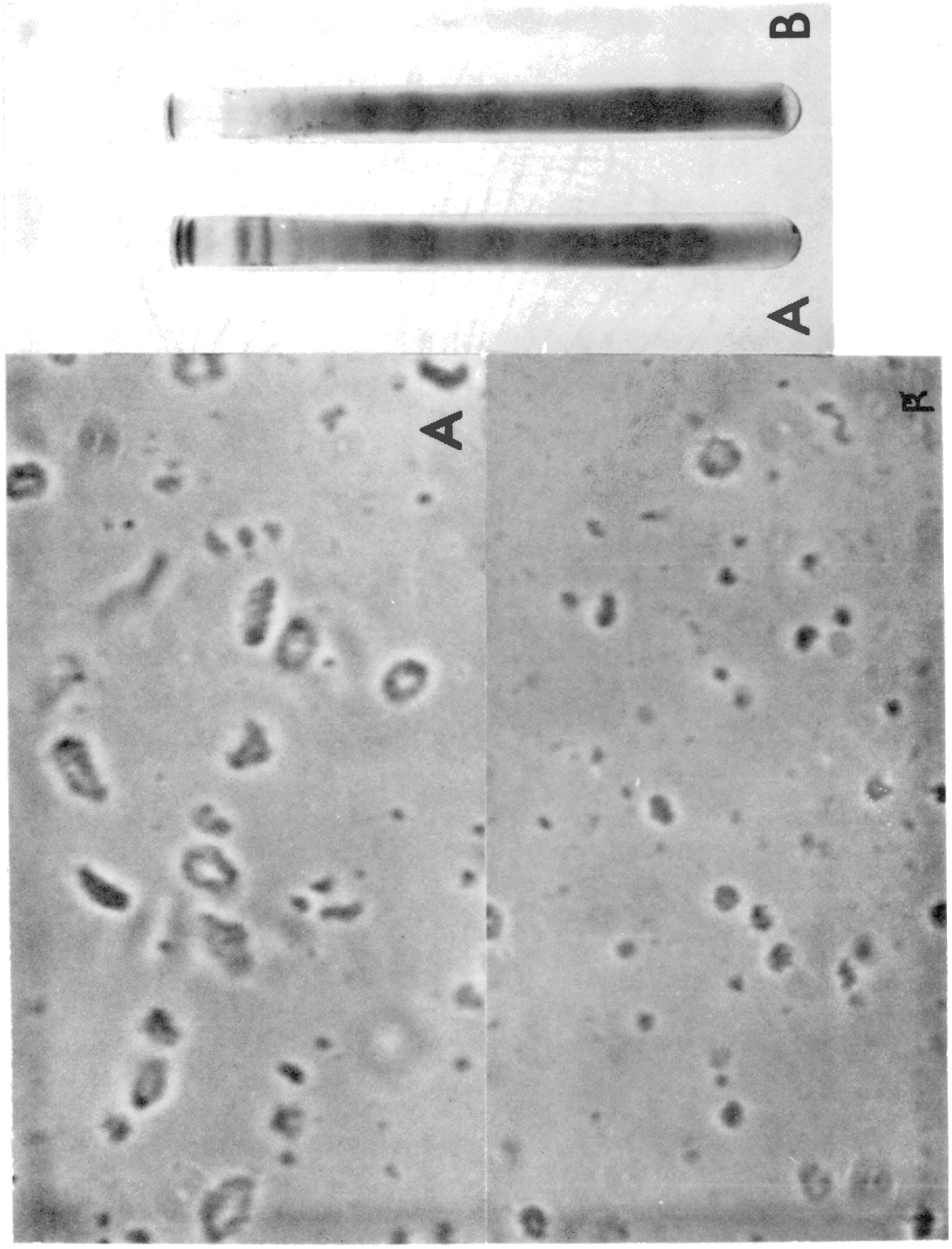

1
2
3
4
5
A
B

Figure 12. Proteolysis of Isolated Membranes with Trypsin.

A) Whole membranes.

B) Trypsin treated membranes for 20 min. at 5 μg/mg protein.


B
A
A
B

Loss of E, AMP, and myosin occurred and in the order listed. As can be seen in both figures, fracturing and vesiculation of the membranes occurred concomitantly with this cleavage. In time course studies with the three enzymes using reaction times of two minutes up to 30 minutes, fracturing appeared to correlate most closely with loss of ABP.

The lower molecular weight proteins, including α-actinin and actin were not efficiently or extensively cleaved by these levels of enzyme. It has been shown that partially polymerized actin solutions show a reduced trypsin digestibility and this is apparently dependent on the F-actin content (87). This suggested that the actin was most likely present in a form that is at least partially polymerized. Since α-actinin was also not cleaved, it was possible that these F-actin filaments subtended the α-actinin (i.e. α-actinin was closer to the membrane) and protected it from cleavage. These proteins could be extracted from the membranes (see below) under appropriate conditions. When this membrane extract was proteolyzed with a similar level of enzyme (5 μg/mg protein) these proteins were efficiently and completely cleaved within a two minute reaction time.

An additional experiment was designed to further show that the α-actinin was most likely protected by the membrane itself on one side and the presumed F-actin lengths on the other. Membranes were first extracted with Triton-X100 before proteolysis, however the proteins did not become more susceptible to cleavage. For reasons which will be discussed in a later section, it seems likely that the extraction procedure did not completely expose the cytoskeletal complex.

Extractions. Isolated plasma membranes were extracted with several media of varying ionic strength and pH. Some of the media are those used by other workers to solubilize myosin and actin. The media tested are listed in Table III together with their ability to cause fragmentation and/or extraction of the cytoskeletal proteins. It is noteworthy that neither actin nor myosin depolymerizing buffers were effective.

The most effective agent was the low ionic strength, high pH media used to extract spectrin and actin from erythrocyte ghosts (88). When the pH of this medium was lowered to 8.5, fragmentation and extraction still occurred although to a lesser extent. If EDTA was excluded from the medium, some fragmentation was observed but long filamentous-like material was seen in the samples via phase microscopy. Lowered amounts of the cytoskeletal proteins were extracted although they were still released together. A time course of extraction with the GEM, pH 9.5 buffer is given in Table VIII. Membranes were centrifuged and the pellets and supernatants collected and solubilized for SDS PAGE gels at intervals from 15 minutes to 2 hours. As can be seen the proteins were not sequentially released but were extracted as a group in approximately the same relative ratios over this time period.

Media which were isotonic caused neither fragmentation nor extraction. If the membranes were treated first with a low ionic strength buffer at pH 7.4, ABP was released along with smaller amounts of actin and some other proteins (Figure 13). Little fragmentation was observed via phase contrast microscopy. If the membranes were recovered and incubated in a second buffer of isotonic strength but at pH 9.5, many of the membranes appeared to fracture into smaller

TABLE VIII

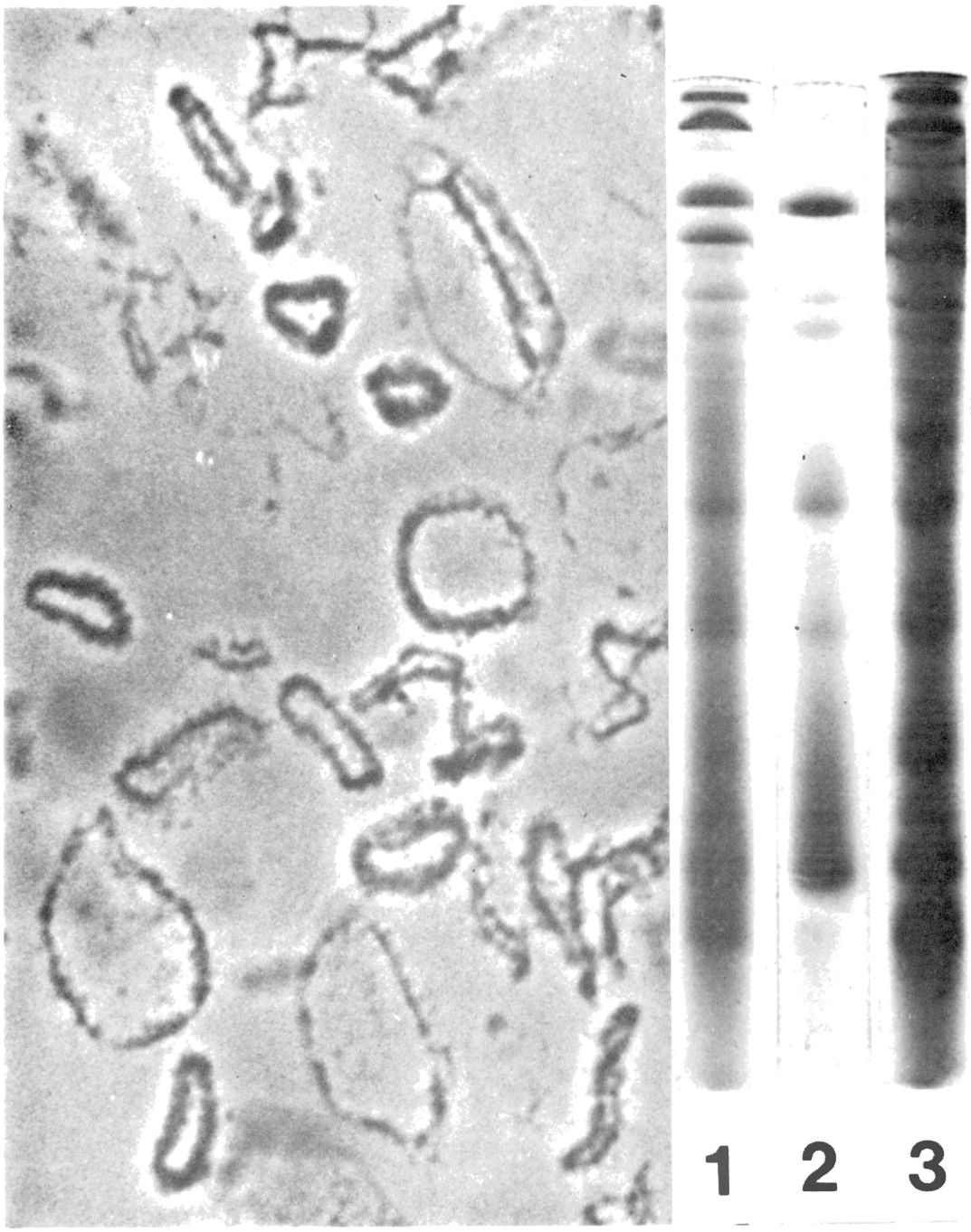
CORRELATION OF RELEASE OF CYTOSKELETON PROTEINS
FROM SA 180 MEMBRANES

Time	% of Total Protein in Each Band			
	E	D	B	A
15 min	1.4	1.0	8.8	11.9
30 min	2.4	1.2	8.8	12.7
45 min	2.5	2.9	15.2	19.4
60 min	2.7	2.7	13.7	17.5
180 min	5.2	5.3	17.5	27.7

Figure 13. Extraction of SA 180 Membranes with Distilled HOH.

Left. Phase microscopy of extracted membranes.

Right. 1) Control whole membranes, 2) distilled water extraction supernatant, 3) distilled water extraction pellet.


1
2
3

pieces spontaneously (Figure 14). Reagents such as cytochalasin B which disrupts actin filament bundles (89), 0.6 M KCl ± MgATP which depolymerizes myosin (90) and 10 mM Tris containing MgATP, which depolymerizes actin filaments (91), failed to cause either fragmentation or extraction. This was interpreted to suggest that these proteins are complexed in such a manner that extensive uncoupling must occur for extraction or fragmentation.

Shin and Carraway (55) had shown that plasma membranes isolated by techniques which did not use the "membrane stabilizer" $ZnCl_2$ were deficient in the high molecular weight proteins but retained α-actinin and actin components. The data just presented suggested that ABP could therefore be a key protein in the complex underlying the membrane.

Data presented by Hartwig and Stossel (Table I) showed that ABP from macrophages was solubilized by treatment with 2 mM EDTA and a pH of 9.0. These conditions are approximated by the GEM extraction medium.

Membranes could be extracted with isotonic Triton X-100 without disrupting the envelope structure, as observed by phase contrast microscopy, or depleting the cytoskeletal proteins (Figure 15). Chemical analyses showed that the membranes were depleted by approximately 50 percent of their lipid phosphate and cholesterol content (Table IX). Additionally, cells were labeled with $Na^{125}I$ by the lactoperoxidase method (55) and membranes isolated. Extraction with Triton X-100 resulted in loss of about 70% of radioactivity, which was shown to be predominantly protein by SDS PAGE. The results indicate that the extraction procedure removed substantial amounts

Figure 14. Fracturing of SA 180 Membranes.

Membranes extracted with distilled water when placed into PBS pH 9.5 buffer spontaneously fracture and vesiculate.

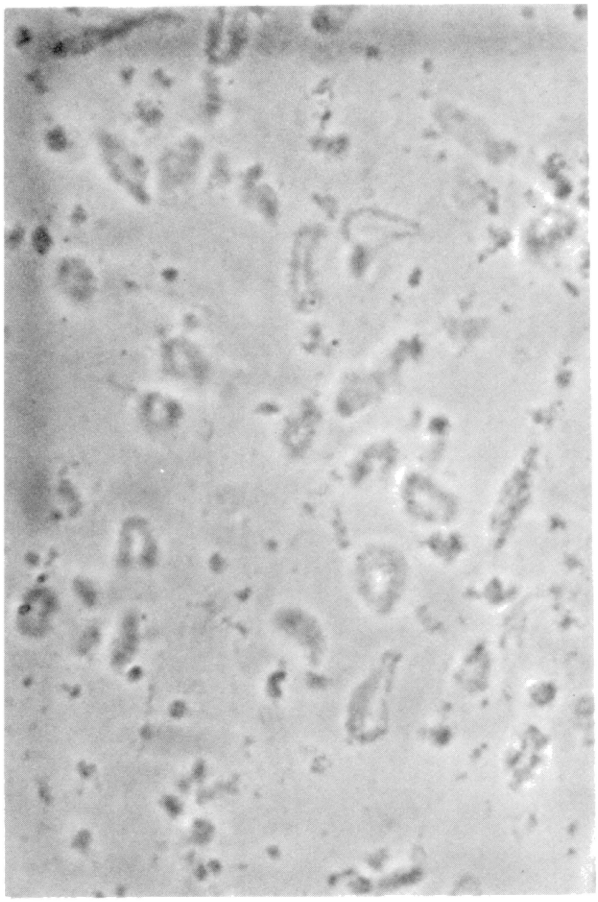


Figure 15. SA 180 Membranes Extracted with Triton X-100.

Left. Phase microscopy of extracted membranes.

Right. SDS PAGE gels of whole membranes and Triton extracted membranes.

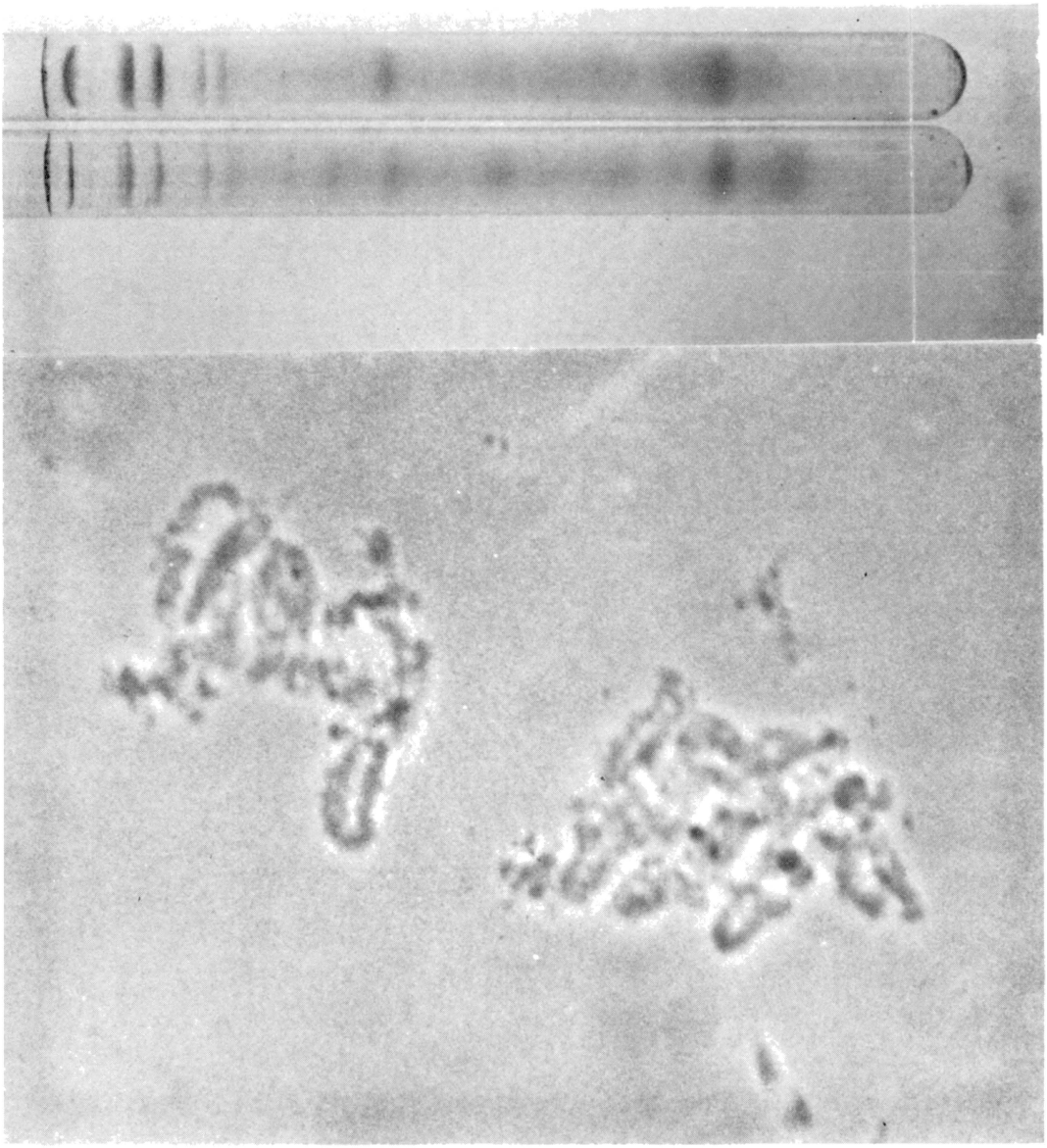


TABLE IX

LIPID AND SURFACE GLYCOPROTEIN CONTENTS OF ENVELOPES AND RESIDUES FROM TRITON EXTRACTION OF SARCOMA MEMBRANE ENVELOPES

Sample	Lipid P µg/mg protein	Cholesterol µg/mg protein	^{125}I cpm
Original Membranes	40	122	-
Residue	14	53	7,100
Triton Supernatant	NA	NA	22,400

of the integral membrane components without destroying the envelope structure, which must be provided by the cytoskeletal framework. Since partial retention of the lipid and cholesterol components remained after extraction, it is likely that in the protease experiments on extracted membranes, the enzyme was still not able to cleave from the membrane side and hence no enhancement of α-actinin proteolysis was obtained.

Extraction with Triton X-100 buffer followed by extraction with GEM 9.5 and KCl resulted in depletion of the cytoskeletal proteins from the "membrane residue" consistant with previous extraction data. The final residue consisted predominately of actomyosin (Figure 16).

Cross-Linking Studies. Several attempts were made to selectively cross-link the cytoskeletal components using one of the bi-functional agents commercially available.

The reagents were generally used at a concentration range from 0.1 to 3X the protein concentration of the membranes. As shown in Table IV the amount of cross-linking with most reagents was extensive. Additionally, there appeared to be a threshold concentration below which little reaction occurred and above which extensive reaction occurred. Better selectivity was not achieved by decreasing the concentration of the cross-linker (Figure 17).

More selectivity of reaction was attempted by chosing reagents with various distance between the reactive end groups. As can be seen also in Table IV and Figure 17, again little reaction occurred or complete reaction occurred as a function of length.

Since a very large complex containing all the cytoskeletal

Figure 16. Extraction of SA 180 Membranes with GEM and KCl after Triton Extraction.

1) GEM extract.

2) KCl extract.

3) KCl residue.

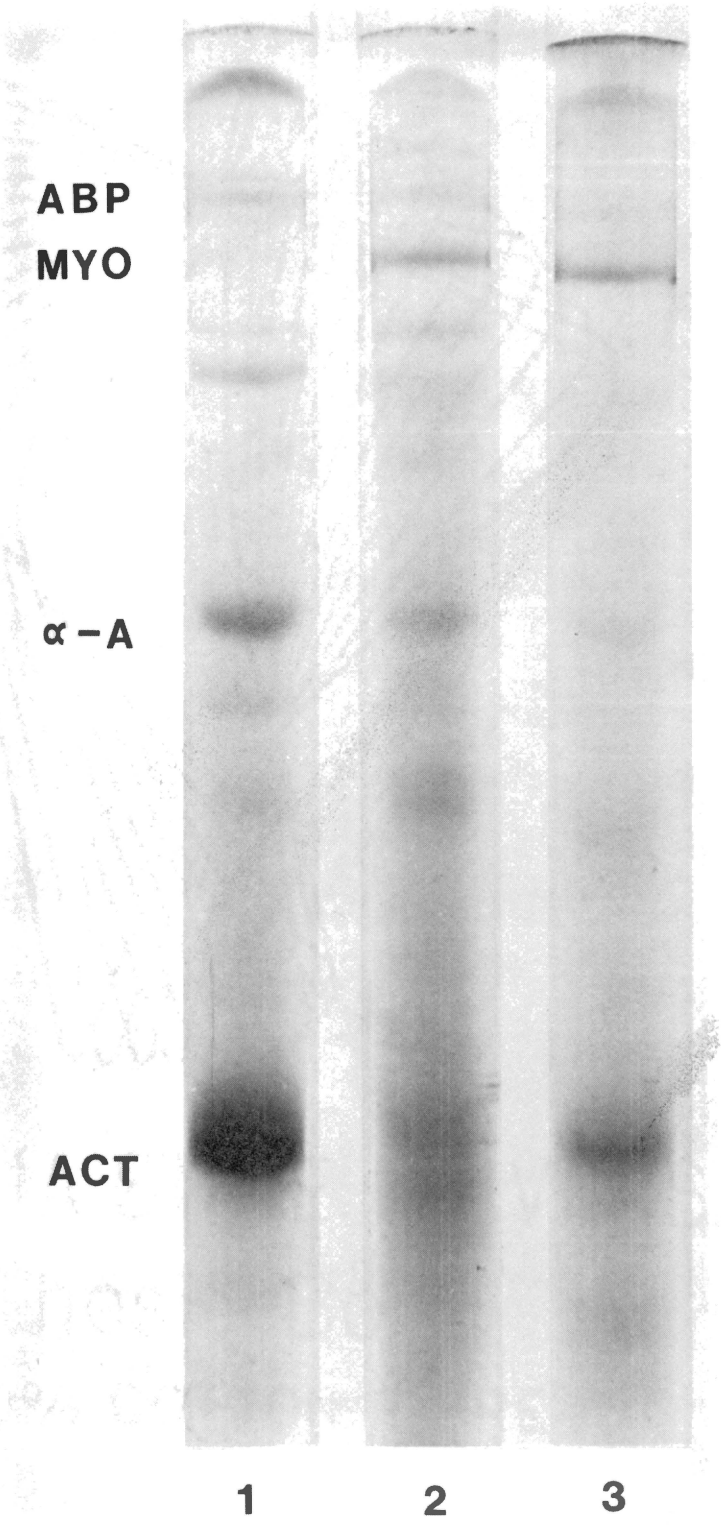

ABP
MYO
α-A
ACT
1
2
3

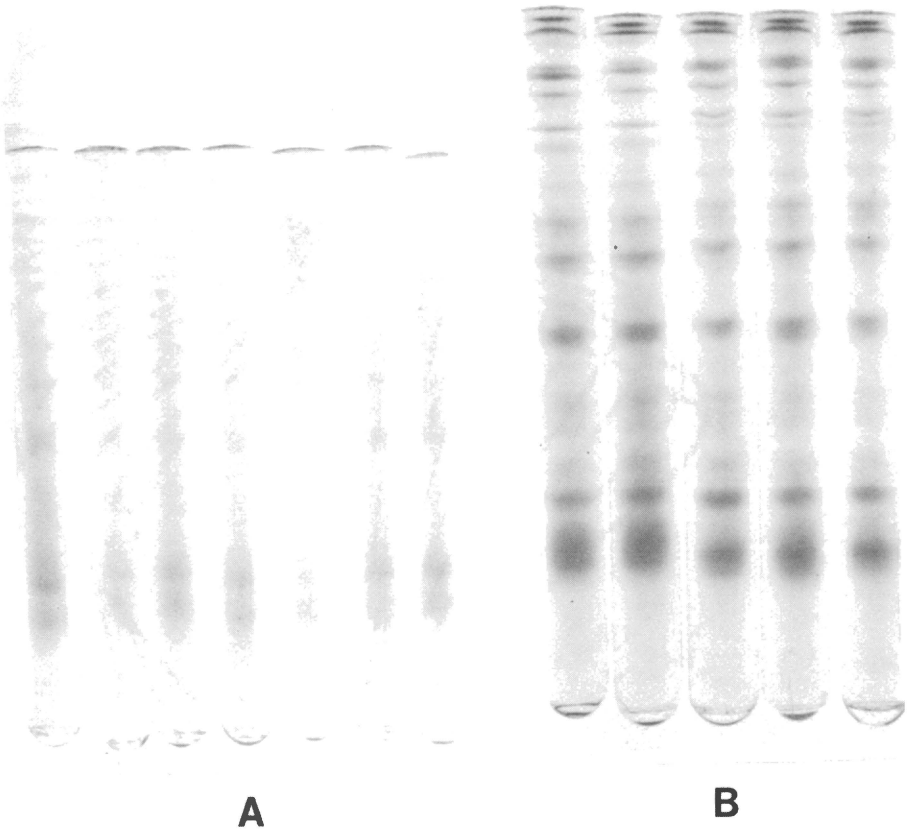
Figure 17. SDS PAGE of Cross-Linked SA 180 Membranes.

A. Adipimate - 5% Na_2HPO_4 gels.
Left to right: Control, 0.5 mg/mg, 0.8 mg/mg, 1.2 mg/mg, 1.8 mg/mg, 2.4 mg/mg, 3.0 mg/mg.

B. Malonimidate - 5% Na_2HPO_4 gels.
Left to right: Control, 0.3 mg/mg, 1.0 mg/mg, 2.0 mg/mg, 3.0 mg/mg

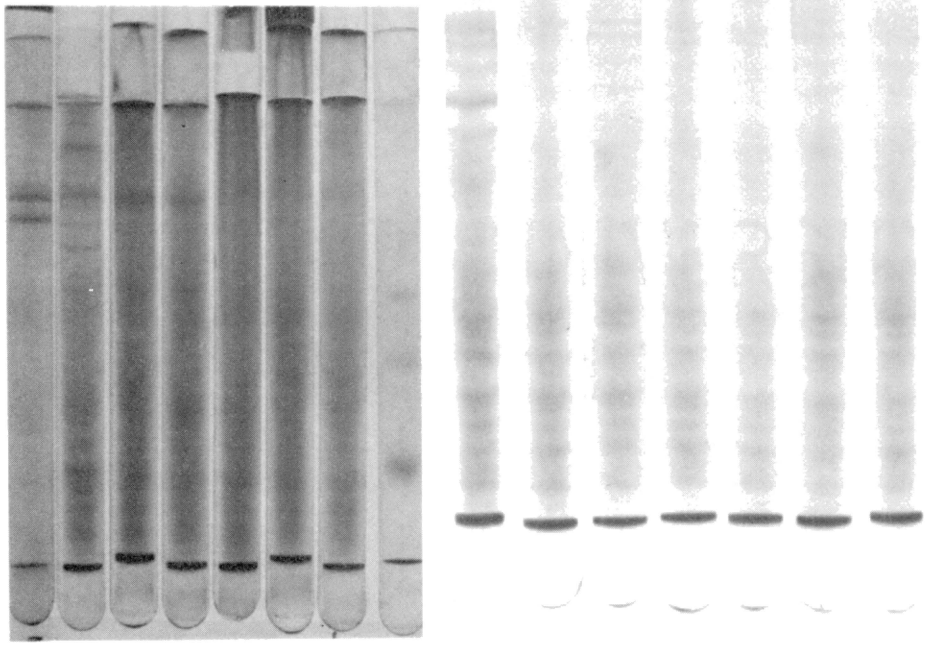
C. DTBP - 5% Tris Laemmli gels.
Left to right: RBC ghosts, control, 0.5 mg/mg, 0.65 mg/mg, 0.8 mg/mg, 1.0 mg/mg, 1.25 mg/mg.

D. CuPA (3:1) - 5% Tris Laemmli gels.
Left to right: Control, 5λ, 6λ, 7λ, 8λ, 9λ, 10λ/ml membranes.



A

B



C

D

proteins appeared to be selectively generated rather than dimers, trimers, tetramers, etc., the results suggested that these proteins were in a complex in sufficiently close relationship so that they were cross-linked completely or not at all.

Leakiness of Cells During Membrane Isolation

Since no Zn^{++} transport system has been as yet described, the question arose as to whether the Zn^{++} was able to cross the membrane or its action was at the outer cell membrane. Results with fluorescence polarization by D. R. Anderson (92) suggested cells suspended in 40 mM Tris became permeable to the external environment.

Cells were initially tested for viability using uptake of Trypan Blue dye as indication of cell death. Viability decreased progressively during the various washing stages. Cells swollen in 40 mM Tris only partially recovered if transferred again to isotonic media.

Additional information was obtained by direct analysis of the cell media. Cells were treated with isotonic HEPES, 40 mM Tris, $ZnCl_2$ in water or homogenized and centrifuged. The supernatants were recovered and tested for a cytoplasmic enzyme (lactate dehydrogenase), a mitochondrial enzyme (succinate dehydrogenase) and ribose (RNA) to determine leakage from the cells into the external media. Cell counts were made on the media to be sure they were minimally present before assays were performed.

Table X shows that cells are maximally permeable in $ZnCl_2$ in water and therefore the Zn^{++} most likely freely passes the membrane. In gelation experiments (see below) high speed supernatants do not undergo syneresis unless ATP is added to the system. Therefore ATP

TABLE X

LEAKINESS OF SA 180 CELLS

Treatment	RNA	SDH	LDH
HEPES	1.0	1.0	1.0
40 mM Tris	1.5	4.9	1.0
$ZnCl_2$ in HOH	4.1	6.3	3.5
Cell Homogenate	8.8	23.0	22.0

Data is average of three experiments.

Data expressed as factor of HEPES values which were set to 1.0.

was not assayed but assumed to be lost at this step if in a free state.

Mechanism of $ZnCl_2$ Action

It was of interest to discover how $ZnCl_2$ exerted its effect of stabilization on the membrane thus allowing isolation of large sheeted envelopes. Its action was not confined to the cell surface due to the swelling of the cells and induced permeability just discussed.

In the first experiment, radioactive $ZnCl_2$ ($^{65}Zn^{++}$ as a chloride salt) was added at the stabilization step and membranes isolated as usual. The $^{65}Zn^{++}$ isolated with the membranes but did not appear to be tightly bound. If cells were treated with $^{65}Zn^{++}$, recovered, washed, and treated with cold $ZnCl_2$, very low levels of radioactivity could be detected. If $^{65}Zn^{++}$ membranes were washed in isotonic buffers, the $^{65}Zn^{++}$ partitioned between the media and the membranes. $CHCl_3$:MeOH (3:1) extraction and GEM (9.5) extraction both released much radioactivity into the soluble fraction. Much of the release was probably due to exchange for cold Zn^{++} salts occurring as contaminants in the media.

It has been suggested that membrane constituents such as phosphatidylinositol can sequester Ca^{++} by chelation. Such has not been shown for Zn^{++}. However, some cell surface complex glycosaminoglycans (93) have been shown to bind zinc. For reasons discussed below, this binding is probably not the important action in stabilization.

An additional experiment designed to delineate the mechanism of Zn^{++} stabilization was to homogenize cells under various conditions. Isotonic HEPES served as the control for normal partitioning of the

proteins. Cells were treated with HEPES, 40 mM Tris, HEPES then $ZnCl_2$, or 40 mM Tris followed by $ZnCl_2$. The homogenates were centrifuged at 2000 rpm in a Sorvall RC2-B to pellet nuclei and large membrane fragments. The supernatants were re-spun at 10,000 rpm to pellet intermediate-sized particles and again at 26,000 rpm in a Beckman L5-65 to pellet F-actin or polymeric proteins. Quantitation from densitomeric scans of SDS PAGE is shown in Table XI. It was clear that the Zn^{++} treatment caused more of the cytoskeletal proteins to be sedimented at the early centrifugation step.

To substantiate these results, sarcoma cells were extracted with Triton X-100 in HEPES or treated with 1 mM $ZnCl_2$ prior to extraction. The upper portion of Table XII shows results for experiments in which only the pellets from extraction were subjected to electrophoresis. In the lower portion of the table, cells or extracted cells were centrifuged and supernatants filtered through a 0.45 μ Millipore filter prior to SDS PAGE. Phase microscopy showed that cell debris had been removed from the supernatants so that soluble proteins or soluble complexes remained. In both cases, the Zn^{++} treatment has caused larger amounts of these proteins to be pelleted; in particular, ABP shows a greater increase in sedimentability than the other proteins.

Electron Microscopy of Membrane Preparation and Cells

Samples of the sarcoma cells were fixed for electron microscopy at the several stages during preparation of membranes. Isolated membranes as well as extracted membranes were also prepared for both

TABLE XI

EFFECT OF Zn^{++} ON TRITON EXTRACTION OF CYTOSKELETAL PROTEINS
AVERAGE % PROTEIN

Fraction	Protein	HEPES Control Cells	HEPES + Zn^{++} Cells	Tris Treated Cells	Tris + Zn Cells
2,000 rpm pellet	ABP	0.95	0.98	0.53	1.03
	Myo	0.85	0.99	0.69	0.95
	α-A	2.46	2.53	2.61	2.57
	Act	7.82	9.07	9.04	10.46
10,000 rpm pellet	ABP	0.78	2.16	0.61	1.91
	Myo	0.96	2.08	1.12	1.47
	α-A	3.05	2.95	2.27	2.46
	Act	4.36	5.29	8.61	8.11
26,000 rpm pellet	ABP	0.49	0.58	0.47	0.34
	Myo	1.45	1.71	0.87	1.34
	α-A	4.63	2.91	4.28	3.94
	Act	9.86	8.77	10.27	11.94
26,000 rpm supernatant	ABP	0.84	0.22	1.03	0.41
	Myo	0.77	0.35	0.73	0.45
	α-A	5.46	3.60	3.99	6.19
	Act	13.59	17.32	16.51	13.07

TABLE XII

EFFECT OF Zn^{++} ON TRITON EXTRACTION OF CYTOSKELETAL PROTEINS

Fraction and Treatment	% of Total Protein			
	ABP	Myosin	α-Actinin	Actin
SET I				
Whole Cells	0.94	0.82	4.66	11.26
Triton Extracted Whole Cells	0.60	0.98	2.68	7.49
Zn^{++} Cells	1.37	1.18	4.69	8.31
Triton Extracted Zn^{++} Cells	2.00	2.11	3.61	8.96
SET II				
Triton Extracted Whole Cells				
Supernatant	1.17	0.90	3.35	14.54
Pellet	0.77	1.12	4.69	8.25
Triton Extracted Zn^{++} Cells				
Supernatant	0.99	1.14	5.39	10.82
Pellet	1.15	1.24	6.57	9.24

scanning and transmission study.

The HEPES washed cells by SEM had irregular surfaces with long microvilli and numerous folds (Figure 18). During the swelling step they became rounder and smoother with apparently only the tips of the microvilli extending from the surface. After treatment with zinc, the cells still appeared to have folds and projections and some blebbing may have occurred by increased background material seen in the preparations.

By TEM, cells washed in HEPES showed the presence of substantial amounts of "fuzz" associated with the inner surface of the plasma membrane. Cells which had been swollen or swollen and zinc treated showed loss of intercellular contents, swollen mitochondria and endoplasmic reticulum and perhaps loss of part of the filamentous meshwork underlying the plasmalemma. No additional materials that had become associated with this network were apparent via this treatment.

Isolated membranes also appeared to have irregular surfaces although this was not the general observation (Figure 19). Membranes which had been sedimented tended to be rolled and folded into compact structures. TEM showed large amounts of a "fuzz" material in the inner surface. Only occasionally were filamentous structures seen. These structures could not be traced to direct attachment to the plasmalemma but they did closely oppose the membrane. Actual filaments were not present in quantities great enough to account for the protein concentrations observed via SDS PAGE electrophoresis of whole membranes. However, the "fuzz" network could account for the protein levels observed.

Figure 18. TEM and SEM of Sarcoma 180 Cells.

Panels A and B. HEPES washed cells.

Panels C and D. 40 mM Tris, pH 7.4 washed cells.

Panels E and F. 1 mM $ZnCl_2$ treated cells.

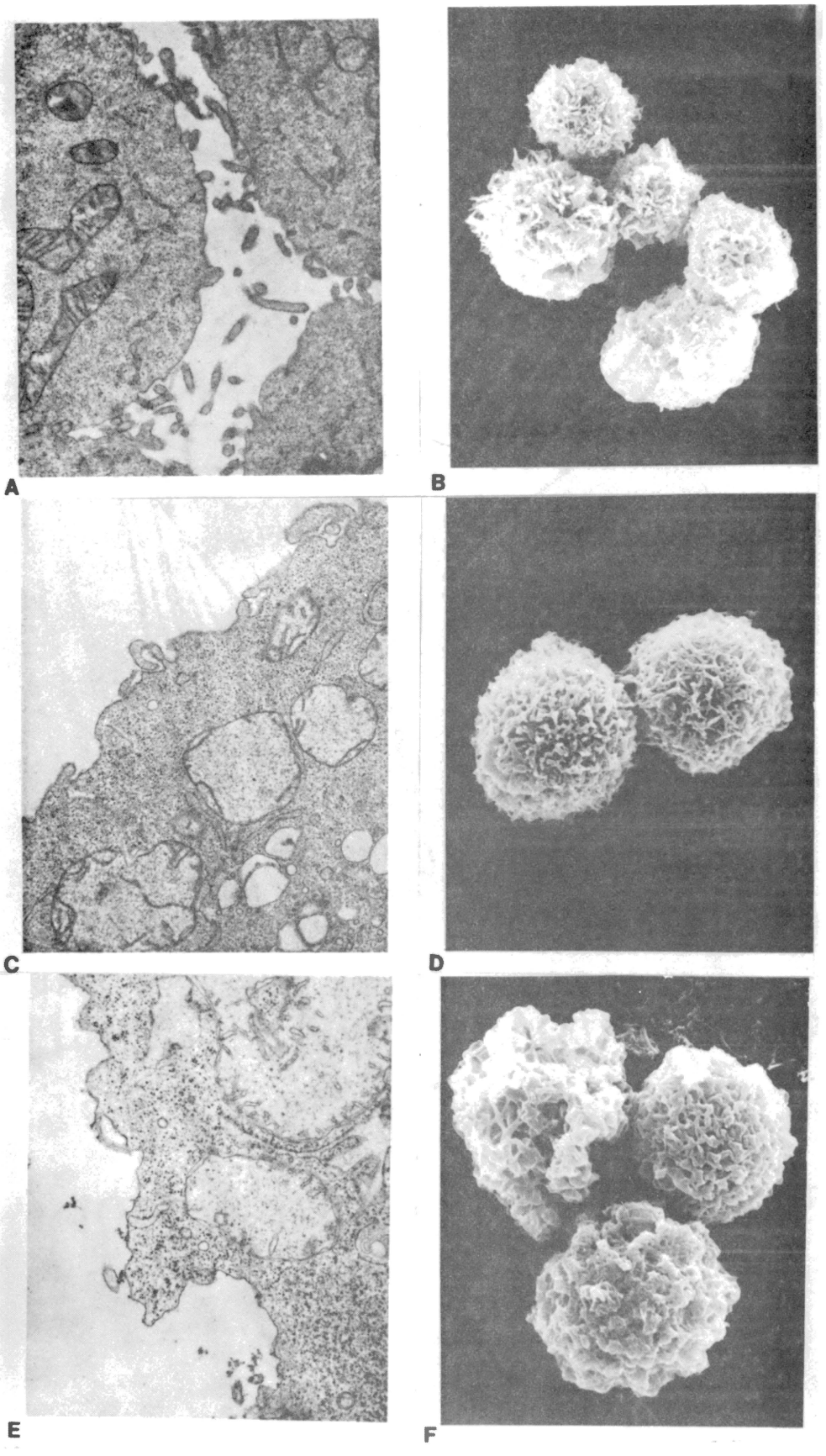
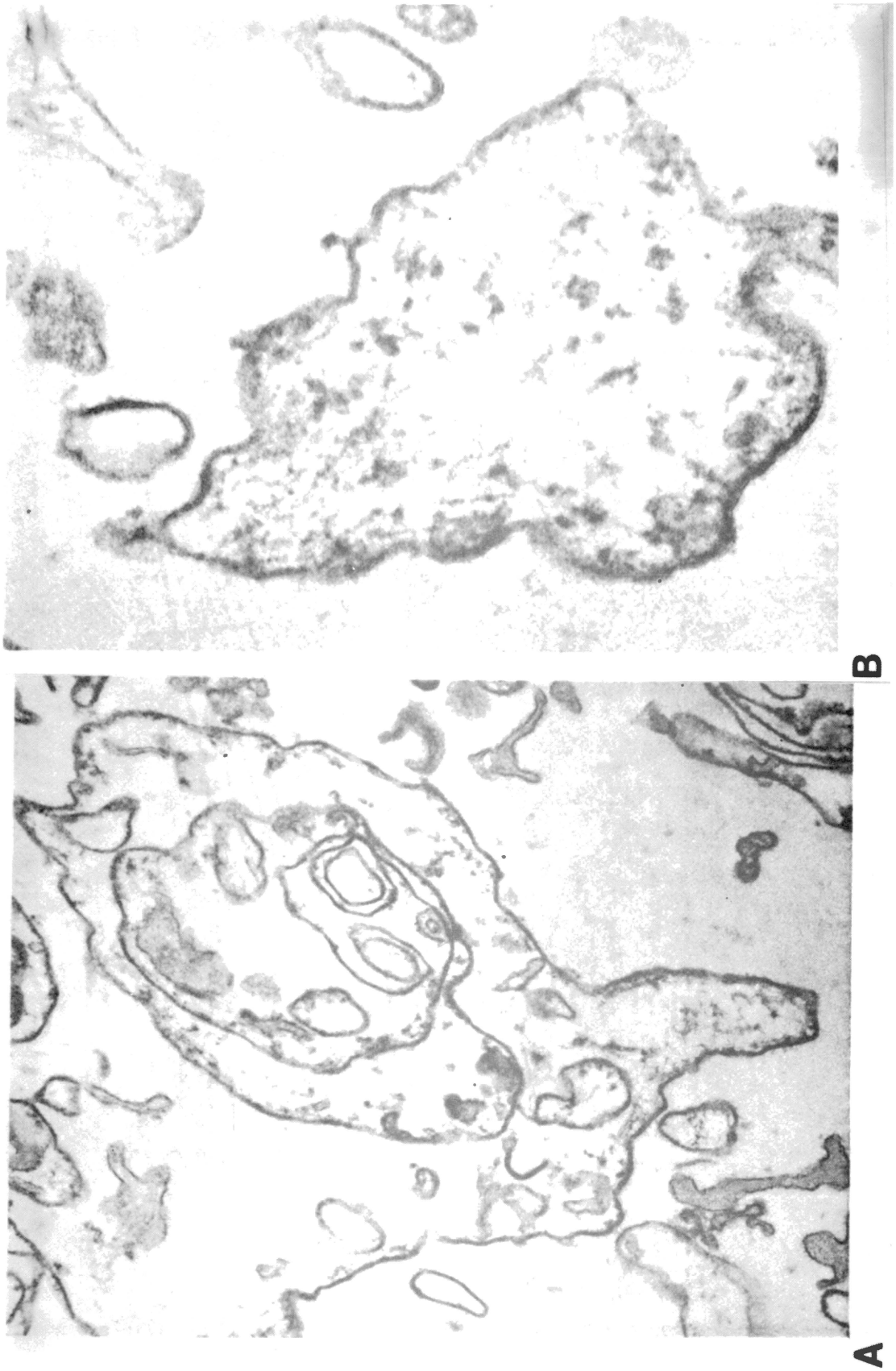

A
B
C
D
E
F

Figure 19. SA 180 Membranes Isolated via Zinc Stabilization.

Panel A. TEM of isolated membranes showing "fuzz" associated with the inner membrane surface.

Panel B. Higher magnification of membrane showing filamentous networks.


B
A

Under extraction conditions using the GEM, pH 9.5 media, TEM showed fragments of membranes and vesicles which had been depleted of this "fuzz" material (Figure 20, left).

Extraction with Triton X-100 and recovery of the "ghost" membranes showed that the trilamellar structure had been abolished but some heavily stained material remained (Figure 20, right). These ghost membranes likewise retained the whorled appearance similar to that of the intact membranes.

Together these results also suggested this material is the cytoskeletal complex itself since it correlates with the protein composition of samples subjected to SDS PAGE electrophoresis.

Effect of Cytochalasins on Membrane Isolation and Extractability

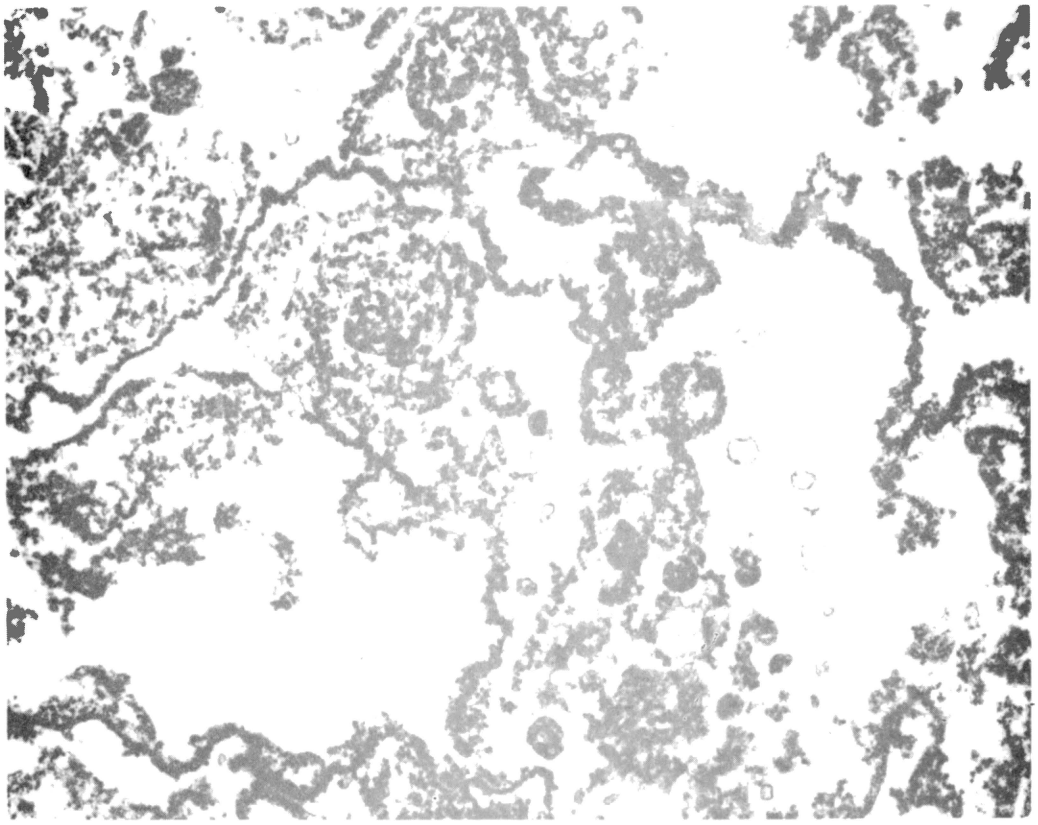
Several attempts were made to pre-treat SA 180 cells with cytochalasin B to determine if this agent would prevent the Zn^{++} stabilization of the plasma membranes. Due to the ease of reversibility of cytochalasin B effects (94), it was necessary to incorporate the agent into all media. Membranes prepared by the Zn^{++} method after drug treatment appeared to sheet normally and contain quantities of the cytoskeletal proteins relatively equivalent to those found via the Zn^{++} stabilization method (Table XIII).

To ascertain if the Zn^{++} itself was reversing any effect of cytochalasin B, an attempt was made to isolate membranes without the addition of $ZnCl_2$. In this case, the cells were not readily homogenized but membranes which were obtained appeared to sheet off in larger fragments and had a morphology more similar to those using

Figure 20. SA 180 Membranes Extracted Under Conditions Releasing Cytoskeletal Proteins and Lipids.

Panel A. Membranes extracted with GEM, pH 9.5 showing loss of the "fuzz" material associated with the inner surface.

Panel B. Membranes extracted with Triton X-100 showing loss of trilamillar structure but retention of the "fuzz" material.



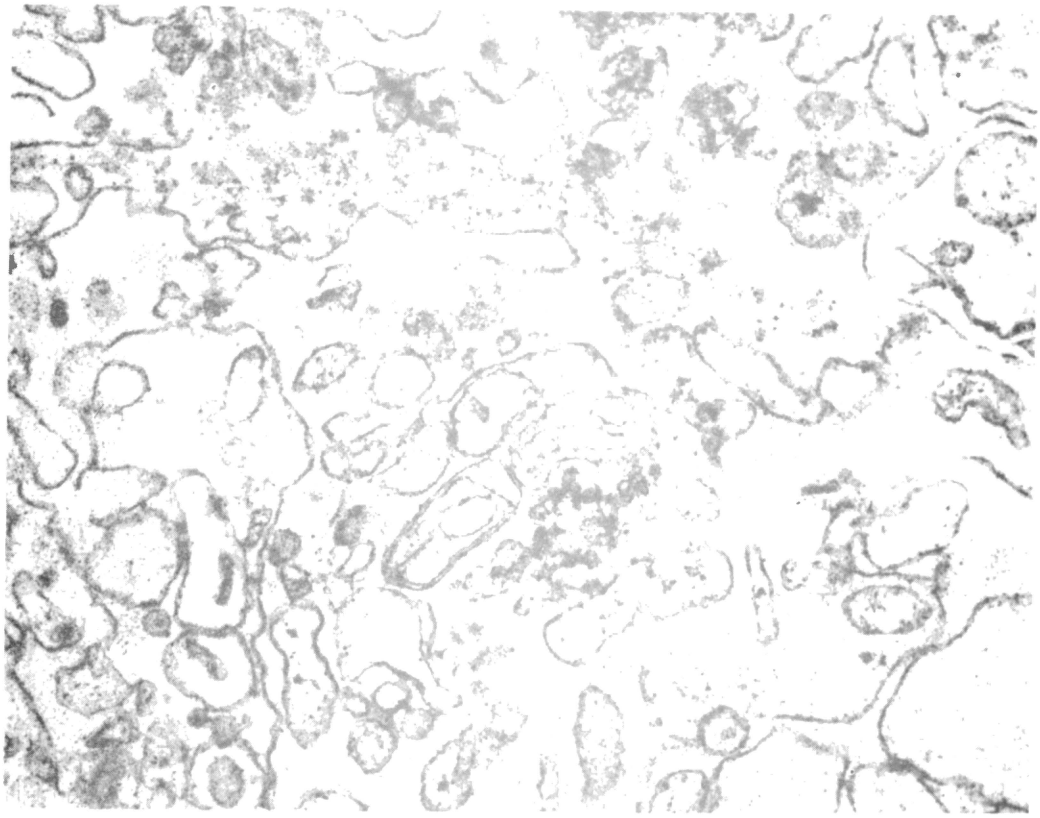


TABLE XIII

CYTOSKELETAL PROTEIN COMPOSITION OF SA 180 MEMBRANES

	% of Total Protein
Zn^{++} Membranes	
Actin	16.1
α-Actinin	7.2
Myosin	2.8
ABP	3.8
Cytochalasin B Followed by Zn^{++} Membranes	
Actin	15.9
α-Actinin	6.6
Myosin	2.5
ABP	3.4

the conventional preparation technique. Membranes from control samples (no addition or DMSO) were small and appeared to have outward projecting spikes spaced around the membrane (Figure 21). By densitometry, the amounts of cytoskeletal proteins were lower than those from membranes isolated after Zn^{++} stabilization (Table XIV).

Membranes isolated in the presence of cyto B showed an increased content in all proteins except ABP when compared to membranes isolated without Zn^{++} (Table XIV). This result is consistent with preliminary data for cyto B or cyto D treatment of platelets. There appears to be a loss of ABP but retention of actin and myosin in the Triton insoluble fraction of platelets when compared to controls not treated with the drug (data not shown).

Phase microscopy of the cells during the experiment showed that cells treated only with DMSO became aggregated into large clumps with Triton treatment. Also most appeared to have intact nuclei with collapsed material surrounding them. Cells treated with the cytochalasins were much less aggregated and were mainly single cells. Nuclei appeared to have retained a network at a distance from them so that a denser "cytoplasm" was retained.

Figure 21. Membranes Isolated Without Zn^{++} Stabilization.

Panel A. Control membranes.

Panel B. Membranes isolated in the presence of 10 μg per ml cytochalasin B.

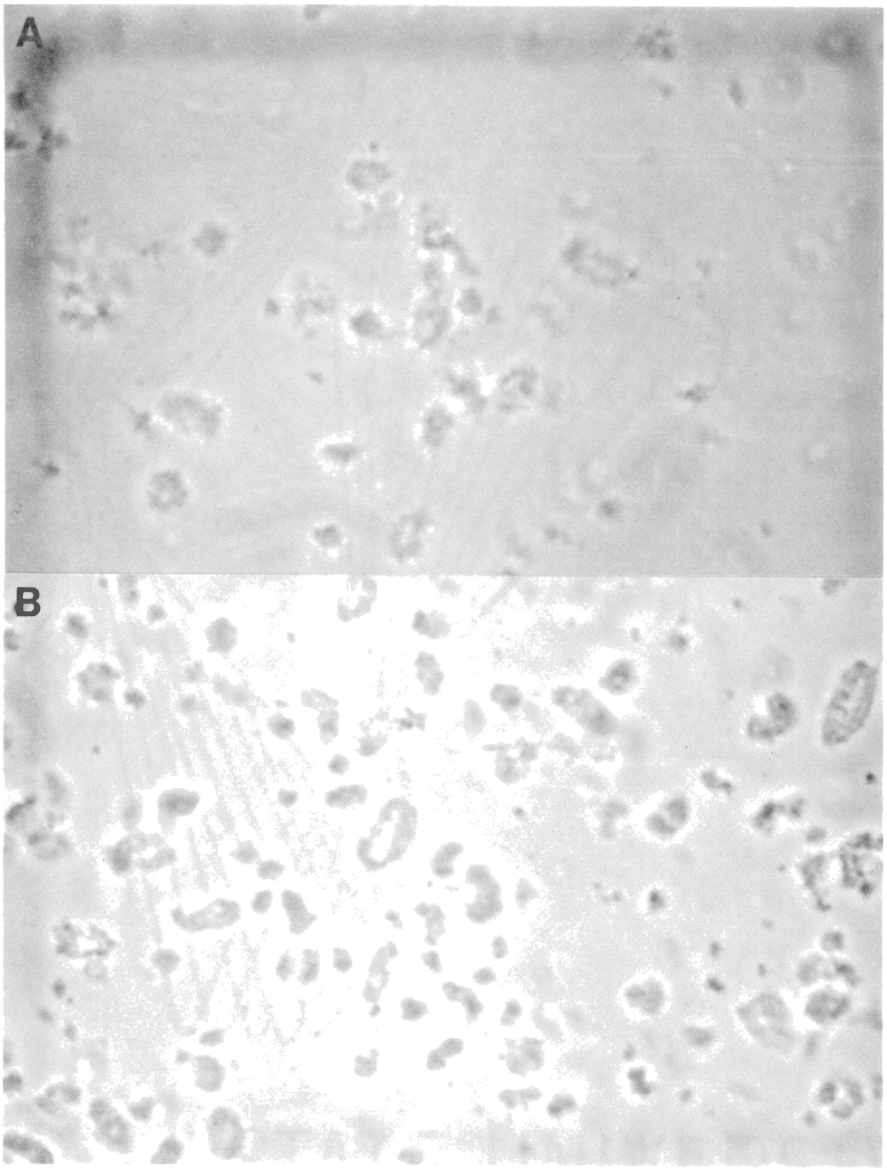

A
B

TABLE XIV

COMPARISON OF CYTOSKELETAL PROTEINS OF SA 180 MEMBRANES ± Zn^{++} AND CYTOCHALASIN B

	Percent of Total Protein	Percent of Actin
Zn^{++} Membranes		
Actin	16.1	100
α-Actinin	7.2	45
Myosin	2.8	17
ABP	3.8	24
Membranes Isolated Without Zn^{++}		
Actin	11.5	100
α-Actinin	4.5	38
Myosin	0.5	4
ABP	2.4	21
Membranes Isolated Without Zn^{++} and With Cytochalasin B		
Actin	12.7	100
α-Actinin	4.6	37
Myosin	0.7	6
ABP	2.1	16

CHAPTER IV

GELATION

Results

Studies in this area were originated for isolation of ABP from the Sarcoma ascites cells following the published methodology of Stossel and Hartwig (60) for macrophages. In this method, the buffer contains 0.34 M sucrose to help stabilize lysosomes during the cell homogenization step. The sucrose prevented sufficient swelling of the tumor cells to be easily broken with the tighter fitting pestle of a Dounce homogenizer. Therefore, the cells were pre-swollen in 40 mM Tris-HCl, pH 7.4 buffer prior to homogenization. Since this step caused the cells to have a greater internal volume, the added volume per packed cell volume was reduced to 1:1.

Cell homogenates were centrifuged in either a Sorvall RC2-B refrigerated centrifuge at 15,000 rpm (27,000 x g) for 50 to 60 min or a Beckman L5-65 ultracentrifuge at 26,000 rpm (90,000 x g) for 60 to 420 min. Floating material, presumably lipid, was removed and discarded prior to decanting the remaining supernatant, which was used in all gelation experiments.

In initial experiments the material was allowed to remain at room temperature until it underwent a transition to yield a consistency of set Jello® (Figure 22). Centrifugation at 9-12 X 10^3 rpm at 25°C

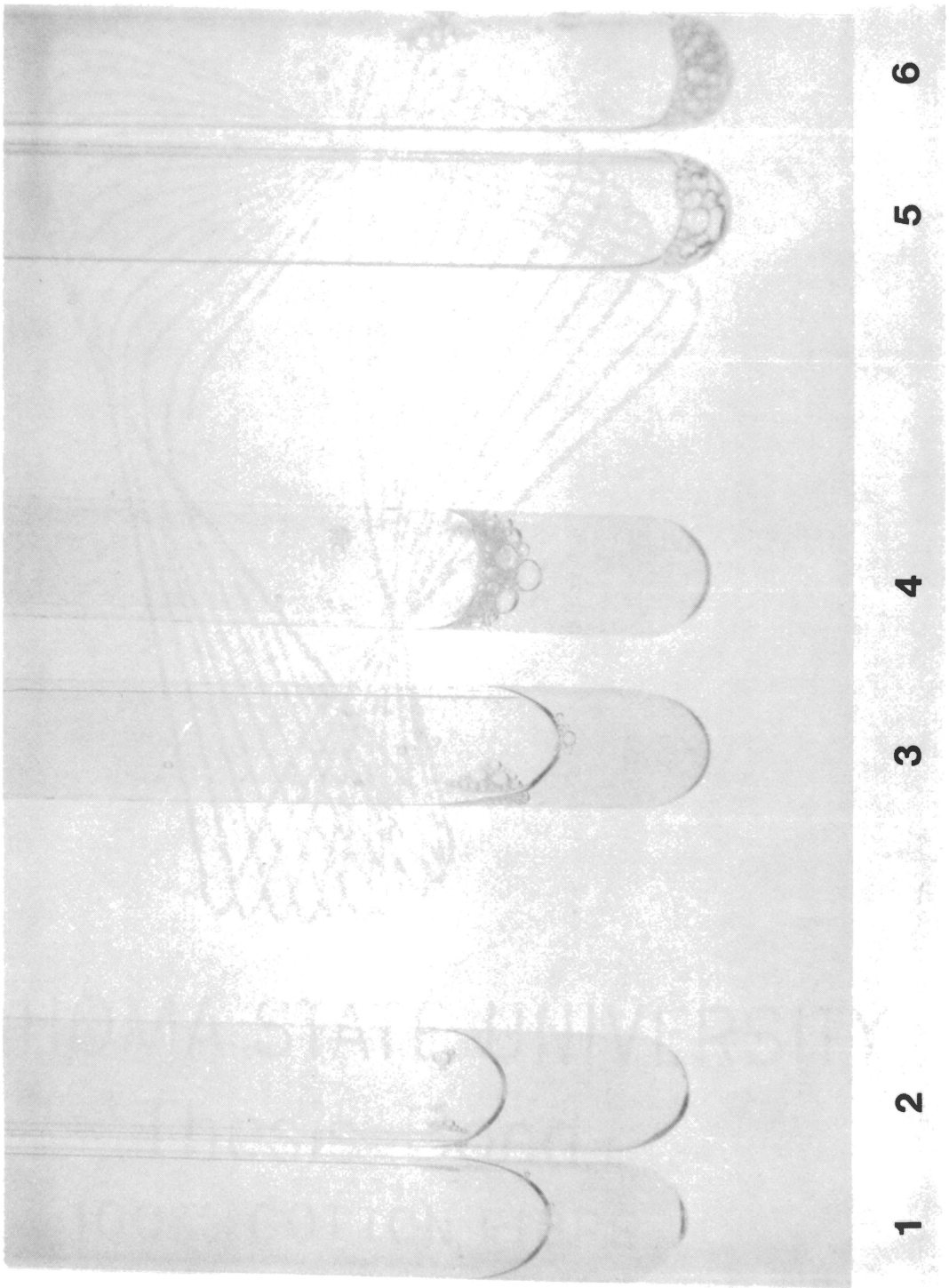
Figure 22. Gel Formation in SA 180 Extract.

Tubes are lying in the plane of the paper.

1 & 2. 5 µg/mg trypsin added, 5 min incubation.

3 & 4. 20 µg/mg trypsin added, 5 min incubation.

5 & 6. Control, no trypsin added, 5 min incubation.


1
2
3
4
5
6

resulted in collapse of the gel to a material having both a gelled consistency and fibrous nature. When treated with 0.6 M KCl, the centrifuged gel became more fibrous but was easily broken into fragments by homogenization. Centrifugation and dialysis of this material against a low ionic strength (0.05 M KCl) buffer yielded a fraction that was depleted in both actin and myosin but enriched in ABP. The ABP was then purified further as outlined in Chapter II and in Table XV.

Proteolysis

Since proteolysis of SA 180 membranes caused fragmentation and depletion of the high molecular weight proteins but seemed to leave the lower molecular weight proteins intact, this technique was used on the high speed supernatant to see if proteolysis of ABP could be correlated with prevention of gel formation. Unexpectedly, the material gelled more quickly than controls by a factor of 8 to 10. Analysis by SDS PAGE suggested that both ABP and myosin were cleaved prior to the onset of gelation, i.e. these proteins were depleted by 2 min of proteolysis at a concentration of 5-20 μg per mg protein (Table XVI and Figure 23). Gelation occurred between 4 to 7 min for these samples in contrast to controls which usually gelled in 35 min. These results are consistent with those of Pollard (44) in that intact ABP did not seem to be required for the phenomenon as well as those of Maruta and Korn (45) who had obtained evidence that four low molecular weight proteins of 20-30,000 daltons produced most of the gelation of whole cell extracts. Proteolysis of ABP could therefore produce fragments capable of cross-linking actin filaments into the

TABLE XV

FLOW DIAGRAM OF ABP ISOLATION

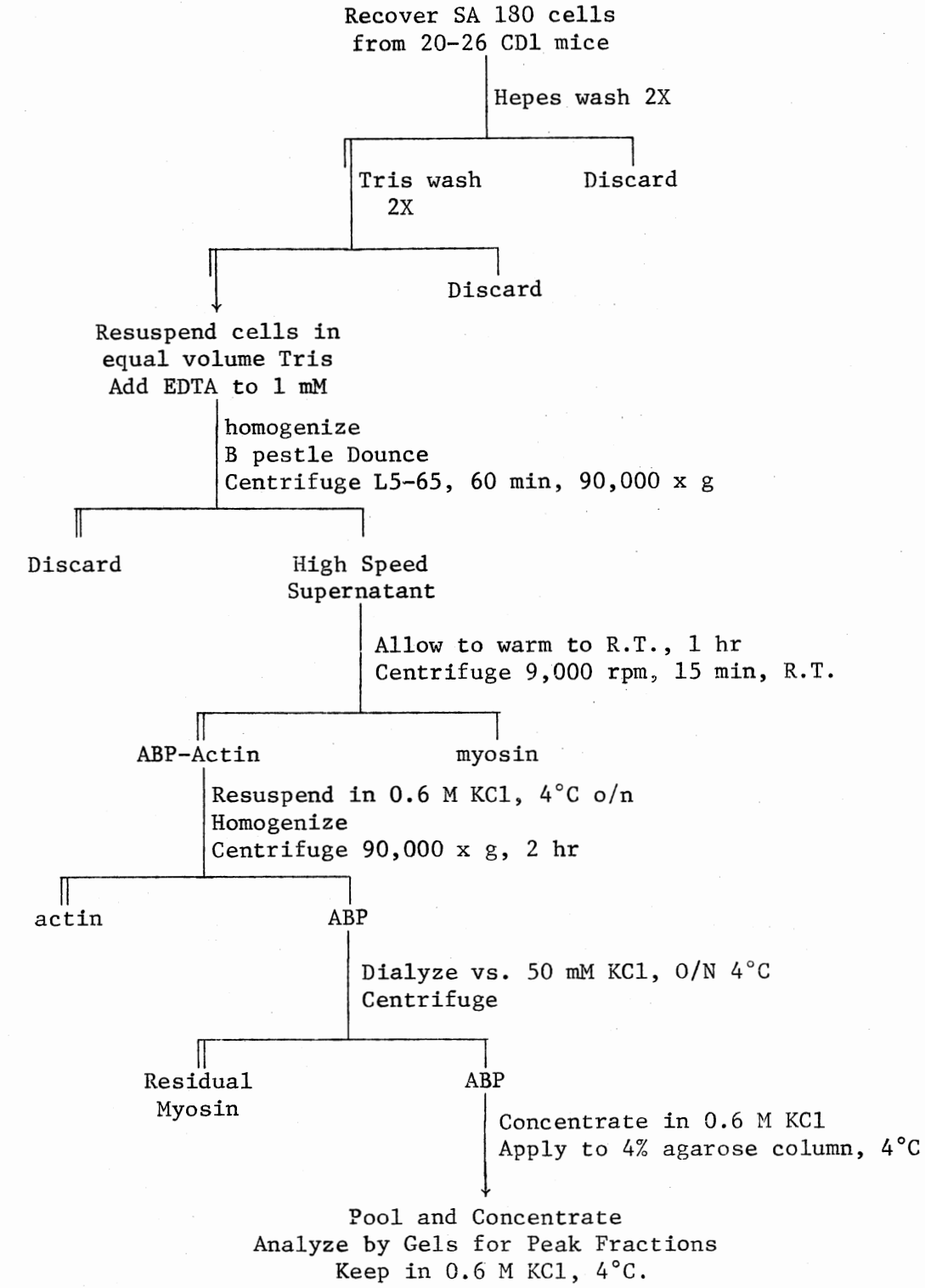


TABLE XVI

PROTEOLYTIC ENHANCEMENT OF GELATION OF SARCOMA 180 CYTOPLASMIC EXTRACTS

Time min	Control	Trypsin 5 µg/mg	Trypsin 20 µg/mg	Papain 10 µg/mg	Cyto B 20 µg/mg
5	-	+	+	+	-
10	-	+	+	+	-
15	-	+	-	-	-
20	-	+	-	-	-
25	-	+	-	-	-
30	-	+	-	-	-
35	+	+	-	-	-
40	+	+	-	-	-
45	+	+	-	-	-
50	+	+	-	-	-
60	+	+	-	-	-

Figure 23. Proteolysis of SA 180 Extract.

1) 2 min control, no trypsin added.

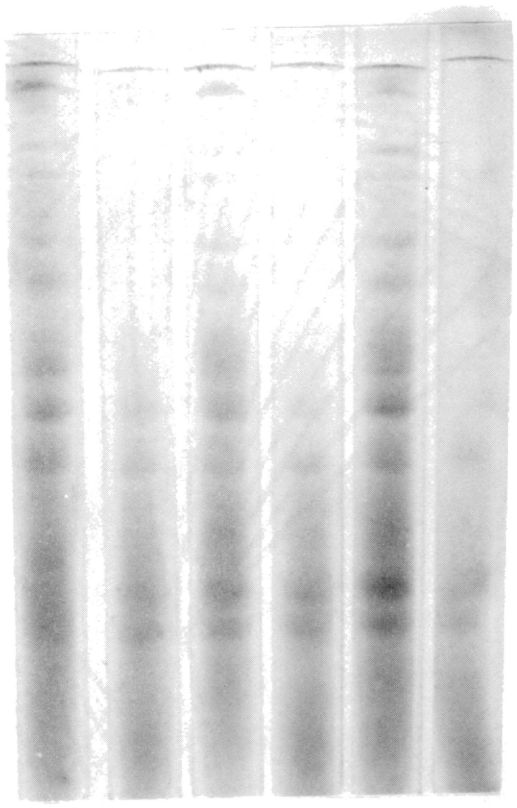
2) 2 min, 5 µg/mg trypsin.

3) 4 min control.

4) 4 min trypsin.

5) 40 min control.

6) 40 min trypsin.


1
2
3
4
5
6

gel matrix. These results also suggested that intact ABP could function as an inhibitor of actin filament formation.

The identical experiment was performed with two rat mammary adenocarcinoma cell lines used in this laboratory (56). One of these lines has an extensive cell surface carbohydrate complex and does not cap when treated with Con A whereas the other line does cap and does not have this surface complex (95). The results showed that the former cell type (C1) underwent gelation similar to the SA 180 cells whereas the latter (B1) gelled only with extended incubation times or not at all (Table XVII). Quantitation by densitometry from SDS PAGE gel scans showed that a protein of molecular weight similar to ABP was incompletely proteolyzed in the B1 cells (Figure 24). One can isolate a protein by the Hartwig-Stossel technique, presumably ABP, from both these cell types. Since the ascites cells are treated with Tris prior to homogenization, levels of erythrocytes and/or contaminating spectrin should be low. The results support the idea that intact ABP initially acts as an inhibitor of actin polymerization.

Gelation as a Function of ABP/Actin Ratio

Additional support for this concept was obtained by extended centrifugation of the high speed supernatant at various speeds. Table XVIII shows there was a marked change in the ABP to actin ratio which correlated with the ease of gel formation and/or the extent of gelation, i.e. whether the gel would hold tightly to the container upon gelation or was easily disrupted by inversion of the container.

TABLE XVII

GELATION OF CYTOPLASMIC EXTRACTS OF MAT-B1 AND MAT-C1 ASCITES CELLS

Cell	Time	5 µg/mg Trypsin	Gelation
MAT-B1	6	-	-
		+	-
	10	-	-
		+	-
	30	-	-
		+	-
	45	-	+
		+	-
	60	-	-
		+	-
MAT-C1	6	-	-
		+	+
	10	-	-
		+	+
	30	-	+
		+	+
	45	-	+
		+	+
	60	-	+
		+	+

Figure 24. Proteolysis of MAT-B1 and MAT-C1 Extracts.

The numeral refers to the time of reaction.

c indicates no enzyme was added.

t indicates 5 µgm/mg protein was added at 0 min.

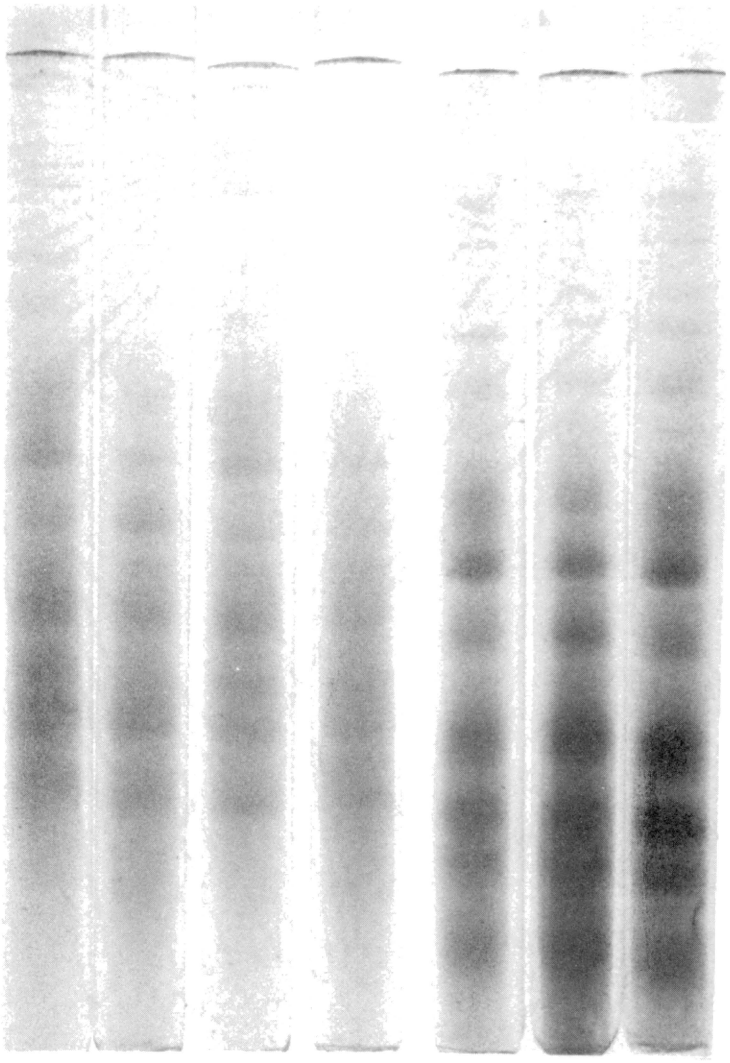

4c
4t
8c
8t
1c
6t
6c
MAT C1
MAT B1

TABLE XVIII

RATIO OF ABP TO ACTIN AND GEL FORMATION

RPM	Time (min)	ABP/A	Minimum Gelation Time
15,000	50	6.26	+ (20 min)
15,000	50	7.82	+ (25 min)
26,000	60	5.59	+ (30 min)
26,000	60	10.57	+ (35 min)
26,000	60	13.08	+ (35 min)
26,000	180	11.67	+ (35 min)
26,000	240	12.17	+ (40 min)
26,000	240	14.98	+ (70 min)
26,000	420	18.17	- (70 min)

Effects of Perturbants

The drug cytochalasin B has been reported to inhibit gelation (96). This result was also obtained with the ascites cells and could result if the drug acts either by stabilizing the ABP inhibition or preventing myosin release from actin lengths such that polymerization of actin filaments or cross-linking into the gel matrix is not allowed.

It has also been noted that the phenomenon does not occur at temperatures of 4°C. It was of interest to see if trypsin could overcome this temperature dependence. Trypsin treated samples held at 4°C gelled in 6-8 minutes while controls did not gel over at least a 6 hour period. Neither was a gel observed after leaving the controls overnight at 4°C. Samples held at 37°C gelled at an accelerated rate, usually in 10-12 min. A possibility for the temperature dependence is a necessary conformational change or phosphorylation-dephosphorylation that could allow correct protein interactions to occur for either filament formation or cross-linking.

Experiments designed to determine if such a possibility existed in this system used several enzyme inhibitors (trasylol, IAA, NEM, and PMSF). Both trasylol and NEM enhanced of gelation while IAA and PMSF did not. Carraway and Shin (97) have shown that the sulfhydryl reagents NEM and IAA may have very different specificities of reaction under identical experimental conditions. Table XIX shows that the NEM effect could possibly be related to protein levels within the high speed supernatant. Alternatively, it could be inhibiting a required enzymatic step such as a kinase or phosphatase to prevent myosin inhibition of gelation. Studies are planned to determine if such a

TABLE XIX

EFFECT OF PROTEIN MODIFYING REAGENTS ON GELATION

Reagent	Sample Gelation Time min	Control Gelation Time min	High Speed Extract Concentration Protein mg/ml
PMSF	35	35	17.0
IAA	45	45	24.4
	35	35	19.4
NEM	5	40	24.4
	5	35	19.4
	5	20	24.4
	13	45	24.4
	20	60	16.6
	30	30	19.6
	45	45	13.6

regulatory event is important for gelation.

Another regulatory mechanism could be oxidation-reduction of sulfhydryls. Gelation occurs with or without added β-mercaptoethanol. An additional experiment would be to add the sulfhydryl oxidant diamide for possible effect. However, the data so far indicate a phosphorylation-dephosphorylation is the more likely of the two mechanisms.

Mechanism of Gelation

The gelation process is probably at least a two step procedure. Kane (40) showed that addition of G-actin in sea urchin egg homogenates did not enhance gelation at room temperature whereas addition of F-actin caused immediate gel formation. This suggests the cold sensitive step could be either formation of nucleation centers or polymerization of G-actin onto pre-formed nuclei which are blocked unless the temperature is raised.

Experiments were therefore designed to test which possibility was more likely. Extracts were passed through several disc filters of decreasing pore size and tested for gelability. Filters of 8 μ to 0.22 μ sizes did not diminish the gelability of the extracts although the protein concentration was decreased at each step (Table XX). The extract also became somewhat clarified by passage through the filters, especially 0.45, 0.22 and 0.1 μ sizes, most likely due to loss of lipid vesicles. Filters of 0.1 μ and below (Amicon XM 300 and XM 50 or 50-300 Å) apparently depleted required material since these filtrates would no longer gel. SDS PAGE analysis (Figure 25) did not show depletion of any protein in particular with the possible

TABLE XX

GELATION OF SARCOMA 180 CELL HOMOGENATES

Sample	Protein Concentration mg/ml	Gelation
100,000 x g supernatant	17.0	+
	19.3	+
7 μ filtrate	ND	+
3 μ filtrate	ND	+
1 μ filtrate	16.8	+
	18.7	+
0.45 μ filtrate	16.3	+
0.22 μ filtrate	13.0	+
	11.6	+
0.1 μ filtrate	12.7	-
XM300 filtrate	ND	-
XM50	ND	-

ND = not determined.

Figure 25. SDS PAGE Analysis of Filtered SA 180 Extract.

1) High speed extract.

2) 7 μ filtrate.

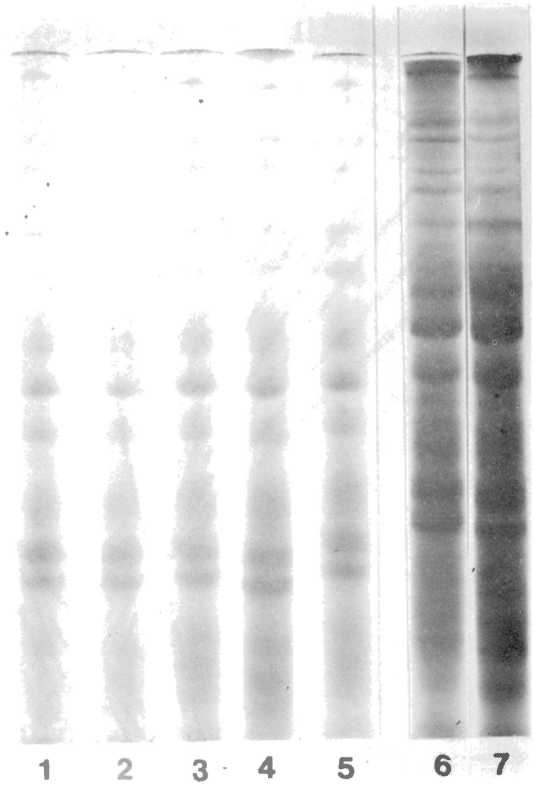
3) 3 μ filtrate.

4) 1.2 μ filtrate.

5) 0.5 μ filtrate.

6) XM-300 filtrate.

7) XM-50 filtrate.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6
7

exception of actin or other low molecular weight factor. Protein concentrations should have been sufficient to allow gelation since unfiltered cell extracts of identical concentration still undergo gel formation.

The most likely explanation for inability of the 0.1 μ or smaller filtrates to gel was the loss of vesicles containing actin nucleation centers. This was investigated by several experimental approaches.

The high speed extract obtained by 1 hour centrifugation at 26,000 rpm was fractionated both prior to warming to room temperature and after recovery of the "gel supernatant" following removal of the collapsed gel. Both were analyzed by sucrose gradient centrifugation (Figure 26) and elution on 4% agarose columns (Figure 27).

Due to the changing sucrose concentration across the gradients, the proteins did not migrate consistently unless the sucrose was reduced by dialysis. Protease inhibitors were incorporated into the dialysis buffer to prevent protein loss over the two day dialysis period. The actin appeared in several fractions, but most migrated suggestive of either a monomeric state or complexed with several other proteins in lipid vesicles (Figure 28). The actin-binding protein, however, was essentially only found in fractions containing actin complexed with vesicles. The ABP content appeared to be depleted upon gel formation, although this was not conclusive.

Likewise, SDS PAGE analysis of these same extract and supernatant samples by 4% agarose chromatography (Figure 29) showed that before gelation essentially all of the ABP was eluted at the void volume along with part of the actin. The supernatant remaining after gelation, however, showed loss of ABP and conversion of most of the monomeric

Figure 26. Sucrose Gradient Fractionation of SA 180 Extracts.

Upper. Before gelation.

Lower. After gelation.

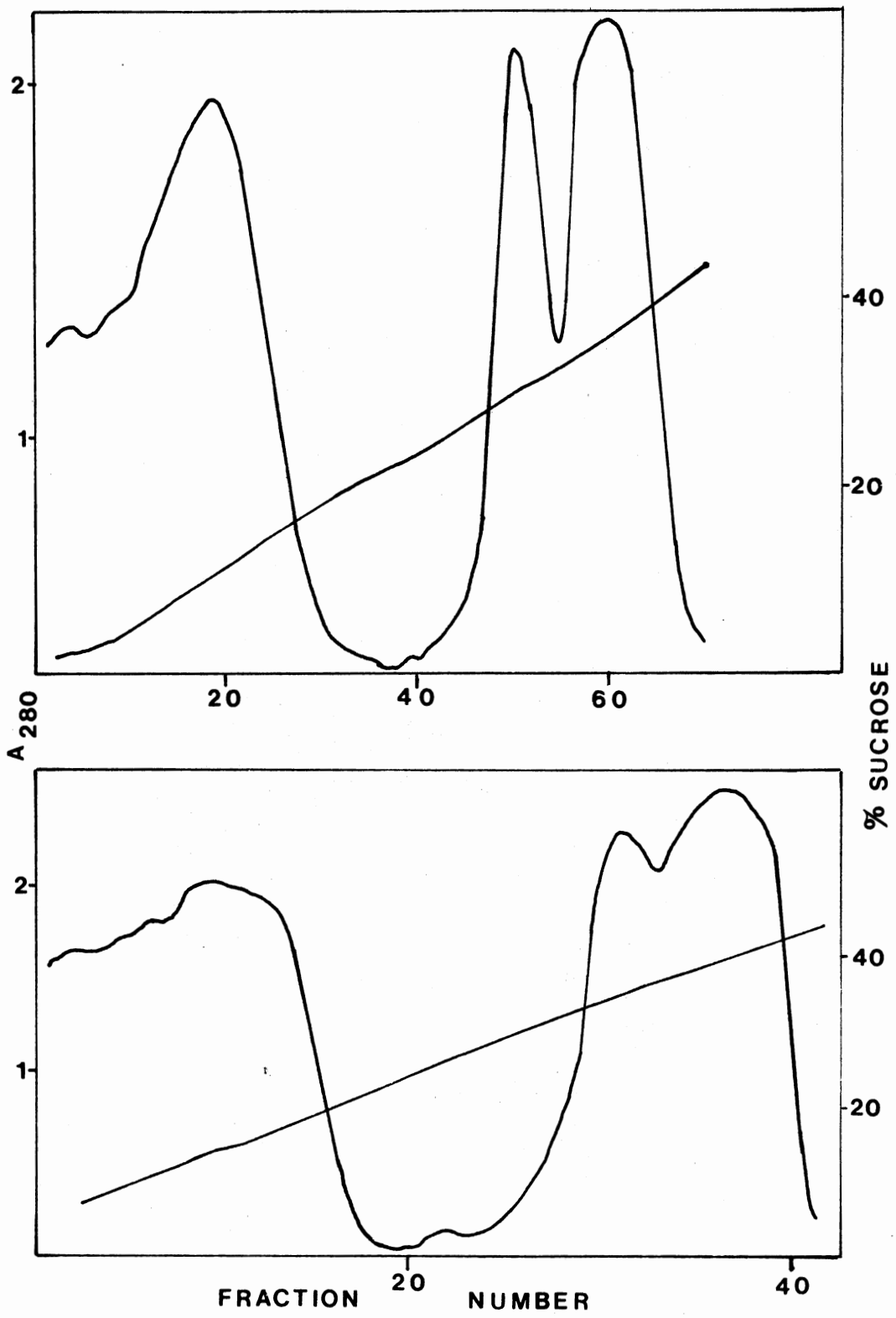

2
1
20
40
60
A_{280}
40
20
% SUCROSE
2
1
20
40
FRACTION NUMBER
40
20

Figure 27. Bio-Gel A 5 M Fractionation of SA 180 Extracts.

Upper. Before gelation.

Lower. After gelation.

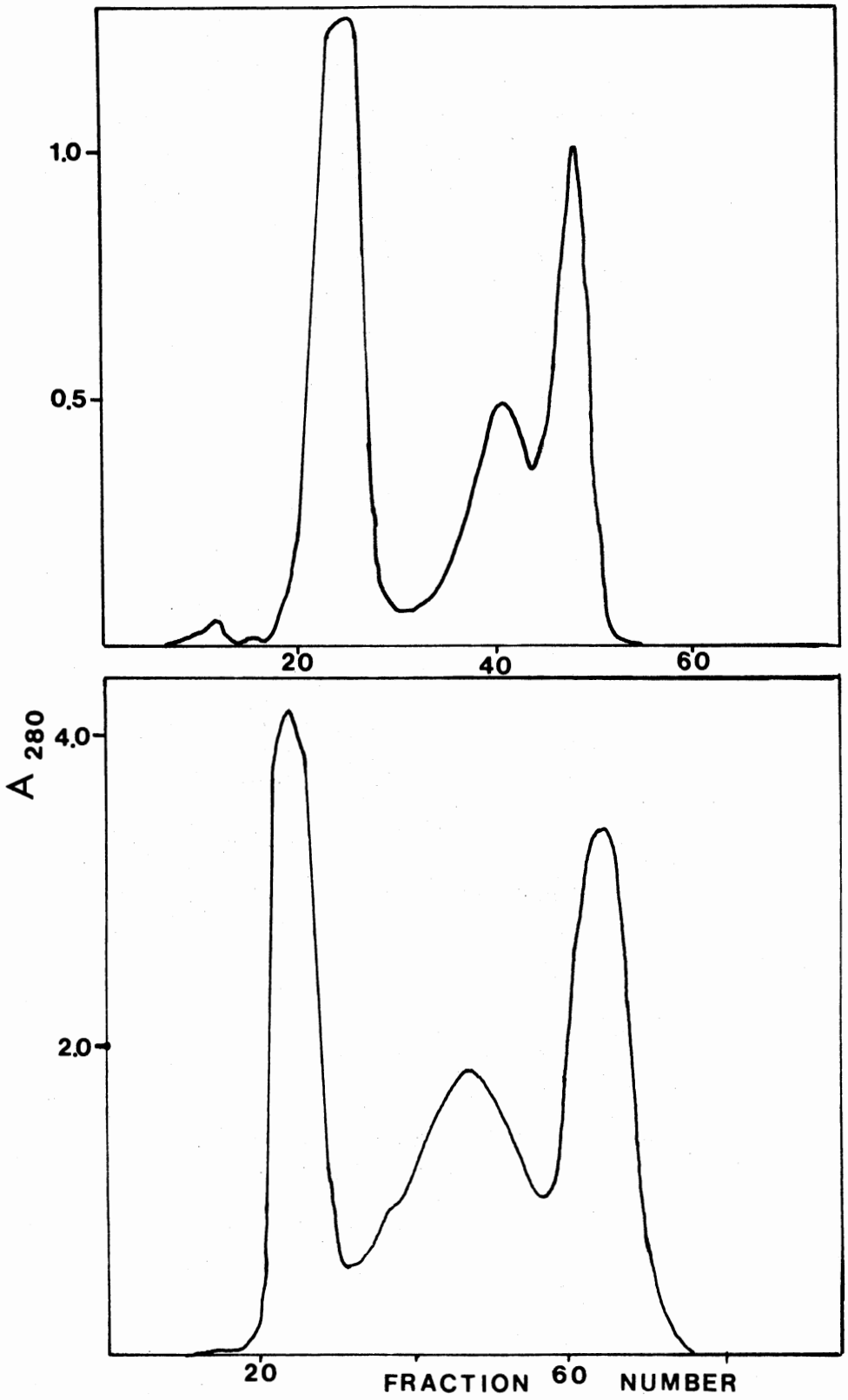

1.0
0.5
20
40
60
A 280
4.0
2.0
20
FRACTION 60 NUMBER

Figure 28. Sucrose Density Gradient Fractionation of SA 180 Extracts.

Left. Before gelation supernatant.
1) 6% sucrose, 2) 7%, 3) 12%, 4) 15%, 5) 31%,
6) 33%, 7) 38%.

Right. After gelation supernatant.
1) 3%, 2) 7%, 3) 10%, 4) 14%, 5) 31%, 6) 35%,
7) 39%.

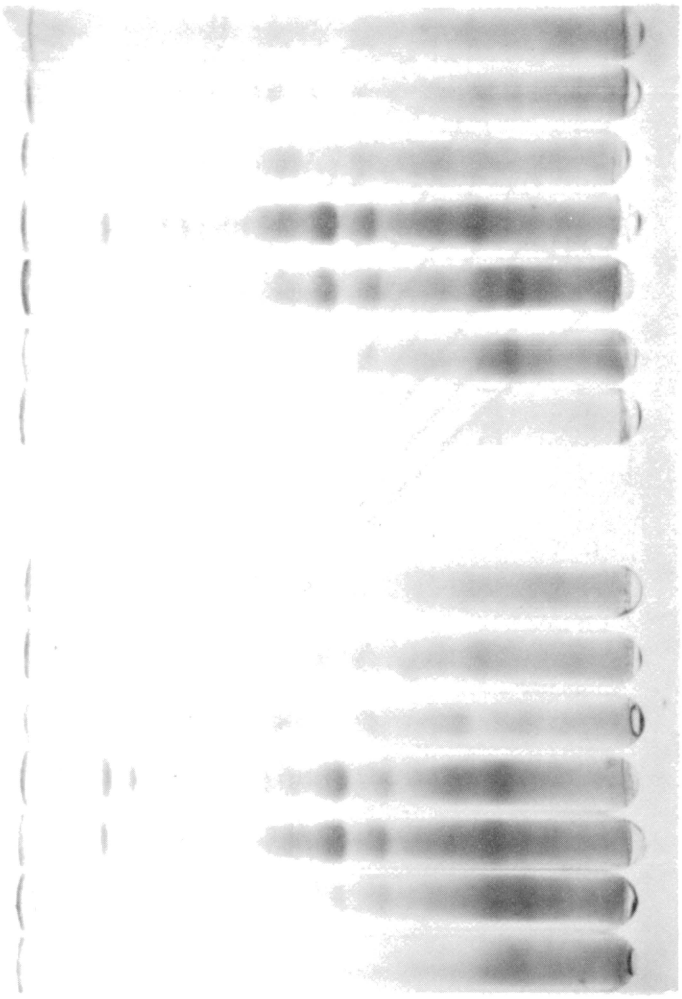
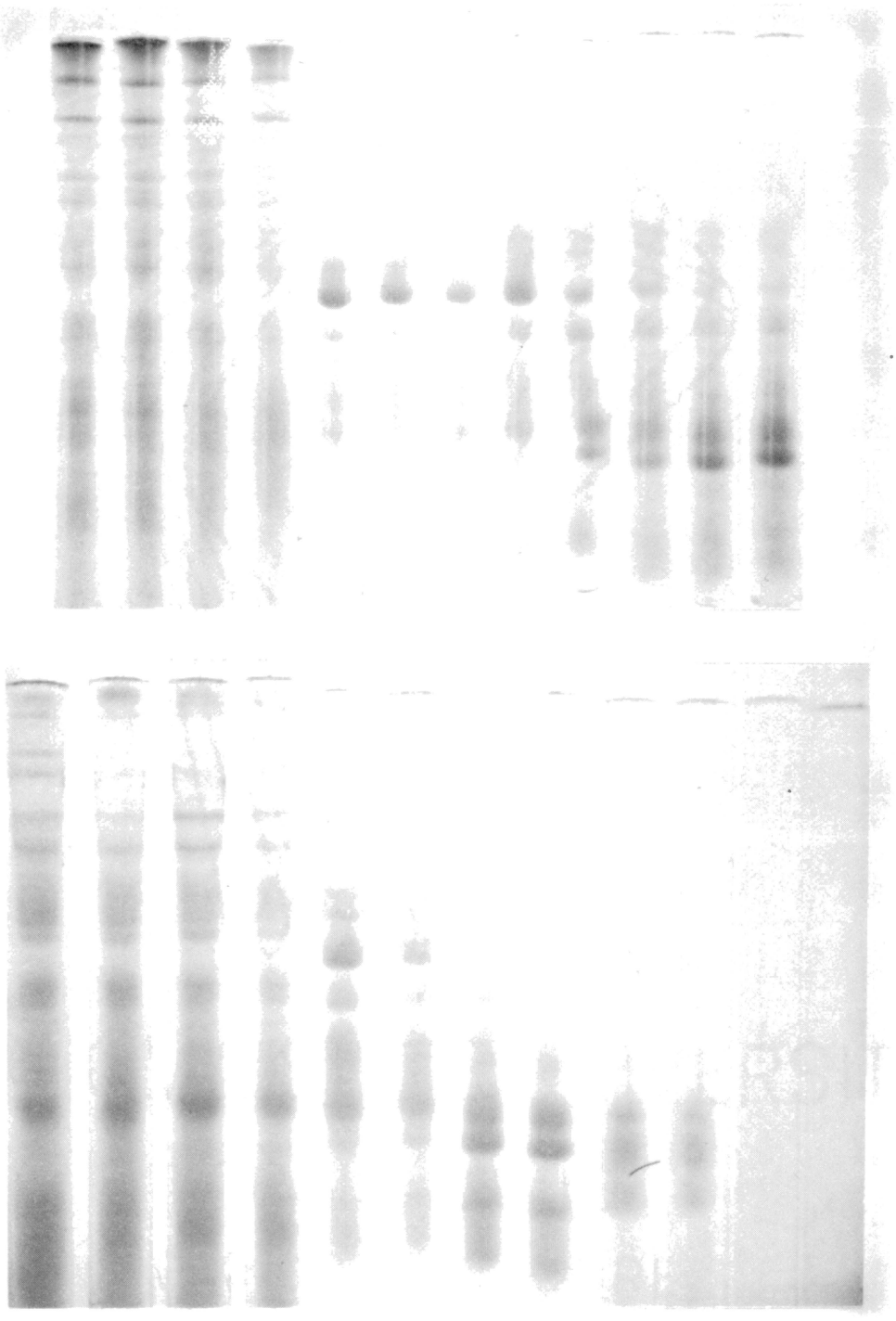


Figure 29. SDS PAGE of Bio-Gel A 5 M Fractions of SA 180 Extracts.

Upper. Before gelation column. Fractions 18, 19, 23, 24, 31, 32, 36, 37, 41, 42, 45, 46.

Lower. After gelation column. Fractions 22, 23, 25, 26, 43, 44, 50, 51, 61, 62, 65, 66.



actin to a size eluting at the void volume, probably indicating conversion to F-actin.

Table XXI lists results of vesicle analysis for the fractions analyzed by SDS PAGE. By comparison with the gradient and column profiles it can be seen that fractions at low sucrose content contained vesicles. Likewise, material eluting near the void volume contained the bulk of the lipid. The membrane marker enzyme, Na^+-K^+ ATPase, was eluted in fractions consistent with the idea that both ABP and part of the actin are likely complexed with vesicles.

In Table XXII, data is presented in which it can be seen that the relative concentration of ABP is increased in the gelatin pellet, myosin is increased in the supernatant while α-actinin and actin are approximately evenly distributed. It is possible that 1) considerable quantities of soluble proteins become trapped within the gel matrix during centrifugation or alternatively, 2) the gel is not completely stable to the force applied and it is partly broken up by the sedimentation conditions used. If one centrifuges the gel at 3500 rpm, the gel barely collapses. However, if one sediments at 12,000 rpm, the gel is usually completely collapsed to a fibrous-like meshwork material. At intermediate speeds, both gel and fibers appear in the pellet. Therefore, the second possibility most likely occurs to some extent. To avoid this as well as to wash the gel free of trapped proteins it is hoped a method can be devised to allow gelation to occur over a layer of sucrose or other dense material and through which the gel can be sedimented. As yet, attempts have not succeeded.

Kane (40) has suggested that the required first step for gelation is a G → F actin conversion. The data just presented are consistent

TABLE XXI

FRACTIONATION OF VESICLES FROM SA 180 EXTRACTS

Treatment	Fraction	Na^+-K^+ ATPase µg Pi/min/mg	Lipid P µg/mg
Sucrose	5% sucrose	4.5	n.d.
Gradient	8	1.4	2.4
Before	10	0.7	0.6
Gelation	33	n.d.	n.d.
	38	n.d.	n.d.
Sucrose	10% sucrose	0.9	2.1
Gradient	13	0.2	0.5
After	27	n.d.	n.d.
Gelation	33	n.d.	n.d.
Bio-Gel A 5 M	5	0.2	11.9
Chromatography	19	0.02	2.1
Before	24	0.31	n.d.
Gelation	32	0.04	n.d.
	37	n.d.	n.d.
	42	n.d.	n.d.
	46	n.d.	n.d.
Bio-Gel A 5 M	22	0.16	2.2
Chromatography	25	0.07	1.2
After	43	0.28	n.d.
Gelation	50	0.12	n.d.
	61	n.d.	n.d.
	65	n.d.	n.d.

n.d. = none detected.

TABLE XXII

PERCENT PROTEIN IN GELATION SUPERNATANTS + PELLETS

Sample	Protein	% of Total
Supernatant	ABP	0.93
		0.48
		0.79
	myosin	0.91
		1.16
		1.35
	α-actinin	4.93
		3.98
		1.83
	actin	15.15
		15.06
		12.78
Pellet	ABP	2.27
		1.87
		2.32
	myosin	0.39
		0.43
		0.38
	α-actinin	3.51
		4.03
		1.60
	actin	14.32
		10.65
		12.64

Data points represent three separate experiments.

with this mechanism as well as one in which ABP must release from the "vesicle complex" in order to allow polymerization to occur.

In addition to the chromatography results, data in support of polymerization occurring for gel formation was obtained by labelling the ascites cells in situ with ^{35}S-methionine injection into the peritoneal cavity of a mouse containing ascites cells near plateau growth (7 days after injection of tumor cells). Cells were recovered after 2 hours and washed and homogenized as usual. The high speed supernatant was obtained and fractionated on a 4% agarose column. Material eluting in the region of monomeric actin was pooled and concentrated for the experiment.

The labelled actin was added to the gelation mixture and allowed to gel. Specific activities in the supernatant and pellet fractions from the centrifuged gel showed an increase of labelled material in the pellet (Table XXIII). If the sample was allowed to proteolyze with added trypsin, again the specific activity was higher in the pellet. KCl was then added to the ^{35}S-actin and it was allowed to polymerize. Addition of this material showed levels of label approximately equal in both fractions.

These results are consistent with a two step reaction in which polymerization preceeds cross-linking and F-actin becomes bound into a sedimentable form.

Addition of a crude gizzard α-actinin preparation enhanced gelation. This was the anticipated result due to the known ability of α-actinin to act as an actin filament organizing center. Again, no enhancement was observed at 4°C.

The preliminary results described above are consistent with a

TABLE XXIII

^{35}S SPECIFIC ACTIVITIES IN SA 180 FRACTIONS

Fraction	cpm/mg
Control	
Supernatant	0.68
Pellet	0.96
KCl treated actin	
Supernatant	0.29
Pellet	0.32
Trypsin treated extract	
Supernatant	0.56
Pellet	0.73

with a required de-binding of ABP and/or filament formation by polymerization of monomeric actin at pre-existing nucleation centers being a necessary step prior to cross-linking into a matrix.

CHAPTER V

PLATELETS

Results

Platelets are a specialized cell type in that they are derived from megakaryocytes as a terminal blood cell. At vessel damage sites, these cells undergo a sequence of events including aggregation, pseudopod formation and contraction to form a net-like plug. They contain large quantities of the contractile proteins studied in the sarcoma cell, hence they are a reasonable auxiliary system for research. In addition, since they undergo a contractile event for retraction of the plug matrix, they can give some insights into the actual role of ABP in the contractile system and its interaction with the other proteins — in particular, actin.

The majority of the actual experiments were concerned with isolation and protocol changes to give working platelet solutions with a minimum of stress on the cells.

A swinging bucket rotor was used exclusively for all centrifugations. The cells were subjected to less stress by drag against the tube walls by use of an HB-4 rotor. When removing the PRP, care was taken not to suction close to the lower red blood cell layer. One wash with Barber-Jamieson (63) buffer was assumed sufficient to allow 75-90% recovery of platelets while minimizing erythrocyte contamination.

Centrifugation speeds were then adjusted to enable a minimal number of sedimentations to recover a pure platelet fraction. An initial centrifugation at 1350 rpm pelleted most of the red blood cells and was not repeated unless absolutely necessary. In most cases, any coloration of the platelets appeared to be adsorbed hemoglobin and not due to intact erythrocytes via phase microscopy inspection. A second spin at 1750 rpm sedimented the platelets. They were washed once only with Barber-Jamieson buffer and resuspended in the same buffer for use. Resuspension was by swirling gently over several minutes. Any material that appeared to remain clumped was discarded by decanting the resuspended platelets away. All manipulations were done at 20-25°C including all centrifugations. Only plastic-ware came in contact with the platelets during preparation. Microscope slides and cover slips were siliconized to minimize aggregation while observing via phase microscopy.

Several attempts were made to treat platelets with Triton or Triton + $CaCl_2$ and ADP to cause aggregation. SDS PAGE gels showed that the two media were causing the same effect, i.e. Triton itself appeared to be allowing release of Ca^{++}-ADP from the granules which in turn was allowing aggregation to occur. This was confirmed by an experiment comparing no Triton treatment with Triton treatment in various media. Citrate buffers were apparently able to chelate the bulk of the Ca^{++} since only moderate aggregation occurred with or without Triton. $ZnCl_2$ was able to stabilize the contractile proteins so that they were not rendered soluble (Figure 30). Barber-Jamieson buffer served as a control media. Without Triton the cells remained as slightly elipsoid single discs. When treated with Triton, the

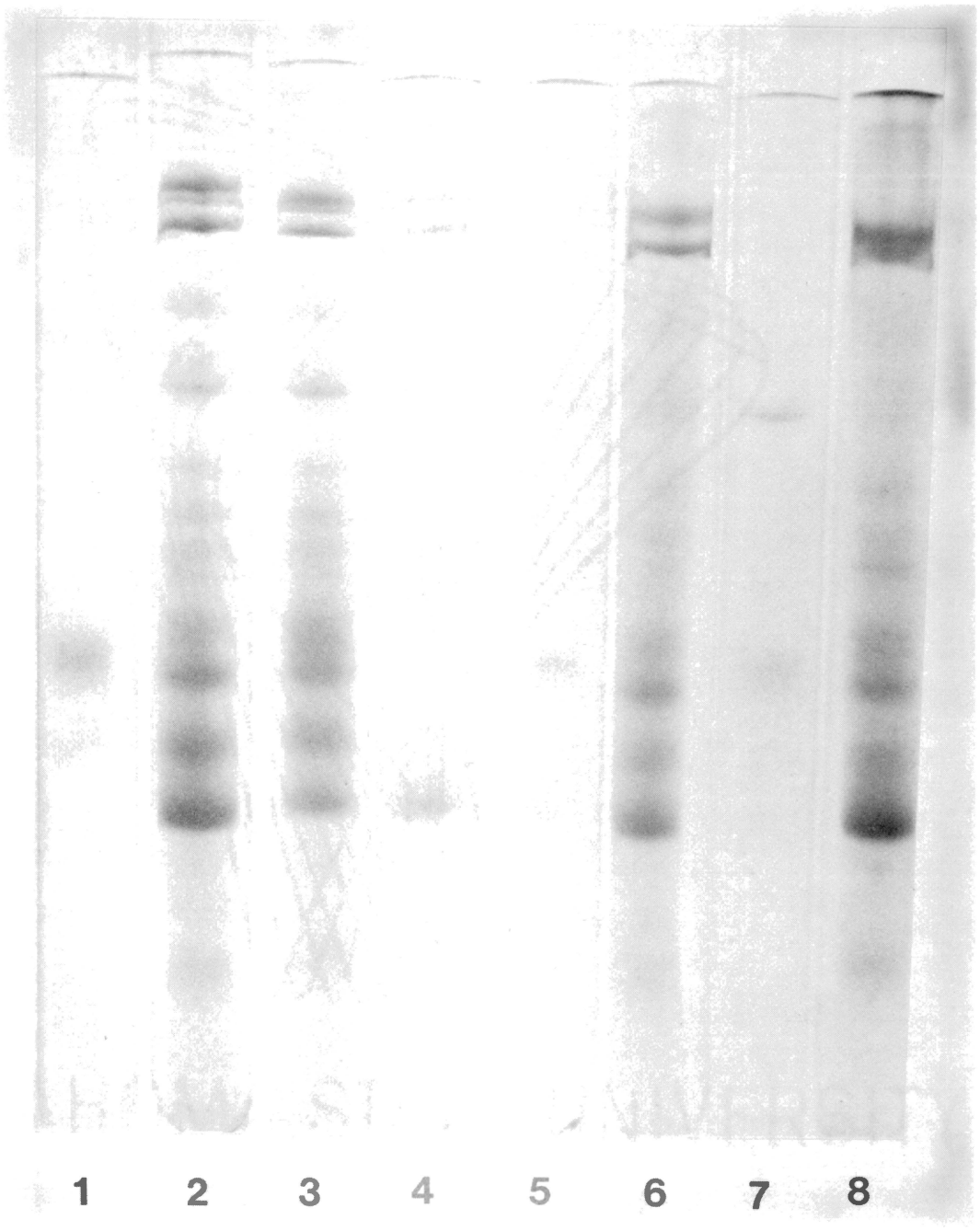
Figure 30. SDS PAGE of Platelets.

1 + 2. B.J. buffer, supernatant and pellet.

3 + 4. B.J. buffer + Triton, supernatant and pellet.

5 + 6. $ZnCl_2$ in HOH, supernatant and pellet.

7 + 8. $ZnCl_2$ in HOH + Triton, supernatant and pellet.


1
2
3
4
5
6
7
8

cells aggregated and most of the proteins were released into the media (also Figure 30).

The refractive index of platelets treated with Triton changed considerably within a few minutes. The solution cleared completely as material composed principally of actin, myosin and ABP precipitated from the sample.

Analysis of various perturbants on aggregation and secretion by platelets with both SDS PAGE and SEM and TEM for correlation of morphological changes to loss of cytoskeletal components is being studied.

CHAPTER VI

DISCUSSION

The results from studies on cytoskeletal protein associations with membranes as well as experiments designed to probe the biochemical basis of gelation were combined into a proposed model of the structure of the submembrane cytoskeletal complex (Figure 31). The model differs from that presented in the literature by Stossel and Hartwig (99) in the placement of ABP and stresses the pre-existence of short oligomeric lengths of actin which can function as polymerization nucleation centers.

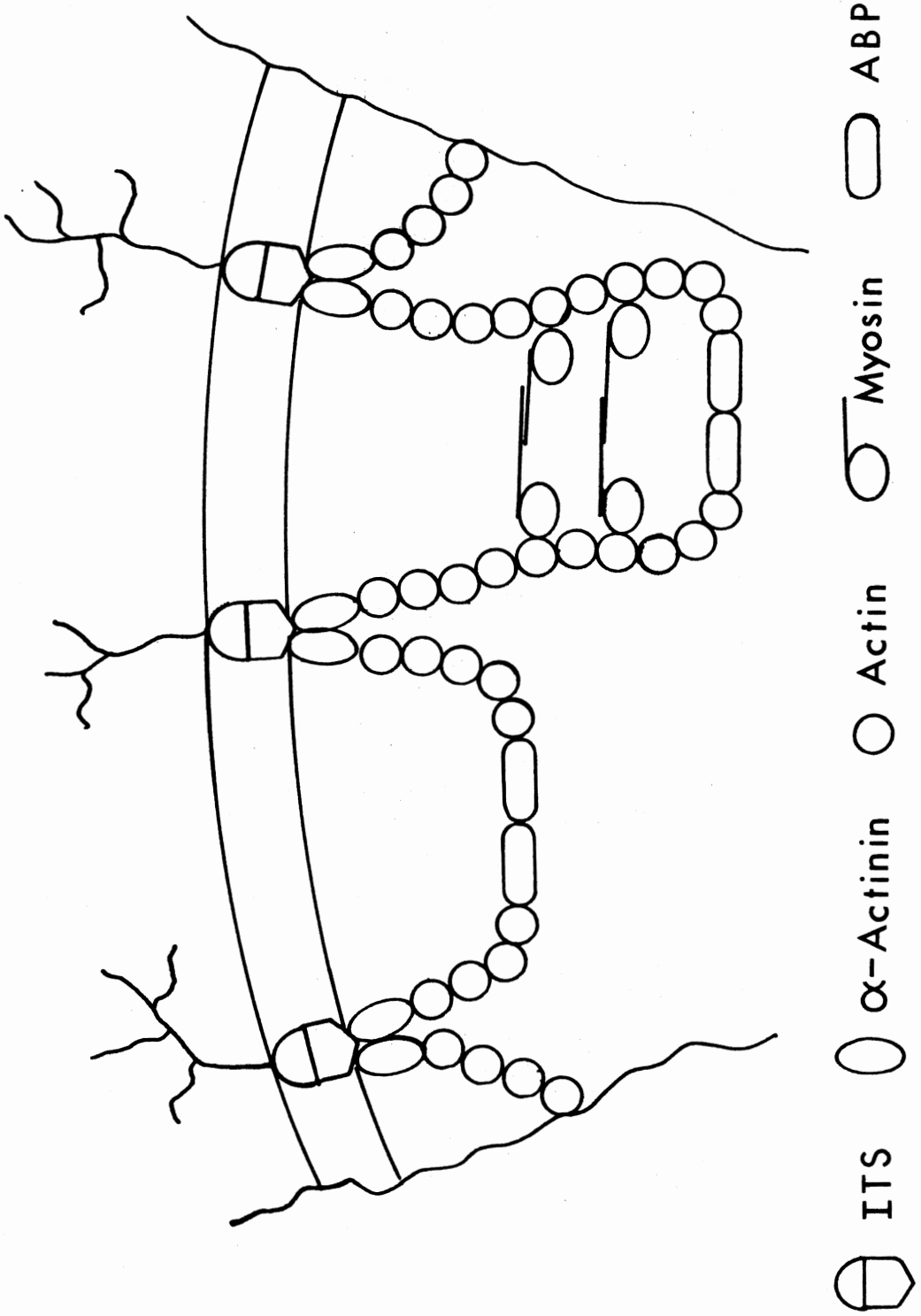
Mooseker and Tilney (84) have suggested that α-actinin located at the tips of intestinal brush-border microvilli may function in an analogous manner to the Z lines of striated muscle which are rich in α-actinin.

In both systems, the actin filaments bind HMM in such a way that the arrowhead arrangement points away from the α-actinin containing region. Some criticism for HMM binding studies has been discussed in the literature since it can induce F-actin formation (presumably with a preferred orientation of the actin monomers).

Therefore, it is imperative that the actin filaments be shown to "pre-exist" in the system under study by other means. Tropomyosin binding has also been shown to cause actin polymerization (100) and so its use is subject to the same considerations. The use of actin

Figure 31. Cytoskeletal-Membrane Interactions in Ascites Tumor Cells.

The figure shows a section of the plasma membrane with cell surface glycoproteins extending outward into the medium and the cytoskeletal complex extending inward into the cytoplasm.


ITS
α-Actinin
Actin
Myosin
ABP

antibody binding requires chemical modification (such as peroxidase coupling) for visualization. If phalloidin (binds only F-actin) can be shown not to cause polymerization it could be an alternative filament probe.

Nonetheless, the idea that α-actinin could cause F-actin orientation and/or polymerization by providing nucleation centers is generally accepted (101). In striated muscle α-actinin embeds one end of the thin filaments to the Z line, hence its placement at the membrane side of the actin oligomeric lengths is justified.

In the gelation experiments α-actinin caused an enhancement if added at room temperature but not if done at 4°C. Likewise it was susceptible to proteolysis only if dissociated from the membranes by GEM, pH 9.5 buffer. Both are consistent with α-actinin being subtended by other proteins and under the lowered temperature conditions being unable to act in its role of inducer of F-actin polymerization due to blockage of this polymerization step.

In the model the key protein which fulfills this "blocking" action is ABP. In gelation experiments, proteolysis of the high molecular weight proteins, including ABP have been shown to enhance gel formation. Incomplete cleavage of ABP (MAT B1 data) blocks gelation. SDS PAGE analysis of the proteins contained in the high speed supernatants of SA 180 cells before and after gelation are consistent with conversion of monomeric actin to an excludable size on 4% agarose columns. That this fraction also shows a net loss of ABP and contains lipid is not conclusive proof, however, that ABP has been released to allow addition of monomeric actin. Likewise, there has been no conclusive proof from other laboratories that ABP is truely bound to actin in

the gel, especially since the conditions used may cause aggregation of ABP. Techniques are needed to isolate the gel matrix which preclude loss due to fracture during centrifugation or trapping of other proteins.

In one attempt to resuspend the gel in 40 mM Tris and fractionate it on a 4% agarose column, the material, for the most part, remained aggregated and did not enter the agarose matrix. The material observed to elute could represent material trapped in the gel matrix only since the protein content was markedly depleted (data not shown).

Lipid analysis was only performed on the material extruded from the gel during collapse by centrifugation. However, there is electron microscopic evidence in the literature that the gel itself is composed of both vesicles and criss-crossed filaments. It is not possible to say for certain whether the filaments originate at vesicle regions or whether the vesicles are merely trapped within a disordered mesh of filaments (99, 103).

Precedence for high molecular weight proteins which can sequester actin in a "storage form" has been provided by Tilney in Thyone sperm (102). He suggested these proteins were related to spectrin of erythrocytes, however. Electron micrographs of red blood cells before and after trypsin treatment show that filaments (presumed to be actin) can be seen in the cell after trypsin treatment but not before (102). In view of the trypsin data with the sarcoma proteins it is possible that the trypsin treatment causes a membrane perturbation (e.g. ion flux) sufficient to release a possible blockage of polymerization.

Kane (40) concluded the cold sensitive step in the gelation process was actin polymerization based on addition experiments. No

data was given, however, on relative protein concentrations and interpretation as to amounts added in excess of endogenous actin or ABP is difficult. The data does not preclude a step prior to polymerization (i.e. unblocking of nucleation centers) as being necessary for G → F actin conversion.

The ITS protein (Integral Transmembrane Site protein) focuses on the demonstrable ability of cell surface perturbants to cause changes in the cytoskeletal proteins. ITS protein is visualized as a multisubunit protein to allow coupling or uncoupling of the cytoskeleton to external events via conformational changes. Ash and Singer (104) have used FITC-Con A cell surface receptor binding coupled with simultaneous rhodamine antimyosin binding to intracellular myosin to demonstrate that cell surface Con A receptors were superimposable on linear arrays of internal myosin and these arrays were a reorganization of diffuse myosin staining apparently induced by the receptor binding.

The model is consistant with available evidence suggesting actin stress fibers do not exist in rounded cells. A reorientation or de-binding of ABP molecules could allow further polymerization to give the characteristic stress fibers observed in spread cells. Release of ABP could also influence the ability of myosin to bind the actin filaments and they could be mutually antagonistic. Under conditions where myosin can compete favorably with the gelation factor(s) for actin filaments, contraction could result (syneresis) with loss in ability to form a gel (44, 61).

The several proteins considered to be cytoskeletal components have been shown to isolate with plasma membranes under conditions of stabilization and are releasable as a group in essentially constant

proportions to each other. Cross-linking studies support the concept they comprise a complex since they are extensively linked to each other above a low threshold concentration of cross-linking reagent. Attempts to generate selectivity of reaction by lowering reagent concentrations or decreasing the length between reactive sites on the linker agent were generally unsuccessful. Labelling the cell surface with ^{125}I before isolation of membranes showed none of these proteins were labelled and therefore presumed to be unavailable for reaction. If membranes were prepared and then labelled, they all showed incorporation of radioactivity, consistent with the idea they reside on the cytoplasmic side of the membrane. One attempt was made to isolate membranes from ^{125}I labelled cells followed by cross-linking. Label did appear in the material not able to enter a 3% gel but could have been generated by cross-linking at the outer surface alone. Less extensive cross-linking is required for further experiments to determine if a transmembrane connection exists. An alternate experiment could be to use receptor specific reagents, such as Con A, prepare membranes, triton extraction and determine if loss of label occurs.

In preliminary experiments with Dr. J. Huggins, whole cells apparently will cap when treated with FITC-Con A. $ZnCl_2$ treated cells and membranes will not, even with added ATP. This is consistent with a transmembrane interaction between the cytoskeleton and cell surface receptors. One would predict that extraction with GEM, pH 9.5, should allow free mobility and capping. Vesicles were prepared but visualization by light microscopy was not adequate and electron microscopy will be needed to determine this.

The model adequately correlates the data presented as well as

stimulating additional areas for further work in understanding the role these several proteins may play in cellular movement.

CHAPTER VII

SUMMARY

Membrane envelopes prepared by Zn^{++} stabilization of Sarcoma 180 ascites cells contain polypeptides which appear to be related to the putative cytoskeletal elements responsible for control of cell shape and mobility. Four of these proteins have identified as ABP, myosin, α-actinin and actin based on their physical and biochemical properties including amino acid analysis, mobility on SDS and non-denaturing gels, enzymatic properties and extractability.

If membrane envelopes are vesiculated by extraction with alkaline EDTA solution at low ionic strength, ABP, α-actinin and actin are released. Myosin is not extracted. Re-extraction with 0.6 M KCl will now effect release of the myosin.

Extraction of the envelopes with distilled water releases actin binding protein, and the envelopes will fragment upon transfer to isotonic salt at pH 9.5. Neither fragmentation nor cytoskeletal protein release is observed if the isotonic extraction is performed without such treatment.

Extraction of the envelopes with Triton X-100 removes 60% of the membrane lipid and 70-80% of lactoperoxidase-iodinated cell surface proteins without removal of significant amounts of the cytoskeletal proteins. The Triton residues maintain the shape of the original envelopes but have lost the tri-laminar membrane structure.

Proteolysis of intact envelopes with trypsin or papain cleaves the high molecular weight polypeptides in the order E > ABP > myosin. Fragmentation occurs with cleavage of E or ABP, but does not appear to require cleavage of myosin. Actin and α-actinin are not appreciably cleaved when associated with the membrane.

Cross-linking of membrane proteins with copper phenanthroline or dithiobispropionimidate led to large aggregates with no evidence of heterologous dimers, trimers or tetramers useful for describing nearest neighbor relationships. All of the cytoskeletal proteins were present in the aggregated material suggesting close interactions among them.

ABP from the ascites is conveniently isolated by allowing cell extracts to undergo gelation. Proteolysis of the extracts with low levels of protease enhanced the rate of gelation and specifically cleaved only the high molecular weight polypeptides. MAT C1 adenocarcinoma cell extracts showed similar behavior while MAT B1 cells did not readily undergo gelation and no gelation was observed with protease. SDS PAGE analysis showed ABP was not readily cleaved in this cell. This suggests ABP may function partly as an inhibitor of gelation.

Studies have shown that nucleation sites apparently pre-exist in lipid vesicles and polymerization of actin onto these sites constitutes the cold sensitive step. It is suggested that release of ABP inhibition is required for polymerization to occur and it is this step and not actual polymerization which is not induced unless the extracts are warmed to room temperature.

A model is presented based on the combined results in which

short actin microfilaments are attached to the membrane interior surface through α-actinin linkages. These filaments are cross-linked by ABP and/or myosin to form a network under the cell membrane, stabilizing the envelopes. Modulation of this network by altering the association of actin-binding protein or myosin with these microfilaments regulates the actin polymerization and contractility of the system, permitting it to affect cell surface receptor distribution.

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