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COMPOSITION AND FUNCTION OF AN INVERTEBRATE ERYTHROCYTE CYTOSKELETON

City University of New York PH.D. 1985

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COMPOSITION AND FUNCTION OF AN INVERTEBRATE ERYTHROCYTE CYTOSKELETON

by

Jacquelyn A. Joseph-Silverstein

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1985

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This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

COMPOSITION AND FUNCTION OF AN INVERTEBRATE ERYTHROCYTE CYTOSKELETON

by

Jacquelyn A. Joseph-Silverstein

Advisor: Professor William D. Cohen

The cytoskeleton of the "blood clam" erythrocyte consists of a cold-labile marginal band (MB) of cross-bridged microtubules (MTs), the function of which in mature cells is unknown, and a surface-associated cytoskeleton (SAC). The identity of the MT cross-bridging proteins or any MB associated proteins (MB MAPs)is not known, nor is the composition of the SAC of invertebrate erythrocytes.

To identify MB MAPs and to study MB function in mature erythrocytes, cells with and without MBs were prepared under otherwise identical conditions. MB reassembly at 18-20^{o}C after 0^{o}C disassembly was blocked by nocodazole or colchicine, while MB disassembly at 0^{o}C was blocked with taxol, thus producing cells with and without MBs at both room temperature and 0^{o}C. In addition, MB assembly was induced at 0^{o}C using taxol.

When subjected to the mechanical stress of fluxing through capillary tubes, cells without MBs buckled and folded while those with MBs remained normal in shape,

demonstrating that the MB functions in maintaining the flattened elliptical shape of the cell during mechanical stress.

The cytoskeletal protein composition of cells with and without MBs was analyzed by SDS-PAGE, which revealed that in addition to tubulin, two proteins with Mrs of 80,000 and 105,000 were present in decreased amounts in cells lacking MBs. These two proteins may be MB MAPs. The Mr80,000 and Mr105,000 proteins, along with tubulin were also found in a soluble fraction from Brij-extracted cells incubated at 0°C to disassemble the MB.

The cytoskeleton of these erythrocytes was further characterized by SDS-PAGE as well as by Western blotting and indirect immunofluorescence microscopy using a panel of antibodies to known cytoskeletal proteins. The major protein components were identified as tubulin (which localized to the MB), actin (which localized to the SAC), and a 240,000Mr protein that comigrated with human α-spectrin, and reacted with an anti chicken erythrocyte"α-spectrin" antibody. This antibody localized to the SAC. Vimentin, a component of intermediate filaments was not identified here.

This work demonstrates a mechanical function for the MB in mature erythrocytes and identifies two proteins which cycle with the MB and therefore behave as MB MAPs. The protein composition of the invertebrate erythrocyte SAC shares some similarities with that of the vertebrate erythrocyte, but they are not identical.

ACKNOWLEDGEMENTS

This thesis represents the culmination of a fruitful and enjoyable doctoral student experience which is a tribute to Dr. William D. Cohen, my thesis advisor. His enthusiasm for research was infectious and I am grateful to him for imparting this sense of enthusiasm to me. I am also thankful to him for his guidance and encouragement, the sustinence of all graduate students. Dr. Cohen encouraged my participation in a summer course at the Marine Biological Laboratory which was a joyful experience in research and aided my growth as a scientist. I will always be grateful for his family's hospitality to me during my summers at the MBL.

I would also like to thank Dr. Peter Lipke, Dr. James Wyche, Dr. Ray Gavin and Dr. Susan B. Horwitz for acting as members of my doctoral thesis committee. The discussion offered by them during my defense made it both an educational and enjoyable experience.

Ms. Monique DeFour was a great help in preparing the photographs for this thesis. My peers, Ms. Mary Ginsburg, Dr. Diana Bartelt, Dr. Iris Nemhauser and Dr. Joanne Carroll provided encouragement throughout work on this thesis. Dr. Rivka Rudner, our graduate advisor, worked to make my stay at Hunter College an enjoyable one. I am grateful to all of them.

I would also like to thank Dr. Judith Spiegel for an enjoyable, educational, and fruitful collaboration.

Finally, I would like to acknowledge my family, who always encouraged any endeavor I might take on. My husband, Dr. Roy L. Silverstein, played an important role during the course of this research as a sounding board for my ideas, and during the preparation of this thesis when he spent interminable hours editing, helping to prepare figures, and word processing. I am grateful to him for this, as well as for his continual encouragement during the course of this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The cytoplasm of eukaryotic cells is highly ordered. Much of this order is provided by a filamentous network termed the cytoskeleton or cytomatrix (Porter, 1984). This cytoskeletal system is involved not only in the organization of intracellular components (Sandoval, 1984), but also in organization of the cell surface (Bourguignon et al (1984), intracellular transport (Beckerle et al.,1983), cellular motility (Taylor et al., 1979) and in the generation and maintenance of cell shape (Dustin, 1978). The cytoskeleton, as reflected by its role in the many cellular properties listed here, is thus a complex and dynamic system.

CYTOSKELETAL COMPLEXITY

Transcellular Elements

Electron microscope studies of thin sections of nucleated nonerythroid cells have revealed that at least three types of filaments, identifiable by their differing diameters and cross-sectional appearances make up the eukaryotic cytoskeleton: microtubules (MTs) (Sandborn et al.,1964;Behnke,1964), microfilaments (MFs) (DePetris et al.,1962), and intermediate filaments (IFs) (Ishikawa et al.,1968). The protein components of each of the filament types is specific, with MTs being composed mainly of tubulin (Dustin, 1978), and MFs of actin (Ishikawa et al., 1969).

There is not a single class of IFs but rather five distinct classes, each of which is composed of different subunit proteins (Lazarides, 1982). Purification of the protein components of these filaments allowed the preparation of specific antibodies which could be used in conjunction with immunofluorescence microscopy to localize a specific type of filament within the cell. Such studies showed that the three major filament systems have a differential distribution within cells (MTs:Brinkley et al., 1975; MFs:Lazarides et al., 1974; IFs:Franke et al., 1978).

Improvements in electron microscope technology, such as the development of the high voltage electron microscope, and improved methods for the preparation of specimens such as freeze-drying have provided a three dimensional picture of the cytoskeleton as a complex system in which all three elements are highly cross-linked to one another (Wolosewick et al., 1976: Wolosewick et al., 1979; Heuser et al.,1980; Schliwa et al., 1981)

Biochemical studies of purified cytoskeletal components have led to the identification of proteins with which they specifically interact, some of which are involved in the bundling of a single cytoskeletal element (Bretscher et al.,:actin filaments) or in the cross-linking of one filament system to another (Griffith et al., 1978:MFs to MTs; Bloom et al., 1983: MTs to IFs). Actin-binding proteins (Goldman et al.,1979), microtubule-associated proteins (Vallee et al.,

1984) and intermediate filament-associated proteins (Lazarides,1982) have all been identified. In addition, many of these associated proteins have also been localized to specific cytoskeletal components by immunofluorescence microscopy, utilizing antibodies raised against the putative associated proteins (Brinkley et al., 1980; Connolly et al., 1980). In addition to providing a linkage between filaments, proteins which co-purify with cytoskeletal elements may serve to link cytoskeletal filaments with cellular organelles or the plasma membrane (Mangeat et al., 1984). Others may have enzymatic activity (Lockwood et al.,1982) or may be proteins involved in the regulation of filament assembly or disassambly such as the STOP proteins which are associated with MTs (Job et al., 1983; Hesketh, 1984).

Surface-associated Cytoskeleton

In addition to the transcellular elements of the cytoskeleton described above, a "surface lamina" or surface-associated cytoskeleton has been described in nucleated non-erythroid cells. When cells such as cultured fibroblasts (Ben-Ze'ev et al.,1979), tumor cells and lymphocytes (Mescher et al.,1981) were extracted in the detergent Triton X-100, a closed matrix resembling the shape of the intact cell was recovered in the Triton-insoluble residue. Detergent insolubility is considered, operationally, to define the cytoskeleton.

This surface lamina may be analogous to the surface-

associated cytoskeleton first described for mammalian red cells in 1973 by Yu et al. Mammalian red cell membrane ghosts prepared by osmotic lysis of whole cells, maintain the shape of the intact cell and contain intact integral membrane proteins, as well as any peripheral proteins interacting with them. The peripheral proteins remaining with the ghosts, form a meshwork underlying the plasma membrane which is Triton-insoluble (Hainfeld et al., 1977; Sheetz et al.,1978). No microtubules, microfilaments or intermediate filaments are present in the mammalian red cell cytoskeletons.

The protein composition and organization of this "membrane skeleton" in mammalian erythrocytes has been well-characterized. Its major components are spectrin (a 240,000MW and 220,000MW heterodimer), actin (42,000MW), band 4.1 (80,000MW), and band 4.9 (48,0000MW) (Cohen, 1983). These four proteins interact with one another and the meshwork they form is attached to the lipid bilayer by the association of spectrin with the major red cell transmembrane protein, band 3. This association is mediated by still another peripheral protein, ankyrin or band 2.1 (210,000MW) (Bennett et al., 1979).

Recently, antibodies against human erythrocyte spectrin have been found to cross-react with proteins in several non-erythroid cell types (Goodman et al.,1981; Bennett et al.,1982a; Lehto et al.,1983; Nelson et al.,1983). These proteins are spectrin-like in that they share a common

antigenic site, similar morphology demonstrated by electron microscopy rotary shadowing (Glenney et al.,1982), the ability to bind to actin and/or ankyrin (Bennett et al.,1982a; Burridge et al.,1982; Glenney et al.,1982), and a basic heterodimer structure (Goodman et al.,1981; Levine et al.,1981; Bennett et al.,1982a; Burridge et al.,1982; Glenney et al.,1982; Glenney et al.,1983). Immunofluorescence microscopy of non-erythroid cells using antibodies prepared against the spectrin-like proteins of the intestinal brush border (TW260/240) or brain fodrin (a heterodimer of Mr240/235), revealed mainly diffuse staining of the cytoskeleton. When tissue slices instead of cells were incubated with the antibodies major localization was to the periphery of the cell (Levine et al.,1981; Burridge et al.,1982; Glenney et al.,1982). This pattern is similar to that for anti-spectrin binding in human erythroid cells (Nicholson, 1971). Similarly, specific antisera to human ankyrin (Bennett et al., 1981), band 4.1 (Spiegel et al., 1984), and band 3 (Kay et al., 1983; Drenckhahm et al., 1984) have also been demonstrated to bind to several non-erythroid cells.

With the demonstration of a "surface lamina" in non-erythroid cells, as well as the identification of spectrin-, ankyrin- , band 3- and 4.1-like proteins in non-erythroid nucleated cells, it is now apparent that our picture of the nucleated cytoskeleton is still more complicated. Non-erythroid cytoskeletons thus contain not only microtubules,

microfilaments, intermediate filaments, and numerous associated proteins, but also a surface-associated lamina or cytoskeleton.

NON-MAMMALIAN RED BLOOD CELL CYTOSKELETONS

Non-mammalian nucleated erythrocytes, like their mammalian counterparts, contain a surface-associated cytoskeleton (Cohen,1978; Cohen et al., 1980; Cohen et al.,1982b; Nemhauser et al.,1980). In addition they also contain a marginal band of microtubules (Meves,1911; Fawcett,1959; Maser et al.,1964; Barclay,1966; Sekhon et al.,1969; Behnke,1970; Cohen,1978; Nemhauser et al.,1980; Cohen et al., 1980; Cohen et al.,1982b) and in cells of some species, such as the chicken, transcellular intermediate filaments (Virtanen et al.,1979; Woodcock et al.,1980; Granger et al.,1982a,b) as well. The similarity of non-mammalian erythrocyte cytoskeletal components to those of non-erythroid cells, along with the relative ease with which blood can be collected and the erythrocytes obtained as a pure population, make these cells extremely useful as a system for the study of cytoskeletal organization and function in nucleated cells.

1. Marginal Bands

Marginal bands (MBs) of microtubules (MTs) are present in all non-mammalian vertebrate erythrocytes (Meves,1911; Fawcett,1959; Maser et al.,1964; Barclay, 1966; Sekhon et al.,1969; Behnke,1970a,b; Cohen, 1978; Cohen,1982; Cohen et

al.,1982b) and in some invertebrate blood cells (Baerwald et al.,1970; Nemhauser et al., 1980; Cohen et al., 1980; Cohen et al.,1983) as well as in thrombocytes (Behnke,1965) and human platelets (Haydon et al.,1965; Behnke,1965; Behnke et al.,1967). The MB was first accurately described as a filamentous ring by Meves in 1911. In 1959, Fawcett described the MB of amphibian red blood cells as a structure consisting of bundled tubular elements. However, it was not until 1964, following the discovery of Sabatini et al. (1963) that improved fixation of tubular elements could be acheived using glutaraldehyde, that MBs were first described as bundles of microtubules (Maser et al., 1964). The relatively simple structure of the MB and its discrete localization within the erythrocyte has led investigators to look here for clues to the generalized spatial organization, temporal regulation, and function of MT systems.

Although the method of MB assembly <u>in vivo</u> is not known, in at least one species there is evidence that a pair of centrioles located at the cell periphery may act as a microtubule-organizing center. The MBs of dogfish (Cohen et al.,1982b), chicken (Barrett et al.,1974; Miller et al.,1984) and "blood clam" erythrocytes (Nemhauser, Joseph-Silverstein and Cohen, 1983) are cold-labile, disassembling when the cells are incubated at 0^{o} C and reassembling upon rewarming of the cells at physiological temperatures. This is not a general property of MBs, since the MBs of all amphibians studied thus far are cold-stable (Behnke,1970a,b). In the

"blood clam", MB reassembly is initiated at a single centriole pair with its associated material (Nemhauser, Joseph-Silverstein and Cohen, 1983). In both the dogfish and chicken, however, reassembly of MTs does not appear to be associated with a single microtubule-organizing center (MTOC) (dogfish:Joseph-Silverstein,unpublished observation; chicken:Barrett et al.,1974; Miller et al.,1984). The cold-lability of the MB of erythrocytes from these organisms has been useful in studying the regulation of spatial organization of the MB, the function of the MB, and the localization of proteins within the cytoskeleton.

When viewed in cross-section by electron microscopy, cross-bridges can be seen between adjacent MTs in the MBs of some species (dogfish:Cohen et al. 1982b; "blood clam":Nemhauser et al.,1983). The demonstration of the existence of these cross-bridges has led to a search for MB microtubule-associated proteins (MAPs) which might act as cross-bridges. Little is now known, however, of such MB MAPs. SDS polyacrylamide gel electrophoresis of isolated dogfish MBs revealed only the tubulin bands, and a minor band seen at approximately 30,000 (Cohen et al.,1982a). It was reported, however, that there is a component of the MB in erythrocytes of the amphibian (Sloboda et al.,1980) and chicken (Murphy et al.,1983a,b) which cross-reacts immunologically with brain MAP 2, a high molecular weight protein co-assembling with tubulin <u>in vitro</u> (Sloboda et al., 1976). However, when MT

protein from chicken erythrocytes was purified and analyzed by SDS-PAGE, tubulin made up 95% of the total protein; the remainder contained spectrin and the tau proteins, MAPs with molecular weights ranging from 55,000 to 60,000 which co-isolate with brain tubulin (Weingarten et al., 1975) but no MAP 2 (Murphy et al., 1983a,b). An anti- MAP 2 reactive antigen has been localized to the MB of salamander and chicken red blood cells by immunofluorescence microscopy using an antibody raised against brain MAP 2 (Sloboda et al.,1980) and Centonze et al. (1984) have further localized this antigen to the cross-bridges between MTs of the bullfrog MB by immunogold electron microscopy.

MB function in non-mammalian erythrocytes has been a subject of some controversy. It has become well accepted that the MB is involved in cellular morphogenesis since the transformation of immature erythrocytes from spherical to discoidal shape is accompanied by the appearance of the MB (Sekhon et al.,1969; Barrett et al.,1972; Barrett et al.,1974; Yamamoto et al.,1975). However, whether it has any function in mature circulating erythrocytes has been questioned. For some microscopists, the fibrous and flexible nature of the MB has implied a continuing role in maintenance of shape in the mature erythrocyte (Meves,1911; Fawcett,1959). However, until recently, there has been no direct evidence for such a role. On the contrary, evidence cited for the lack of a continuing MB function has been the diminishing number of MB MTs as the cells of certain species

mature (Small,1972; Barrett et al.,1974; Yamamoto et al.,1975), and the maintenance of flattened, elliptical cell shape in the absence of the MB (Behnke,1970a,b; Barrett et al.,1974). The latter experiments were done in vitro using erythrocytes with cold-labile MBs and comparing their shape to that of normal cells at room temperature.

We have recently demonstrated that dogfish erythrocytes without MBs responded differently to mechanical stress than those with MBs (Joseph-Silverstein and Cohen,1984). Cells without MBs tended to be buckled or folded while those with MBs appeared normal. This evidence supports the contention that the MB has a continuing mechanical function in mature, circulating erythrocytes. Further evidence for MB function has come from the study of human platelets with and without MBs (White,1984). Platelets without MBs responded differently to partial aspiration into micropipettes than those with MBs. Platelets without MBs were initially more deformable and recovered normal shape more slowly if at all, when released from the micropipette, than platelets with MBs.

Based on the observations that MBs circularize when isolated from the SAC (Bertolini et al., 1976; Cohen, 1978; Cohen, 1982b) and that dogfish erythrocytes with anomalous morphology contain MBs with corresponding anomalous structure (Joseph-Silverstein and Cohen, 1984), we have hypothesized that in the erythrocyte, the MB is a flexible frame under strain, with the shape of the cell corresponding to the shape

of the MB.

2. Surface-Associated Cytoskeleton

The surface-associated cytoskeleton (SAC) of nucleated erythrocytes was first described by Cohen (1978), who viewed it under the electron microscope as "a rough network spanning the space between nucleus and MB and attached to or wrapped around the latter". He suggested that this network might be similar to the spectrin-actin complex found in mammalian erythrocytes. In fact, several investigators studying the plasma membrane components of chicken erythrocytes reported on the similarity of the protein profile of these erythrocyte membranes to those of the mammalian red blood cells (Blanchet,1974; Jackson,1975; Weise et al.,1976; Chan,1977; Sloboda et al.,1980). Proteins comigrating with mammalian erythrocyte α and β spectrin, ankyrin, band 3, band 4, and actin were seen on SDS-polyacrylamide gels of embryonic (Chan,1977; Weise et al.,1978) and adult (Sloboda et al.,1980) chicken erythrocyte membranes. Cohen et al. (1982b) presented evidence for a similar protein profile for dogfish erythrocyte cytoskeletons prepared by lysis of the cells with the detergent Triton X-100.

It was demonstrated, however, that the higher molecular weight component of the "spectrin" doublet in the dogfish erythrocyte cytoskeleton was functionally more similar to brain α- fodrin (Bartelt et al,1982; Bartelt et al.,1984). In addition to this difference between mammalian and non-mammalian erythrocyte SACs, a third high molecular weight

band located between the α and β spectrin bands, and called goblin, was identified in turkey erythrocyte membranes (Beam et al., 1979) and later observed by Cohen et al.(1982b) in dogfish erythrocyte cytoskeletons. The similarity of human erythrocyte band 4.1 to its analog in the chicken erythrocyte cytoskeleton was tested by Granger et al.(1984). Six variants of the chicken analog exist, each of which have almost identical 2 dimensional peptide maps and similar solubility properties to human 4.1. Based on these reports it is apparent that the non-mammalian erythrocyte SAC is structurally similar to, but not biochemically identical to the human erythrocyte "membrane skeleton".

Although, spatially, the MB and SAC are two distinct cytoskeletal components, some evidence exists that they act in concert with one another to bring about the elliptical shape of nucleated erythrocytes. When MBs are isolated by proteases (Cohen,1978) or by citrate-deoxycholate (Bertolini et al.,1976) to remove the SAC, they tend to circularize, rather than maintain their elliptical contours. Based on these observations, it was hypothesized that the SAC, normally under tension, supplies the force necessary to cause the MBs to take on an elliptical conformation (Cohen,1978).

3.Intermediate Filaments

The third component of some non-mammalian erythrocyte cytoskeletons are the intermediate filaments (IFs). That cytoplasmic filaments were present in nucleated erythrocytes,

spanning from the nucleus to the plasma membrane was first demonstrated by Harris and Brown in 1971. Based on their solubility properties and ultrastructural morphology, these filaments were later identified as IFs (Virtanen et al.,1979; Woodcock et al.,1980). The major protein component of these filaments in the red blood cell is vimentin (Granger et al.,1982a,b), an IF protein found in permanently growing cells in culture, cells of mesenchymal origin, and undifferentiated cells (Lazarides,1982). Synemin, a protein usually associated with the IFs of muscle, was found to be associated with vimentin in avian red blood cells, possibly playing a role in cross-linking adjacent filaments (Granger, 1982a,b). Neurofilament protein has also been found in erythrocyte IFs (Granger et al.,1983). The demonstration of IFs in erythrocytes has been limited to those of chicken (Granger et al.,1982a,b) and Xenopus (Gambino, personal communication). However, Cohen et al. (1982) have identified a protein in the dogfish erythrocyte cytoskeleton which comigrated with vimentin on SDS-polyacrylamide gels. The wealth of antibodies now available against purified IF proteins, as well as the development of suitable methods for extraction of avian erythrocytes under conditions which stabilize IFs (Granger et al.,1982a,b) should aid in the search for IFs in erythrocytes of other species.

Intermediate filaments in erythrocytes are thought to play a role in anchoring the nucleus within the cell based on their localization between the nucleus and plasma membrane

(Virtanen et al.,1979; Woodcock et al.,1980; Granger et al.,1982a,b). No direct evidence has yet been reported substantiating this possible role.

INITIAL APPROACHES AND OBJECTIVES

The Blood Clam" Erythrocyte as a Model System

The structural simplicity and discrete location of the MB make it an excellent system for studying the regulation of MT organization within cells, as well as MT function. The MB of the flattened, elliptical "blood clam" erythrocyte is especially interesting and useful in that it is a cold-labile structure which reassembles upon incubation at room temperature in association with a pair of centrioles (Nemhauser, Joseph-Silverstein and Cohen,1983). As yet, there is no evidence of centriole-generated MB reassembly in any other MB system studied, although MB-associated centrioles have been reported in sea cucumber and Sipunculan erythrocytes (Fontaine et al.,1972; Nemhauser,1981). This unique feature of the "blood clam" erythrocyte MB is of particular interest, since mitotic spindles and cytoplasmic arrays of MTs in interphase cells are nucleated by microtubule-organizing centers (MTOCs) consisting of centrioles and their associated material (Brinkley et al, 1980). In addition, electron microscope analysis of thin sections of "blood clam" erythrocytes has revealed that cross-bridges are present between adjacent MTs in the MB (Cohen et al.,1980; Nemhauser, Joseph-Silverstein and

Cohen,1983). Study of the "blood clam" erythrocyte, then is likely to reveal much information on MB MAPs.

The cytoskeletal system of the "blood clam" erythrocyte consists not only of an MB , but also a SAC, which has been observed by phase contrast and electron microscopy (Cohen et al.,1980). The SAC is not a cold-labile structure. When "blood clam" erythrocytes were incubated at 0°C to disassemble the MB and then extracted in Triton, the SAC remained with the insoluble residue (Nemhauser et al., 1983). Thus the SAC can be separated from the MB to simplify its biochemical characterization. To date, no biochemical analysis of an invertebrate erythrocyte cytoskeleton has been reported. Therefore, it was of interest to us to compare the invertebrate SAC with that of the vertebrates to gain some insight into the evolution of erythrocyte cytoskeletons.

Major Objectives

The major objectives of this work were: 1.to identify MB MAPs in "blood clam" erythrocytes; 2.to study whether MBs have a continuing function in mature circulating erythrocytes; 3.to identify and localize the cytoskeletal components of the SAC.

Background and Approach to the Problems

Although the presence of proteins in non-mammalian vertebrate erythrocyte MBs that cross-react with anti-MAP 2 has been reported (Sloboda et al.,1980; Murphy et al.,1983a,b), the co-isolation of MAP 2 (or any other MAP) with MB tubulin has not yet been well documented. The "blood

clam", with its cold-labile MB is well-suited for such a study. The ability to prepare erythrocytes with and without MBs by temperature-cycling, would provide the identification of proteins which cycle with the MB. This could be done by a biochemical comparison of cytoskeletons from erythrocytes incubated at room temperature and 0°C. However, since cold-labile cytoskeletal components other than the MB may be present in these cells, the preparation of erythrocytes with and without MBs at the same temperature would simplify the analysis of data, further substantiating the identification of MB MAPs.

The ability to produce cells with and without MBs at the same temperature would not only aid in the identification of MAPs, but would provide evidence for the localization of other major cytoskeletal proteins. MB function could be studied as well, by comparing the responses of erythrocytes with and without MBs to experimental forces.

Therefore, our initial approach was to test the sensitivity of the MB to various MT drugs, determining whether the system could be experimentally manipulated to provide cells with and without MBs. Immunofluorescence microscopy utilizing an anti-tubulin antibody was the method of choice for observing the drugs' effects on the MB. Colchicine (Taylor et al., 1965) and nocodazole (deBrabander et al.,1976) are two anti-tumor drugs which cause MT disassembly in various cell types. This is believed to occur

by the inhibition of tubulin subunit addition to pre-existing MTs which are dynamic structures in a steady state equilibrium between assembly and disassembly. These drugs can also block MT reassembly of cold-labile MTs following disassembly. Cohen et al. (1982) reported that reassembly of dogfish MBs was blocked by colchicine following 0°C disassembly. The drug taxol, on the other hand is an anti-tumor drug which stabilizes MTs against disassembly, and stimulates MT polymerization and bundling at room temperature (Schiff et al.,1980).

After we established a method for the preparation of such cells with and without MBs at the same temperature, our next approach was to develop a system to allow comparison of the responses of these two cell types to experimental forces.

Because, as described above, the only direct experimental work on MB function in mature circulating cells involved comparing the shape of cells incubated at 0°C (to disassemble the MB) with those incubated at room temperature (Behnke, 1970a,b; Barrett et al., 1974) we felt that a dynamic study was important. The system we chose was based on blood flow through a capillary tube.

MBs do not exist as the sole cytoskeletal element of nucleated erythrocytes, but co-exist with a SAC and in some cases, IFs. Cohen (1978) has hypothesized that the MB is under asymmetric tension generated by the SAC. If this is the case, then the SAC and the MB work together to bring about the flattened elliptical shape of the erythrocyte. Therefore,

it is also of interest to study the composition of the SAC, as well as the MB. Our initial approach was to compare cytoskeletons prepared from cells with and without MBs using SDS-polyacrylamide gel electrophoresis. This would provide an initial identification of SAC proteins as well as proteins that cycle with the MB. Since similarities in protein composition exist between mammalian anucleate erythrocyte cytoskeletons and the nucleated vertebrate erythrocyte cytoskeletons studied thus far, we planned to expand the SDS-PAGE system by transferring the separated "blood clam" cytoskeletal proteins onto nitrocellulose and then probing with antibodies against known erythrocyte cytoskeletal proteins commercially available or obtained from other investigators. The same antibodies could be used for the localization of the proteins within the cytoskeletons by immunocytochemistry.

Our ability to produce cells with and without MBs provided us with a powerful approach for identifying MB MAPs and studying MB function. Our third objective, to study the components of the SAC, also made use of this approach but was greatly facilitated by the use of antibodies for identification and localization of the cytoskeletal proteins.

MATERIALS

Adenosine 5'-triphosphate (ATP); bovine serum albumin, fraction V powder (BSA); 4-chlor-1-naphthol; colchicine; cytochalasin D; dihydrocytochalasin B; dithiothreitol (DTT); deoxyribonuclease 1 (DNase-1); elastase (porcine pancreatic); ethylene glycol-bis-aminoethyl ether N,N'-tetraacetic acid (EGTA); guanosine 5'-triphosphate (GTP); heparin; nocodazole; nonidet P-40 (NP-40); octyl phenoxy polyethoxyethanol (Triton X-100); piperazine N-N'-bis 2-ethane sulfonic acid (PIPES); phenylmethylsulfonyl fluoride (PMSF); polyoxyethylene-20-cetyl ether (Brij-58); tosyl arginine methyl ester (TAME); and Trizma Base (Tris) were all obtained from Sigma Chemical Co. (St. Louis, Missouri). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker Chemical Co. (Phillipsburg, New Jersey).

All reagents for electrophoresis including 2-mercaptoethanol (2-ME) and high molecular weight protein standards were purchased from Bio-Rad (Richmond, California). Nitrocellulose paper was also obtained from Bio-Rad.

Taxol was the gift of Dr. M. Suffness, Natural Products Branch, Division of Cancer Treatment, NCI. Lyophilized human erythrocyte ghosts were the gift of Mr. P. Pederson, Hunter College, New York. Glycerinated rabbit psoas muscle was obtained from Carolina Biological Supply Company. Bovine brain MT protein was purified by the method of Shelanski et al. (1973) in our laboratory.

Rat monoclonal antibody to yeast tubulin was the generous gift of Dr. J. Kilmartin (Medical Research Council, Cambridge, England), and murine monoclonal antibody (IgG-1, kappa) to chicken gizzard actin was a gift of Dr. J.L. Lessard (Children's Hospital Research Foundation, Cincinnati,Ohio). All polyclonal antibodies provided had been raised in rabbits. Polyclonal anti-actin antiserum to chicken muscle actin was purchased from Miles-Yeda (Rehovot, Israel). Polyclonal anti-ankyrin and anti-spectrin IgG to human erythrocyte proteins were gifts of Dr. J. Spiegel (Brandeis University); anti-vimentin antiserum to embryonic chick muscle vimentin was a gift of Dr. B. Granger (Yale University); anti-MAP 2 antiserum to hog brain MAP-2 was generously provided by Dr. R. Sloboda (Dartmouth College); and anti-α-spectrin IgG to chicken erythrocyte α-spectrin was a generous gift of Dr. E. Repasky (Roswell Park Memorial Institute, Buffalo, New York). Fluorescein-conjugated rabbit anti-rat IgG, goat anti-rabbit IgG and rabbit anti-mouse IgG were purchased from Miles-Yeda or Cappel Laboratories (West Chester,Penn.), while the same antibodies conjugated to horseradish peroxidase were purchased from Cappel Laboratories.

METHODS

Maintenance of Animals and Blood Collection

Noetia ponderosa were obtained from Gulf Specimen Company, Panacea, Florida, and *Anadara ovalis* were obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts. Both species were maintained in refrigerated sea water tanks at approximately 20°C. The blood of *Noetia ponderosa* was drawn from the mantle cavity with a Pasteur pipette inserted through a small opening made in the shell and immediately diluted (minimum 1:30 dilution), with stirring, into MBL formula artificial sea water (Cavanaugh,1975). The resulting cell suspension was centrifuged at 1750 g for 2 minutes in an IEC HNS-II centrifuge (International Equipment Corp., Needham Heights, Mass.) yielding an erythrocyte pellet overlaid by a thin white layer of amebocytes and trapped erythrocytes. This layer was dislodged in a single sheet by addition of a small volume of fresh sea water and gentle outward fluxing with a Pasteur pipette. The contaminating layer was then removed and the cells washed two to three times in approximately twice the original blood volume in sea water, or until a white layer was no longer present. The cells were finally resuspended in MBL sea water to a working dilution of 0.3 ml packed cells per 10 ml suspension.

The blood of *Anadara ovalis* was collected and washed in the same manner. However, the initial dilution into MBL sea water was at least 1:100 and in some experiments, 1:200, since these cells tended to undergo morphological changes at

lesser dilutions.

Preparation of Nucleated Cytoskeletons

Cells were lysed in medium containing 100 mM PIPES, 1 mM $MgCl_2$ 5 mM EGTA, 10 mM TAME, 0.1 mM PMSF and 0.4% Triton X-100, pH 6.8 (LyM). In some experiments, Triton X-100 was replaced with 0.6% Brij-58 (Deery et al.,1983). For studies in which large numbers of cells were needed, the erythrocytes were pelleted and then resuspended to their original suspension volume in LyM (an approximately 30-fold dilution of the pellet). For light microscopy, one drop of cell suspension was lysed in 10 drops of LyM.

In the experiments done to determine whether IFs were present in "blood clam" erythrocytes, cell lysis was carried out under IF-stabilizing conditions. The lysing solution used contained 130mM NaCl, 5mM KCl, 5mM $MgCl_2$, 1mM EGTA, 10mM Tris-Cl, 1mM DTT, 0.5mM PMSF, 10mM TAME and 0.4% Triton X-100 (Granger et al., 1982a,b). As above, one drop of cell suspension was lysed in 10 drops of this medium for fluorescence microscopy, or an erythrocyte pellet was resuspended to the original cell suspension volume for samples to be analyzed by gel electrophoresis.

Preparation of Chicken Erythrocyte Nucleated Cytoskeletons

Chicken blood in Alsever's solution (Garvey et al.,1977) was obtained from Pocono Rabbit Farm (Canadensis, Penn.) and was stored at 4^0C. Blood was diluted 1:10 in Hank's Balanced Salt Solution (HBSS) (Humason, 1972) initially, and then

pelleted by centrifugation at 1750g for 2 minutes in the IEC HNS-II centrifuge. Cells were then washed 3 times in HBSS. Following washing, no white cells were present when samples were viewed by phase contrast microscopy.

Cells were lysed under IF-stabilizing conditions as described above, and prepared for fluorescence microscopy or gel electrophoresis. Following protein solubilization, samples contained insoluble chromatin which was solubilized by the addition of 1N HCl. Characteristic SDS-PAGE profiles for chicken erythrocyte cytoskeletons (Blanchet, 1974) were obtained from samples prepared in this way.

Preparation of Anucleate Ghosts and Cytoskeletons

The following procedure was adapted for "blood clam" erythrocytes from Cohen et al (1982b). Pellets of erythrocytes were osmotically lysed by resuspending them to their original suspension volume in 50% LyM wash (LyM wash=LyM lacking Triton X-100 or Brij-58). The resulting red cell ghosts were then fluxed three times through a 22 gauge needle affixed to a 10 ml syringe, freeing the nuclei from the ghosts. The resulting suspension which contained anucleate ghosts, free nuclei, particles, and a small number of nucleated ghosts, was centrifuged at 1750 g for 3 min and the supernatant, containing the anucleate ghosts and particles, was transferred to 15 ml conical centrifuge tubes and centrifuged for 30 minutes at 1850 g in a clinical centrifuge (International Equipment Corp., Needham Heights,

Mass.). The pellet was then washed in a small volume (1-2 ml) of LyM wash and centrifuged for 20 minutes at 1850 g in the clinical centrifuge. The upper layer of the resulting pellet, which contained the anucleate ghosts, was carefully removed, leaving behind any remaining particles. Unfortunately, this technique yielded anucleate ghosts in which the MBs had disassembled. However, it was useful for studying the components of the SAC. To prepare Brij- or Triton- extracted cytoskeletons, pellets of anucleate ghosts were resuspended in a small volume of LyM containing either 0.4% Triton X-100 or 0.6% Brij-58.

Agents tested for their usefulness in MB isolation

Erythrocytes were extracted in modified LyM solutions to determine whether intact MBs could be isolated from the SAC. The success of such a procedure would simplify biochemical analysis of the MB. In some experiments, 1-25mM ATP, 0.5M KCl, 0.5mg/ml heparin or 0.5-4mg/ml DNase was added to LyM containing TAME and PMSF. In others, LyM lacking protease inhibitors was either used alone as an extraction medium or contained 0.1-0.5U/ml elastase. MB isolation in these solutions was monitored by phase contrast or indirect immunofluorescence microscopy using anti-tubulin and anti-actin antibodies.

Polyacrylamide Gel Electrophoresis

Protein composition of erythrocyte ghosts and cytoskeletons was analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970).

Proteins were separated on 7.5% polyacrylamide slab gels, 0.75 mm in thickness. Gels were stained with .075% Coomassie Brilliant Blue R-250 and destained by diffusion. In some cases, Coomassie stained gels were scanned using an LKB Bromma 2202 Ultroscan Laser Densitometer with a 2210 2-channel chart recorder (LKB Instruments, Inc., Bromma Sweden).

Since many "blood clam" erythrocyte proteins comigrated with human erythrocyte proteins in this gel system, human erythrocyte ghosts were routinely used as molecular weight standards. However, a standard curve for the determination of molecular weights was generated using Bio-Rad high molecular weight standards. Gel samples were solubilized in sample buffer containing 2% SDS and 3% 2-ME and boiled for 5 minutes.

Electrophoretic Transfer and Antibody Binding

Proteins separated on 7.5% SDS-polyacrylamide slab gels were routinely transferred overnight at 0.1 amps to nitrocellulose paper according to the method of Towbin (1979), in electroblot buffer containing 24 mM Tris, 192 mM glycine and 20% (vol/vol) methanol at pH8.3 using a Hoeffer electroblot apparatus (Hoeffer Scientific Instruments, San Francisco, Calif.). This lengthy transfer time facilitated the transfer of high molecular weight proteins.

Following transfer, the nitrocellulose was cut into appropriate lanes and in some experiments incubated for 2

hours in blocking buffer containing 10% fetal calf serum (FCS) and 2.5% BSA in phosphate-buffered saline (PBS=0.15M NaCl, 0.041M Na_2HPO_4, 0.01M NaH_2PO_4, pH7.4). The nitrocellulose strips were then rinsed in several changes of PBS (100 ml, total) and incubated for 2 hours at room temperature on a horizontal shaker (New Brunswick Co., Edison, New Jersey) in the primary antibody diluted in PBS containing 2% FCS and 0.5% BSA. Following this treatment, the nitrocellulose was again rinsed in several changes of PBS and incubated for 2 hours at room temperature in the appropriate dilution of secondary antibody conjugated to peroxidase. Following a final rinse in PBS, the nitrocellulose strips were developed in 0.05% 4-chlor-1-naphthol in PBS containing 0.01% H_2O_2. The reaction was stopped by submerging the strips in distilled H_2O (Zackroff et al., 1984).

In some experiments an alternate method for antibody binding was used. In these cases, the nitrocellulose strips were incubated in blocking buffer containing 5% non-fat dry milk (Carnation Inc., Los Angeles, California: commercial food product obtained in a local market) and 0.1% NP-40 in PBS (pH7.4) for 1 hour. Antibody incubations were also carried out for 1 hour in these experiments. This blocking buffer proved superior to FCS and BSA-containing buffers for blocking non-specific protein binding on the nitrocellulose paper, while the 1 hour incubation times proved to be efficient as well as convenient.

For immunoblotting, rat monoclonal anti-tubulin antibody

was diluted 1:200 (approx. final conc. 15μg/ml) (Kilmartin et al.,1982) and murine monoclonal anti-actin antibody was diluted 1:400 (approx. 5μg/ml). Polyclonal anti-actin antiserum was diluted 1:25; polyclonal anti-ankyrin and anti-spectrin (human) antibodies were diluted 1:80 (approx.12.5μg/ml); polyclonal anti-MAP 2 antiserum was diluted 1:50; polyclonal anti-vimentin antiserum (Granger et al., 1979) was diluted 1:100; and polyclonal anti-α spectrin (chicken) antiserum was diluted 1:100. The secondary antibodies conjugated to peroxidase (goat anti-rabbit, rabbit anti-rat, and rabbit anti-mouse IgGs) were all diluted 1:1000.

Microscopy

Phase Contrast Microscopy

Erythrocyte and cytoskeletal morphology was monitored during the course of an experiment by phase contrast microscopy using a Zeiss microscope equipped for photomicrography. To monitor erythrocyte morphology, cell suspensions were diluted 1:1 in "MBL" formula artificial sea water containing 2% glutaraldehyde. To verify whether MBs were present or absent in populations of erythrocytes, one drop of cell suspension was lysed in 10 drops of LyM.

Immunocytochemistry

Immunofluorescence microscopy was used to study the configuration of the MB MTs under various experimental conditions. In these experiments, monoclonal anti-tubulin

diluted 1:100 was used to visualize MTs in cytoskeletons prepared as described above. Immunocytochemistry was also used to determine whether molecules cross-reacting with other known cytoskeletal proteins were present, and if so, to localize them within the cytoskeleton.

Cytoskeletons were prepared for immunofluorescence microscopy in the following manner: lysed cell samples were incubated on ethanol-cleaned coverslips for 5 minutes to promote adherence, rinsed with LyM wash, incubated for 5 minutes in 1% glutaraldehyde in LyM wash, rinsed three times in 0.05M sodium phosphate buffer, pH7.4, and then incubated with 50μl of primary antibody diluted in PBS containing 1% BSA, at pH7.4 for 30 minutes at 37^{o}C in a moist chamber. Coverslips were then rinsed in the phosphate buffer and subsequently incubated for 30 minutes at 37^{o}C in 50μl of fluorescein-conjugated secondary antibody. Following final rinsing in phosphate buffer, coverslips were mounted in 50% glycerol in phosphate buffer. A cytoskeleton sample was incubated with nonimmune serum, when available, to verify that the fluorescence pattern seen was due to specific antibody binding. Samples of cytoskeletons were also incubated with secondary antibody only, to further demonstrate specificity. The preparations were examined in a Zeiss microscope equipped with epifluorescence illumination.

For immunofluorescence, monoclonal anti-tubulin was diluted 1:100, monoclonal anti-actin was diluted 1:200,

polyclonal anti-actin was diluted 1:25, polyclonal anti-vimentin was diluted 1:100, polyclonal anti-chicken αspectrin was diluted 1:300, polyclonal anti-MAP 2 was diluted 1:50, anti-ankyrin was diluted 1:5 and 1:20, anti-human spectrin was diluted 1:5 and 1:20. Secondary antibodies (IgG) conjugated to fluorescein (rabbit anti-rat, goat anti-rabbit and rabbit anti-mouse) were all diluted 1:50.

Transmission Electron Microscopy

For thin section electron microscopy, cells were simultaneously lysed and fixed for 1 hour in LyM containing 1% glutaraldehyde. Fixed cells were then washed in two changes of sodium phosphate buffer (pH6.8 and pH6.0, respectively), post-fixed in 0.1% osmium tetroxide in sodium phosphate buffer (pH6.0) for 45 minutes (on ice), rinsed in distilled water, and then carried through alcohol dehydration (50%-100% ethanol). Post-fixation in osmium tetroxide at low temperature preserves actin filaments where present (Maupin-Szamier et al., 1978). Cells were embedded in Epon 812, sectioned on a Dupont-Sorvall MT-2 ultramicrotome, stained with saturated uranyl acetate in 50% ethanol and Reynold's lead citrate and viewed with a Hitachi HS-8 electron microscope.

For negative staining of MTs, samples were incubated on Formvar-coated grids for 5 minutes, fixed in 2.5% glutaraldehyde in LyM wash for 5 minutes, rinsed with LyM wash, and stained with 2% aqueous uranyl acetate.

Scanning Electron Microscopy

Cells were fixed in suspension in 1% glutaraldehyde in MBL artificial sea water for 1 hour, placed on polylysine-coated glass coverslips (0.1%polylysine) and incubated for 10 minutes. Coverslips were then carried through alcohol dehydration, critical point drying (Tousimis SAM-DRI 780) and coating with gold/palladium (Tousimis SAMSPUTTER). Samples were viewed with the JEOL T300 scanning electron microscope (20 kV).

Temperature-cycling of Erythrocytes

A single erythrocyte suspension was divided into three equal-sized samples containing equal numbers of cells. One sample, designated the room temperature (RT) control, was extracted in LyM containing Triton. The two remaining samples were incubated at 0°C for 3 hours to disassemble the MBs. Following this treatment, one of the two samples was lysed, while the final erythrocyte sample was rewarmed for 1 hour at 18-20°C to reassemble MBs. Following RT incubation, this sample was also lysed. All cytoskeleton samples were ultimately prepared for either gel electrophoresis or stained with monoclonal anti-tubulin as described above, for indirect immunofluorescence microscopy.

Time course studies of MB reassembly and disassembly were also carried out on Anadara ovalis and Noetia ponderosa erythrocytes. Red cell samples were incubated for 3 hr at 0°C to disassemble the MBs, then rewarmed at 18-20°C. Cell samples were then removed and lysed at several time points of

rewarming from 0 to 60 min. The resulting cytoskeletons were incubated with monoclonal anti-tubulin for immunofluorescence microscopy as described above. Similarly, cell samples were taken every 30 min for 3 hr from an erythrocyte suspension incubated at $0^{o}C$ to study MB disassembly. These cell samples were lysed in LyM containing Triton and prepared for immunocytochemistry using monoclonal anti-tubulin.

Preparation of Erythrocytes With MBs at $0^{o}C$

Taxol-stabilized MBs: The MT-stabilizing drug taxol (prepared in DMSO and stored frozen as a 5mg/ml stock) was added to cell suspensions to a final concentration of 10μg/ml ($1.2X10^{-6}M$) (Schiff et al.,1980). These cell suspensions were then incubated for 3 hours at $0^{o}C$ (Fig.1). Control cell suspensions contained an equivalent concentration of DMSO without taxol. The taxol-treated and control cell suspensions were lysed in LyM containing Triton and prepared either for immunocytochemistry with monoclonal anti-tubulin (to verify the presence or absence of MBs) or for gel electrophoresis. Mechanical stress experiments were carried out on suspensions of intact cells with and without MBs.

Taxol-induced MB reassembly: Cells were incubated at $0^{o}C$ for 3 hours to disassemble MBs. Taxol was then added to the

Figure 1. Experimental protocol for the preparation of cells with and without marginal bands (MBs) at 0°C: Taxol stabilization of MBs.

①

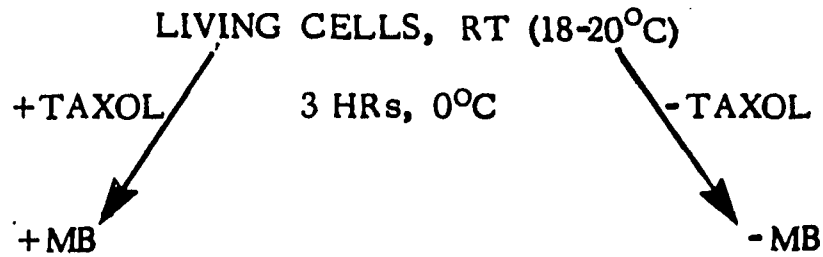
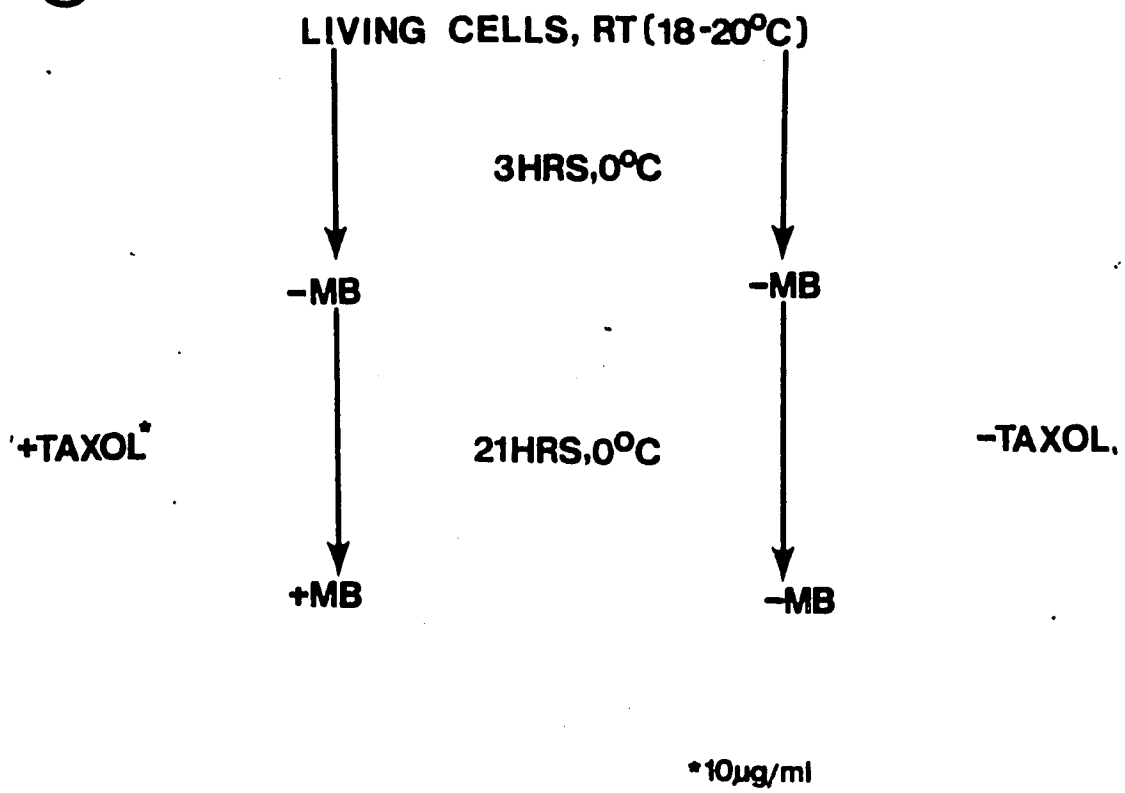


Figure 2. Experimental protocol for the preparation of cells with and without MBs at $0^{o}C$: Taxol induction of MBs.

(2)



*10μg/ml

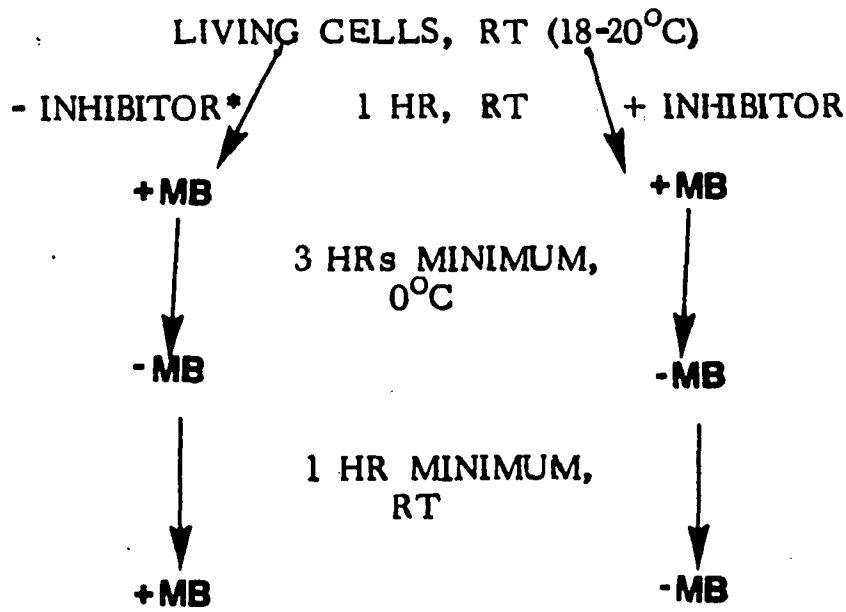
cell suspensions (final conc. ranging from 1-10μg/ml) and incubated for 21 hours at 0°C (Fig. 2). Control cell suspensions were incubated in DMSO without taxol. Samples of taxol-treated and control erythrocytes (with and without MBs) were prepared for gel electrophoresis, immunocytochemistry with monoclonal anti-tubulin, or embedded and sectioned for transmission electron microscopy to study MB reassembly at 0°C. The 21 hr incubation period was found to be adequate for reassembling MBs. Mechanical stress experiments were carried out on cell suspensions with and without MBs.

Preparation of Erythrocytes without MBs at Room Temperature

Erythrocytes were incubated with either 0.1mM colchicine (prepared in H_2O and stored frozen as a 0.1M stock) (Taylor, 1965) or 10 μg/ml ($3.3X10^{-6}M$) nocodazole (prepared in DMSO and stored frozen as a 2mg/ml stock) (deBrabander et al., 1976) for 1 hour at room temperature (20-23°C and subsequently incubated at 0°C for 3 hours to disassemble the MBs. These cell suspensions (which still contained inhibitors) were then rewarmed for 1 hour at 18-20°C (Fig. 3). Control samples were incubated with equivalent amounts of DMSO (without colchicine or nocodazole) and were temperature-cycled in the same manner. Samples of these erythrocytes were then lysed in LyM containing Triton and prepared for immunocytochemistry with monoclonal anti-tubulin to verify the presence or absence of the MB. Lysed cell samples were

Figure 3. Experimental protocol for the preparation of cells with and without MBs at 20°C: Colchicine and nocodazole induction of MB reassembly.

(3)



*0.1mM colchicine, or 10ug/ml nocodazole

also prepared for gel electrophoresis, while whole cell samples were used for mechanical stress experiments.

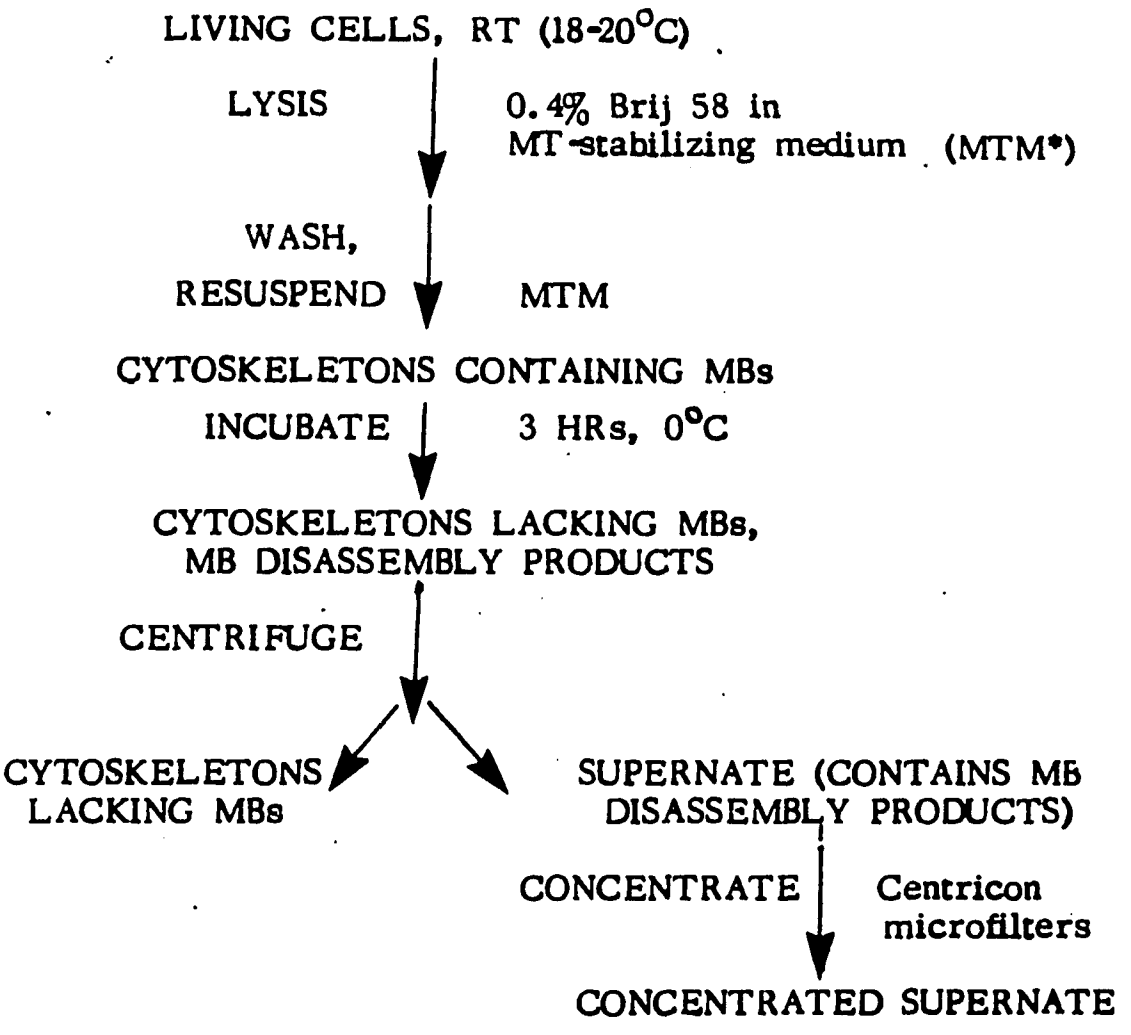
0°C MB Disassembly in a Lysed Cell Model

Washed erythrocytes (approximately 0.5ml packed cells was obtained from 2 large clams) were centrifuged for approximately 1 minute at 1850g in an International clinical centrifuge. The resulting cell pellet was resuspended to the original cell suspension volume in LyM containing 0.6% Brij-58, and then centrifuged at approximately 900g for 15 minutes to collect the cytoskeletons. The hemoglobin-containing supernatant was removed, and these cytoskeletons were then washed in LyM wash containing 0.1mM PMSF, resuspended in 1 to 2 ml LyM wash and incubated at 0°C for 3 hours. The cytoskeletons were then centrifuged at 1850g for 20 minutes at 4°C and the supernatant concentrated at 4°C by ultrafiltration (Centricon microconcentrators; Amicon Corp., Danvers, Mass.). Supernatants were concentrated approximately 10 times by this method (Fig. 4), which provided samples with sufficient protein concentration for visualization by Coomassie stains of polyacrylamide gels.

In some experiments, Brij-lysed cell suspensions were divided so that one-half the sample contained 10μg/ml taxol while the other contained an equivalent volume of DMSO. Both samples were incubated at 0°C as described above. The cytoskeletal composition of these samples following low temperature treatment was analyzed by SDS-PAGE.

Figure 4. Experimental protocol for isolation of MB disassembly products.

④


LIVING CELLS, RT (18-20°C)
LYSIS
0.4% Brij 58 in
MT-stabilizing medium (MTM*)
WASH,
RESUSPEND
MTM
CYTOSKELETONS CONTAINING MBs
INCUBATE
3 HRs, 0°C
CYTOSKELETONS LACKING MBs,
MB DISASSEMBLY PRODUCTS
CENTRIFUGE
CYTOSKELETONS
LACKING MBs
SUPERNATE (CONTAINS MB
DISASSEMBLY PRODUCTS)
CONCENTRATE
Centricon
microfilters
CONCENTRATED SUPERNATE

In other experiments, 1mM GTP was added to the concentrated supernatant and the solution was incubated for 1 hour at 37°C in an attempt to polymerize MB tubulin. Samples of the supernatant were taken before and after incubation and were negatively stained for electron microscopy. Following incubation, the supernatant was centrifuged at room temperature (at approximately 15,000g) in a Beckman microfuge (Beckman Spinco, Palo Alto, California) for approximately 15 minutes. The resulting pellet and supernatant were both prepared for gel electrophoresis.

The Effect of Mechanical Stress on Cells with or without MBs

Suspensions of cells with or without MBs were fluxed ten times through 10μl capillary tubes (400μm diameter, Dade Diagnostics, Aguada, Puerto Rico). The flow rate was approximately 0.5ml/sec on efflux. Cells were immediately fixed by diluting 1:1 in 2% glutaraldehyde in MBL sea water and viewed by phase contrast microscopy. A minimum of 100 cells were scored for normal versus folded appearance for each experimental condition.

Observations on Cells in Flow

Clumps of aggregated amebocytes and trapped red cells obtained during blood collection served as convenient material for observations on cells in flow. Cell clumps were flattened under coverslips and viewed with phase contrast optics. Narrow channels formed spontaneously within the aggregates, in which the deformation of individual moving

erythrocytes could be followed.

Production of Anadara ovalis Erythrocytes With Abnormal Morphology

Erythrocytes with abnormal morphology were obtained in one of two ways: 1.) Cells were collected without dilution. Approximately 15-30 minutes following collection, these cells had abnormal morphology. This procedure was used for experiments in which cytoskeletal morphology of normal and abnormal cells was compared by immunofluorescence microscopy and thin section electron microscopy. 2.) Normal washed erythrocyte pellets were incubated in supernatant from abnormal cell samples. This procedure was devised for experiments in which the role of the MB in the generation of abnormal cell morphology was studied. To prepare suspensions of abnormal cells in this way, normal, washed erythrocytes were pelleted by centrifugation for 2 minutes at 1850g in the clinical centrifuge. The cell pellet was then resuspended to its original suspension volume in supernatant from unwashed erythrocytes (see below). Incubation of erythrocytes for 15 minutes in this supernatant provided maximum numbers of cells with abnormal morphology.

To test the effect of the supernatant on cells with and without MBs, samples containing normal cells with and without MBs were prepared using the colchicine procedure described above. Cell samples were incubated in supernatant for 15 min and were then either fixed in glutaraldehyde and

viewed by phase contrast microscopy or lysed in Triton and prepared for immunofluorescence microscopy.

Preparation of supernatant from Anadara ovalis blood: Anadara ovalis blood was collected without dilution and allowed to stand at room temperature for at least 20 minutes. Following verification by phase contrast microscopy that cells were no longer normal in shape, but had become amorphous or "potato" shaped, they were pelleted at 1850g for 5 minutes. The supernatant was then transferred to 1.5ml polypropylene tubes (Eppendorf) and centrifuged for 15 minutes in an Eppendorf microfuge (Brinkman Instruments, Westbury, N.Y.) to pellet any remaining insoluble material. This supernatant was used to induce morphological changes in normal Anadara ovalis erythrocytes.

Effect of Cytochalasins on Erythrocyte Morphology

Erythrocyte suspensions were incubated with 10μg/ml dihydrocytochalasin B (Atlas et al., 1978) or 10μg/ml cytochalasin D (Carter et al., 1967) for 3 hours at room temperature. (Both drugs were stored frozen as 10mg/ml stocks in DMSO.) Following incubation, cell samples were diluted 1:1 with 2% glutaraldehyde in MBL sea H_2O to fix whole cells. Other samples were lysed in LyM containing Triton X-100 following incubation, and prepared for immunocytochemistry with polyclonal anti-actin or monoclonal anti-tubulin. Control studies substituted equivalent amounts of DMSO without cytochalasin for the drug treatments.

RESULTS

PROTEIN COMPOSITION OF ERYTHROCYTE CYTOSKELETONS

Cohen et al. (1980) reported that the cytoskeletons of "blood clam" erythrocytes consist of a marginal band (MB) of microtubules (MTs) and a surface-associated cytoskeleton (SAC) (Figs.5,6,7). The protein composition of these cytoskeletal elements was characterized as part of this thesis work.

Nucleated Cytoskeletons

As a first approach to characterizing the cytoskeletal components of "blood clam" erythrocytes, nucleated cytoskeletons prepared by Triton extraction under microtubule-stabilizing conditions of cells from either Noetia ponderosa or Anadara ovalis, were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 8, the major bands seen on gels stained with Coomassie Brilliant Blue migrated with bovine brain tubulin (approx. 56,000Mr), human erythrocyte α-spectrin (240,000Mr) and human erythrocyte actin (43,000Mr) (Steck, 1974). Many minor bands were also present. Analysis of cytoskeletal components using nucleated cytoskeletons was complicated by the presence of nuclear proteins.

Anucleate Ghosts and Cytoskeletons

Anucleate ghosts were prepared from erythrocytes of

Figure 5. A model showing major cytoskeletal components of the clam erythrocyte. a) Face view: The MB is enclosed by and in contact with the cell surface-associated cytoskeleton (SAC), represented by two layers of netting (arrowheads) between which the nucleus (N) is suspended. b) Edge view: The MB acts as a frame across which tension is generated within the sac. The SAC is deformable by the MB, and conforms to MB shape.

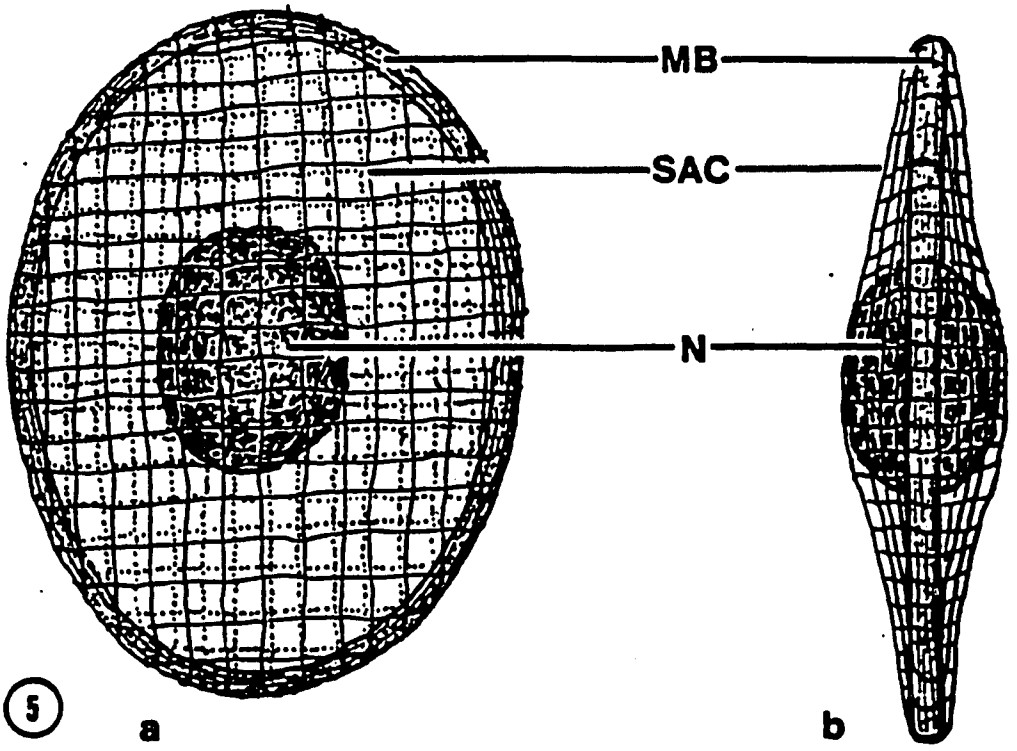


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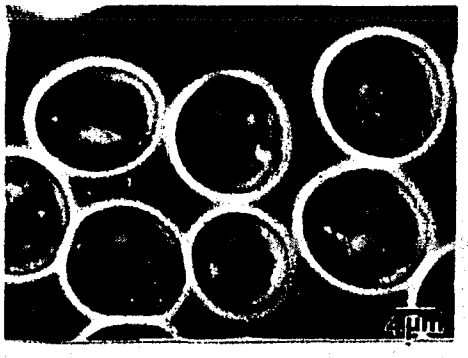
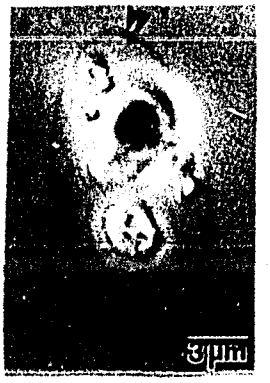
Figure 6. Noetia ponderosa erythrocytes and their cytoskeletons. a) Whole cells fixed in 1% glutaraldehyde showing typical flattened, elliptical morphology. (X 1500). b) Nucleated cytoskeletons prepared by Triton-extraction under microtubule stabilizing conditions. The MB and associated centriole pair (double arrowheads) are visible, as is the nucleus (N). Phase contrast microscopy (X 2560).

Figure 7. Anadara ovalis erythrocytes and their cytoskeletons. a) Whole cells fixed in 1% glutaraldehyde showing typical flattened, elliptical morphology (X 900). b) Nucleated cytoskeletons prepared by Triton-extraction under microtubule stabilizing conditions. Arrowhead points to centrioles, N=nucleus, MB=marginal band. Phase contrast microscopy (X 1700).

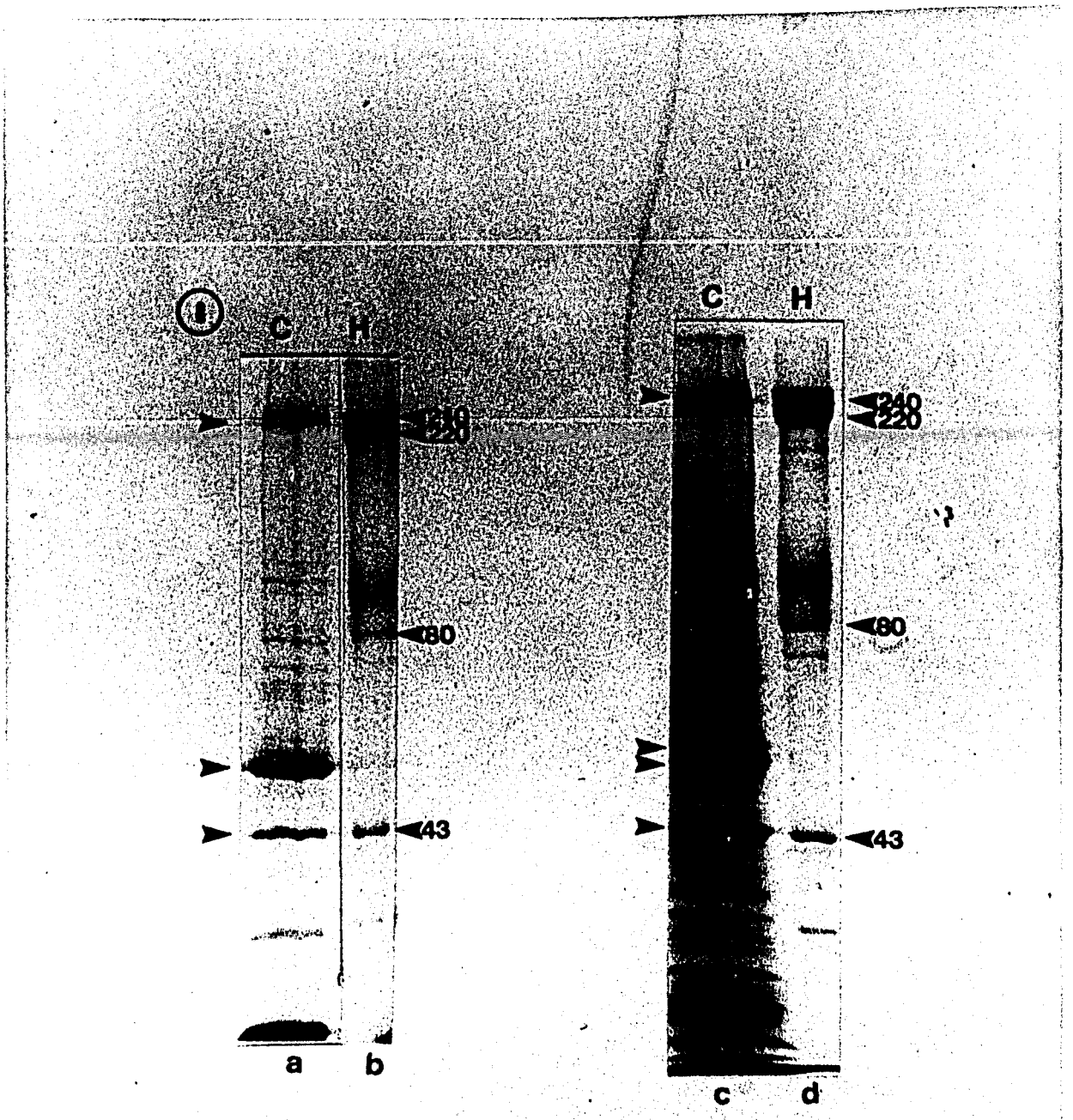
⑥





⑦

Figure 8. Protein composition of nucleated cytoskeletons prepared from "blood clam" erythrocytes (C). SDS-polyacrylamide gels (7.5% polyacrylamide) stained with Coomassie Blue. Noetia ponderosa erythrocyte cytoskeletons (lane a) and Anadara ovalis erythrocyte cytoskeltons (lane c) show 2 major bands that co-migrate with human (H) erythrocyte ghost α-spectrin (Mr 240K) and actin (Mr 43K) (lanes b and d). In addition the blood clam erythrocyte cytoskeletons contain band(s) migrating at Mr 56K (tubulin).


C
H
240
220
80
43
a
b
C
H
240
220
80
43
c
d

Noetia ponderosa by osmotic lysis in 50% LyM wash and subsequently extracted in LyM containing Triton or Brij.

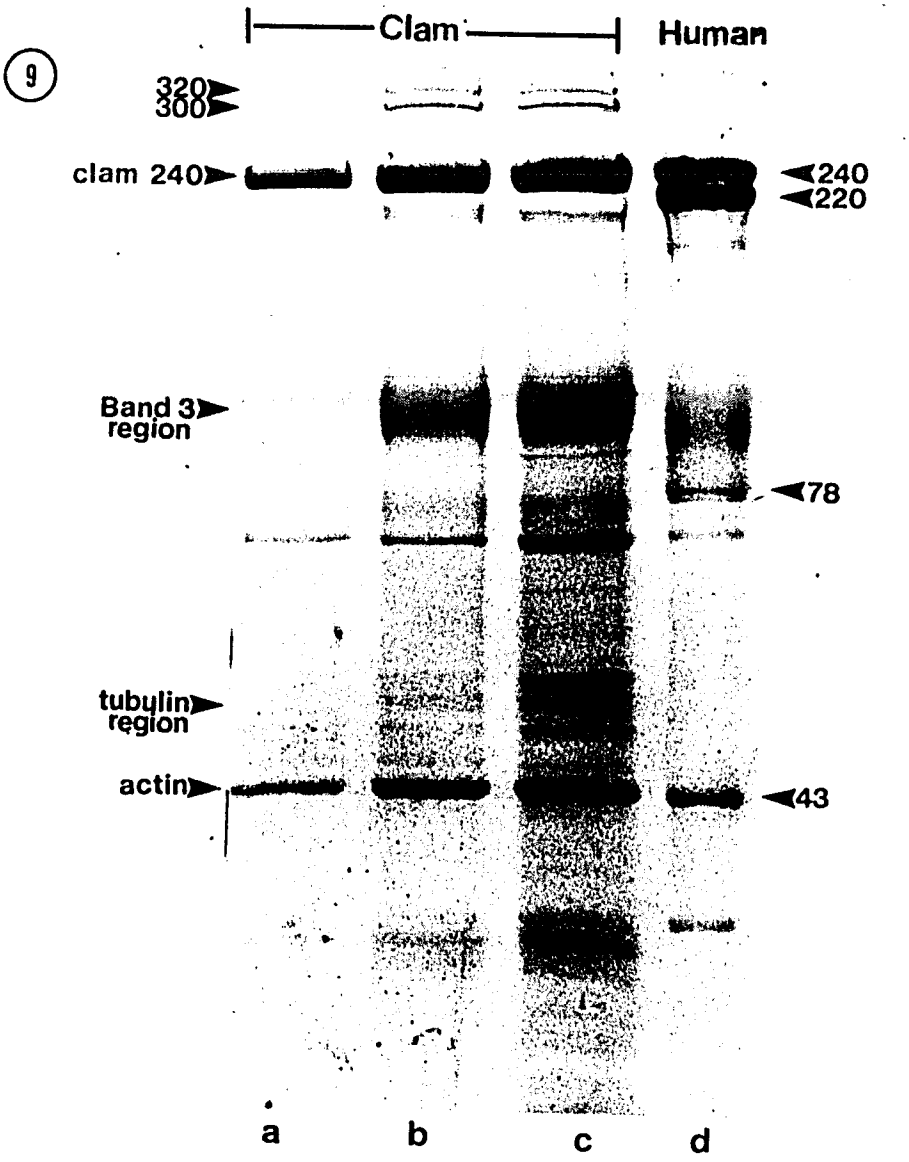
When analyzed by SDS-polyacrylamide gel electrophoresis, the protein profile of the anucleate ghosts was much simplified compared to that of the nucleated cytoskeletons described above. Fig.9c shows that the major bands which comigrated with human erythrocyte α-spectrin and actin were still present. In addition, two minor bands with apparent molecular weights of 320,000 and 300,000 were seen. Little or no tubulin was apparent in gels of anucleate ghosts or cytoskeletons (Fig.9a,b,c). Although these cytoskeletons were prepared from erythrocytes containing intact MBs, the anucleate ghosts, when viewed by phase contrast microscopy, were wrinkled and possibly contained broken MBs (Fig.10).

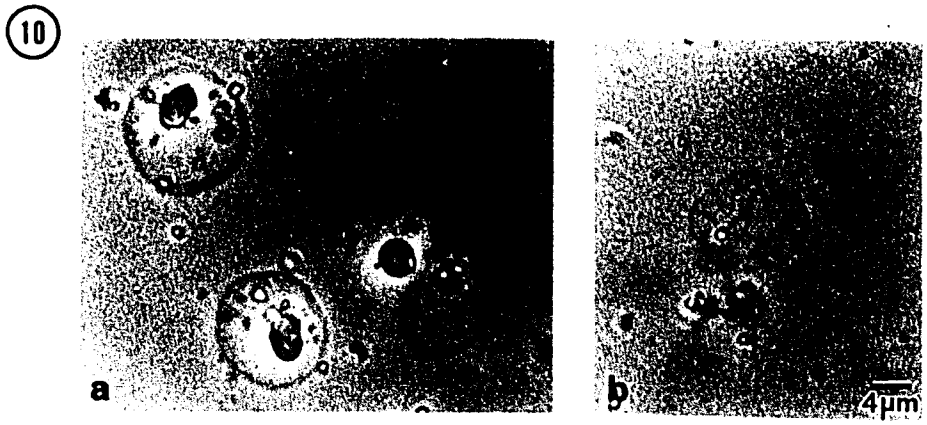
One major difference was seen in the protein profile of anucleate ghosts (Fig.9c) compared to that of anucleate cytoskeletons prepared by lysis of ghosts in MT-stabilizing medium containing 0.4% Triton X-100 (Fig.9a). A diffuse band (85,000-120,000Mr) similar to human erythrocyte band 3 was present in the ghosts but not in cytoskeletons prepared by Triton-lysis. Cytoskeletons prepared by Brij-58 (0.6%) extraction of the ghosts also contained this protein (Fig.9b).

In an attempt to optimize conditions for the retention of MTs in anucleate cytoskeletons, several modifications of the

Figure 9. Protein composition of Noetia ponderosa erythrocyte ghosts and cytoskeletons prepared with different extraction procedures. Cells were osmotically lysed, enucleated and then extracted with (a) Triton X-100, (b) Brij-58, or (c) no extraction. The major cytoskeletal components under all 3 conditions are proteins with Mr 240K and 43K (actin). Little tubulin is present. A protein behaving similarly to human erythrocyte band 3 (lane d) is seen in the anucleate ghosts (lane c) and the Brij extracted cytoskeletons (lane b) but not in the Triton extracted cytoskeletons (lane a). In addition, 2 high molecular weight proteins of Mr 320K and 300K are present under all 3 conditions.

Figure 10. Nucleated and anucleate ghosts of Noetia ponderosa erythrocytes. a) Ghosts prepared by osmotic lysis are smooth and elliptical. One of the ghosts has lost its nucleus (arrowhead). b) Ghosts prepared as in (a), then enucleated by fluxing through a 22g needle are collapsed, with short, straight, phase dense structures which may represent broken segments of the MB (arrow). Phase contrast microscopy (X 1250).


9
Clam
Human
320
300
clam 240
240
220
Band 3 region
78
tubulin region
actin
43
a
b
c
d


10
a
b
4μm

original procedure for their preparation were implemented. As shown in Table I, neither washing cells in Ca^{++}-free MBL sea H_2O prior to lysis nor increasing the EGTA concentration in 50% LyM wash from 2.5 to 10mM facilitated MB retention. Pre-incubation of cell suspensions with 10μg/ml taxol prior to osmotic lysis in medium containing taxol did not stabilize the MB. Use of a 20 gauge needle for the enucleation step did not supply sufficient force for enucleation, nor did use of a size A Dounce homogenizer unless more than 50 strokes were used, and in this case the MBs were not retained in the cytoskeletons. When any mechanical force sufficient to enucleate ghosts was used, MBs were disassembled.

Identification of Cytoskeletal Proteins by Antibody Binding

Since "blood clam" erythrocyte cytoskeletal structure was similar to that of mammalian and non-mammalian vertebrate erythrocytes, and since protein profiles also appeared similar by SDS-polyacrylamide gel electrophoresis, we determined whether any of the "blood clam" erythrocyte cytoskeletal proteins were antigenically similar to the known cytoskeletal proteins with which they co-migrated (Table II). "Blood clam" erythrocyte cytoskeletal proteins were separated on SDS-polyacrylamide gels and then transferred to nitrocellulose where they were probed with antibodies against cytoskeletal proteins.

TABLE I

METHODS ATTEMPTED TO PREPARE ANUCLEATE GHOSTS

CONTAINING MARGINAL BANDS

Cell washing media	Osmotic lysis media	Mechanical enucleation	Result
MBL sea H_2O	LyM wash*	22g needle	No MB
Calcium-free sea H_2O	Increased EGTA (15mM)	22g needle	No MB
Calcium-free sea H_2O	LyM wash	20g needle	Nuclei and MB present
Calcium-free sea H_2O	LyM wash	Homogenizer	No MB
Calcium-free sea H_2O	Increased EGTA (10mM)	Homogenizer	No MB
Calcium-free sea H_2O + taxol	Increased EGTA (10mM) + taxol	22g needle	No MB

*LyM wash= 50mM PIPES, 0.5mM $MgCl_2$, 2.5mM EGTA, 5mM TAME, 0.1mM PMSF, pH6.8.

TABLE II

ANTIGENIC SIMILARITY OF CLAM CYTOSKELETAL PROTEINS TO THOSE OF OTHER ORGANISMS

Antibody to:	Reactivity by Western Blot	Immunofluorescence Localization
Avian RBC α-spectrin	Mr240K	SAC
Human RBC spectrin	neg	ND
Human RBC ankyrin	Mr200K and Mr>300K	MB; SAC; Centrioles
Yeast tubulin	Mr60K	MB
Porcine brain MAP2	Mr240K;56K; and 60K	MB; SAC
Avian muscle actin (monoclonal)	Mr43K	neg
Avian muscle actin (polyclonal)	Mr43K	SAC
Avian muscle vimentin	neg	neg

MB=marginal band; SAC=surface-associated cytoskeleton;

ND=not done; neg=no reactivity

A monoclonal anti-tubulin antibody reacted with the upper band of a doublet (Mr56,000 and Mr60,000) in the clam red blood cell cytoskeleton (Fig.11b). In the gel system used here, this band comigrated with bovine brain tubulin that also reacted with the monoclonal antibody (Fig.11e). Both monoclonal anti-chicken gizzard actin antibody and polyclonal anti-chicken muscle actin antibody reacted with a single 43,000Mr band in "blood clam" cytoskeletons (Fig.11c and 12b, respectively) although reaction with the monoclonal antibody was weak. A single band at Mr43,000 was also seen in the rabbit psoas muscle preparation used as a positive control for the monoclonal antibody (Fig.11f).

An anti-human ankyrin antibody reacted with a band at 200,000Mr in the clam erythrocyte cytoskeletal preparation, which was not visible on Coomassie stained gels. Two minor bands with apparent molecular weights greater than 300,000 also reacted weakly with the anti-ankyrin antibody (Fig.13a). A preparation of human erythrocyte ghosts was probed with this antibody, revealing a band at 215,000 (ankyrin) as well as an array of bands with molecular weights less than that of ankyrin (Fig.13c).

No antibody binding occurred when blood clam erythrocyte

Figure 11. Binding of monoclonal anti-yeast tubulin and monoclonal anti-chicken actin to Noetia ponderosa erythrocyte cytoskeletal proteins. Proteins from nucleated cytoskeletons were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Lane a) Amido Black stain of transferred proteins. Arrowheads denote tubulin (upper) and actin (lower) region of the blot. Lane b) Anti-tubulin binds to a single band at Mr 60K. Lane c) Anti-actin binds to a single band at Mr 43K. Lane d) A control purified IgG does not bind. Lane e) Binding of the anti-tubulin to bovine microtubule proteins. Lane f) Binding of anti-actin to rabbit psoas muscle proteins. Antibody binding was detected by incubating the nitrocellulose strips with horseradish peroxidase-conjugated rabbit anti-rat or anti-mouse IgG followed by color development with 4-chlor-1-naphthol and H_2O_2.

(11)

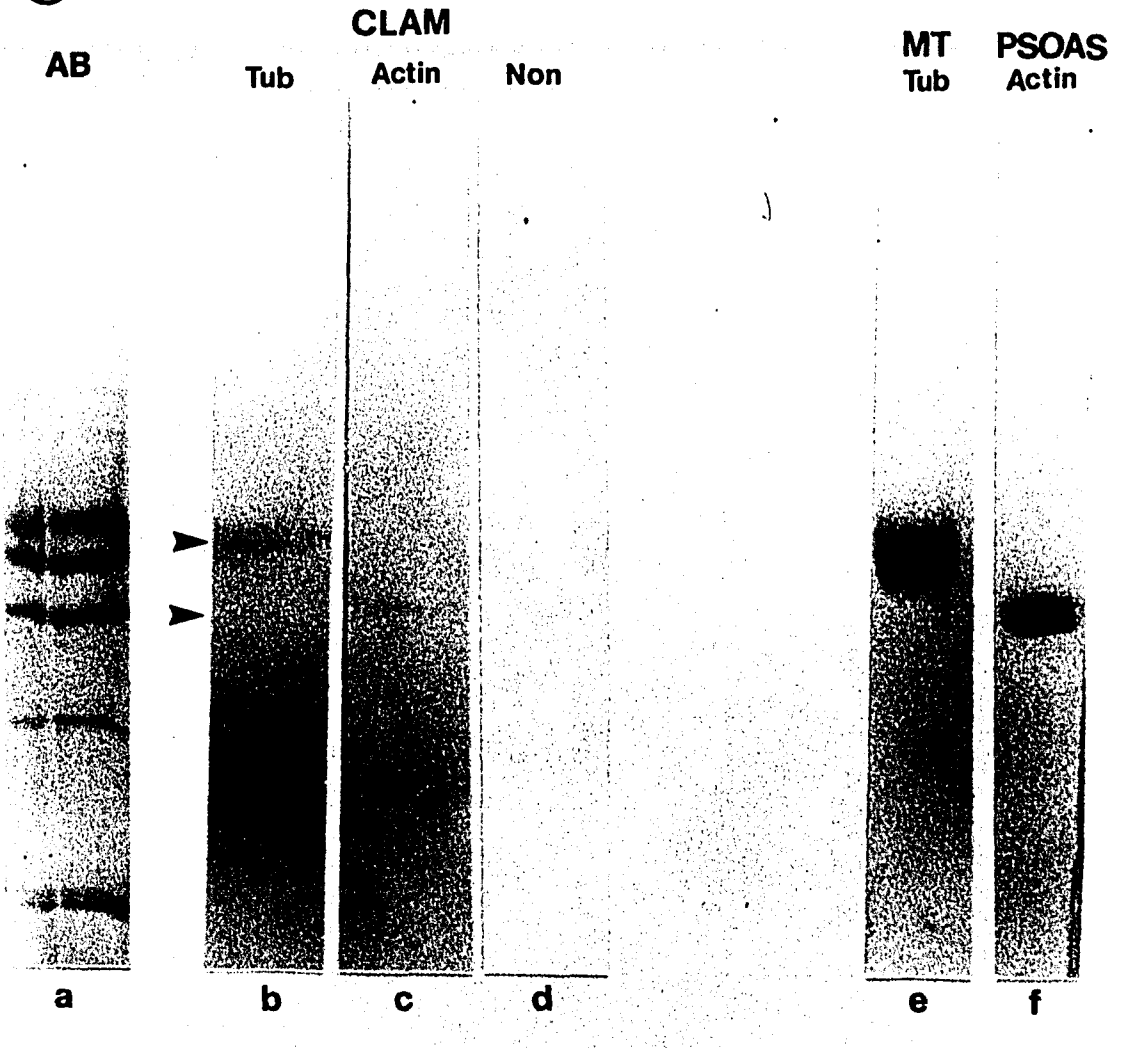
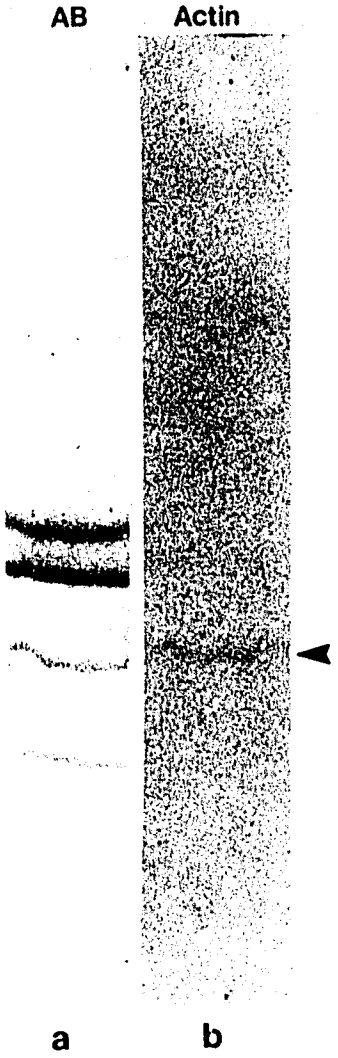


Figure 12. Binding of polyclonal anti-chicken actin to Noetia ponderosa erythrocyte cytoskeletal proteins demonstrated by the immunoblot technique. Lane a) Amido Black stain of transferred proteins. Lane b) Anti-actin binds to a single band at Mr 43K (arrowhead).

Figure 13. Binding of anti-human ankyrin and anti-human spectrin IgGs to Noetia ponderosa erythrocyte cytoskeletal proteins demonstrated by the immunoblot technique. Lane a) Anti-ankyrin binds to a band of Mr 200K (arrowhead) and to 2 bands of Mr greater than 300K (double arrowhead). Lane b) Anti-spectrin does not bind. Lanes c and d show binding of anti-ankyrin (c) and anti-spectrin to human erythrocyte ghost proteins.

(12)


AB
Actin
a
b

(13)


CLAM
HUMAN
Ankyrin
Spectrin
Ankyrin
Spectrin
a
b
c
d

cytoskeletal preparations were probed with antibodies raised against human erythrocyte spectrin (Fig.13b). Human red blood cell ghosts probed with the same antibody served as a positive control (Fig.13d).

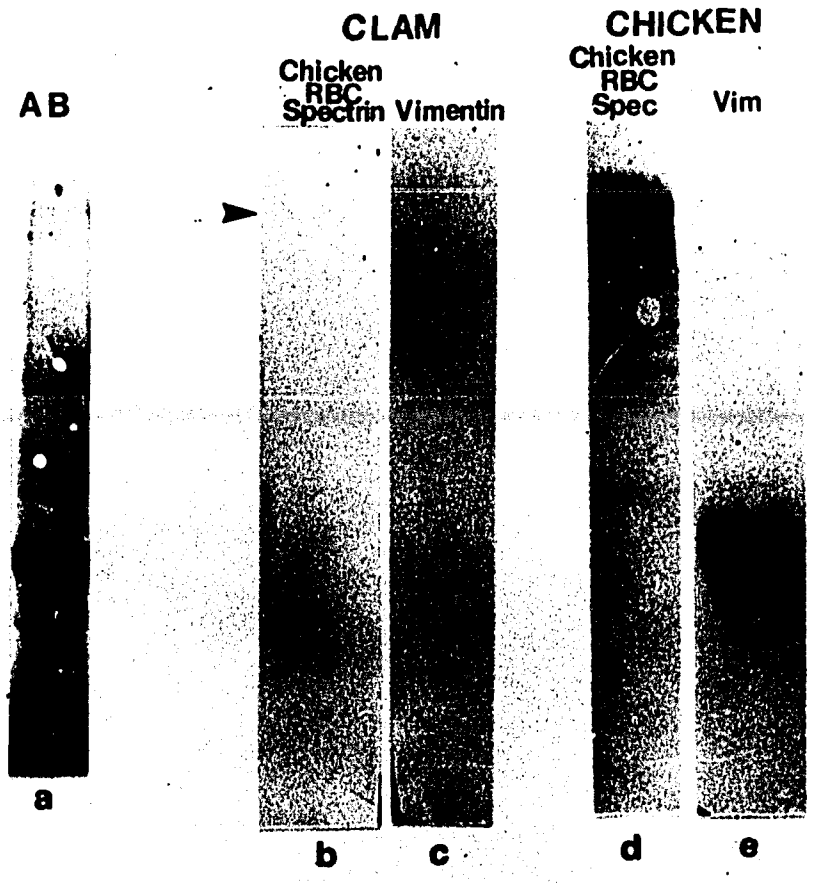
An antibody against chicken erythrocyte " α-spectrin" however, reacted weakly with the Mr240,000 band in a preparation of clam anucleate cytoskeletal proteins separated by SDS-PAGE and transferred to nitrocellulose (Fig.14b). When chicken erythrocyte cytoskeletal proteins were probed with this antibody the α-spectrin band, as well as several minor bands with apparent molecular weights of less than 240,000 bound the anti-"α-spectrin" antibody (Fig.14d).

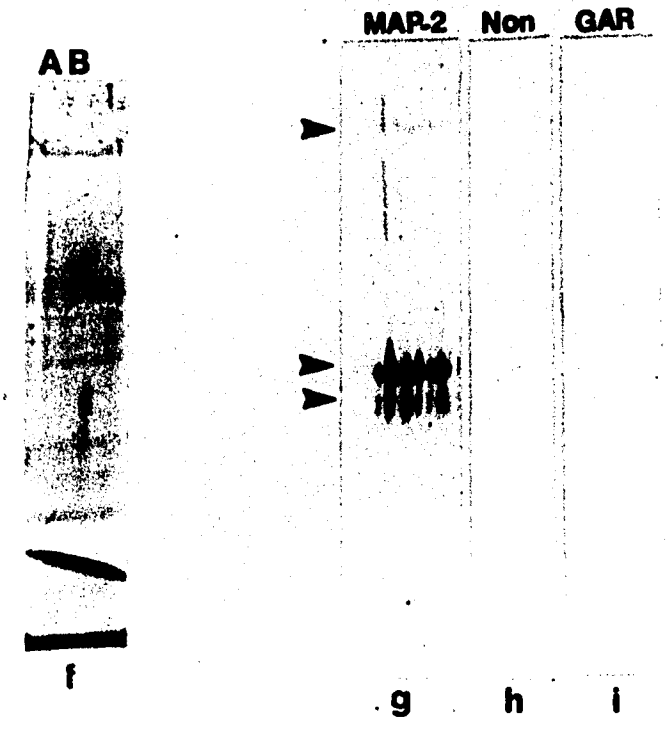
When anti-embryonic chicken muscle vimentin antibody was used to probe blood clam erythrocyte cytoskeletons prepared under IF-stabilizing conditions, no binding occurred (Fig.14c), although chicken erythrocyte cytoskeletons prepared in the same way contained a band at approximately 52,000Mr in the polyacrylamide gel system used here, which reacted with the antibody (Fig.14e).

Anti-hog brain MAP2 antibody reacted with a band at Mr240,000 as well as with two bands in the tubulin region of the gel in anucleate cytoskeleton preparations (Fig.14g). Experiments in which a preparation of purified bovine brain MT protein was probed with this antibody as a positive control revealed binding to 2 bands with apparent molecular weights of greater than 300,000.

Figure 14. Binding of anti-chicken erythrocyte α-spectrin IgG, anti-chicken vimentin IgG and anti-hog brain MAP2 to Noetia ponderosa erythrocyte cytoskeletal proteins demonstrated by the immunoblot technique. Lane a) Amido Black stain of transferred proteins from nucleated cytoskeletons. Lane b) Anti-chicken erythrocyte "α-spectrin" IgG binds to a single band of Mr 240K (arrowhead) in a clam erythrocyte anucleate cytoskeletal preparation. Lane c) Anti-chicken vimentin does not bind to clam cytoskeletal proteins prepared under intermediate filament stabilizing conditions. Lanes d and e) Binding of the anti-"α-spectrin" (d) and anti-vimentin (e) IgGs to chicken erythrocyte cytoskeletal proteins. Lane f) Amido Black stain of transferred proteins from anucleate cytoskeletons. Lane g) Anti-hog brain MAP2 binds to a band of Mr 240K (arrowhead) and to 2 bands of Mr approximately 56K and 60K (double arrowheads) in clam erythrocyte anucleate cytoskeletal proteins. Non-immune IgG (lane h) or the goat anti-rabbit IgG (i) do not bind clam erythrocyte cytoskeletal proteins.

(14)


CLAM
CHICKEN
AB
Chicken RBC Spectrin
Vimentin
Chicken RBC Spec
Vim
a
b
c
d
e


AB
MAP-2
Non
GAR
f
g
h
i

Second antibody alone or non-immune serum did not react with the cytoskeleton preparations in the Western blots.

MB REASSEMBLY:ANTI-TUBULIN IMMUNOFLUORESCENCE OBSERVATIONS

We have reported previously that the MBs of the blood clam erythrocyte disassembled upon incubation at 0°C and reassembled upon rewarming at room temperature in association with a centriole pair. This was initially shown by whole mount and thin section electron microscopy (Nemhauser, Joseph-Silverstein, and Cohen, 1983). Immunofluorescence microscopy using an anti-tubulin antibody verified the reassembly sequence and allowed us to survey more cells than possible with electron microscopy. After incubation of Noetia ponderosa erythrocytes at 0°C for 4 hours, greater than 99% of cells counted contained no microtubules (Fig.15b). Cells were rewarmed at 18-20°C and studied in time course samples. Reassembly was not tightly synchronized, so that various stages were present in a given sample. At the earliest time points (5-10 minutes), all of the cytoskeletons in which reassembly had begun contained microtubules focused on the centrioles, producing a pointed or pole-like appearance (Fig.15c and d). The number of such cytoskeletons reached a maximum at 5-10 minutes, after which

time recognizable MBs began to appear, many of which were still pointed (Fig.15e and f). Centriole presence at the point of microtubule "focus" was verified in each case by phase contrast microscopy.

MB reassembly in the erythrocytes of Anadara ovalis was also monitored. In this species, some microtubules or thin MBs were seen in 25% of the cells following 0°C incubation and made analysis of the data more complicated. However,a similar reassembly sequence was seen with short MTs initially radiating form the centrioles creating a pole-like appearance (Fig.16a-d), followed by the reappearance of a complete MB (Fig.16e).

Disassembly of the MB at 0°C in the erythrocytes of Anadara ovalis was also monitored by immunofluorescence microscopy using the monoclonal anti-tubulin antibody. As shown in Fig.17, the MB became thinner and thinner during disassembly until it had completely disappeared. There was no intermediate "pointed" stage present at any of the sampling times, such as that seen during reassembly,.

Figure 15. Noetia ponderosa MB disassemby and reassembly visualized by antitubulin indirect immunofluoresence of cytoskeletons. a) Noncooled control. b) Disappearance of MB and all MTs after incubation of cells for 4h at 0°C: only nuclei are visible. c) and d) Cytoskeletons after rewarming of cells at 18°C for 5 and 10 min, respectively. Early stages of MB reassembly, with MTs emanating from centriolar "points". The extent of assembly varies within a given time point. In the lower example in (d) there appears to be an extremely thin MB on the verge of continuity. e) and f) Phase contrast/fluorescence photomicrograph pair , 20 min rewarming. The MB is continuous at end of cell distal to centrioles, but it is still pointed. The centrioles lie at the point (e, arrowhead), with part of another cytoskeleton nearby. g) and h) Phase/fluorescence pair , 45 min rewarming. As observed by immunofluorescence (h), the MB is no longer pointed: it approximates the control (a) in appearance except that MTs still rediate from the centrioles (g, arrowhead) to join MB some distance away. (X 2500).

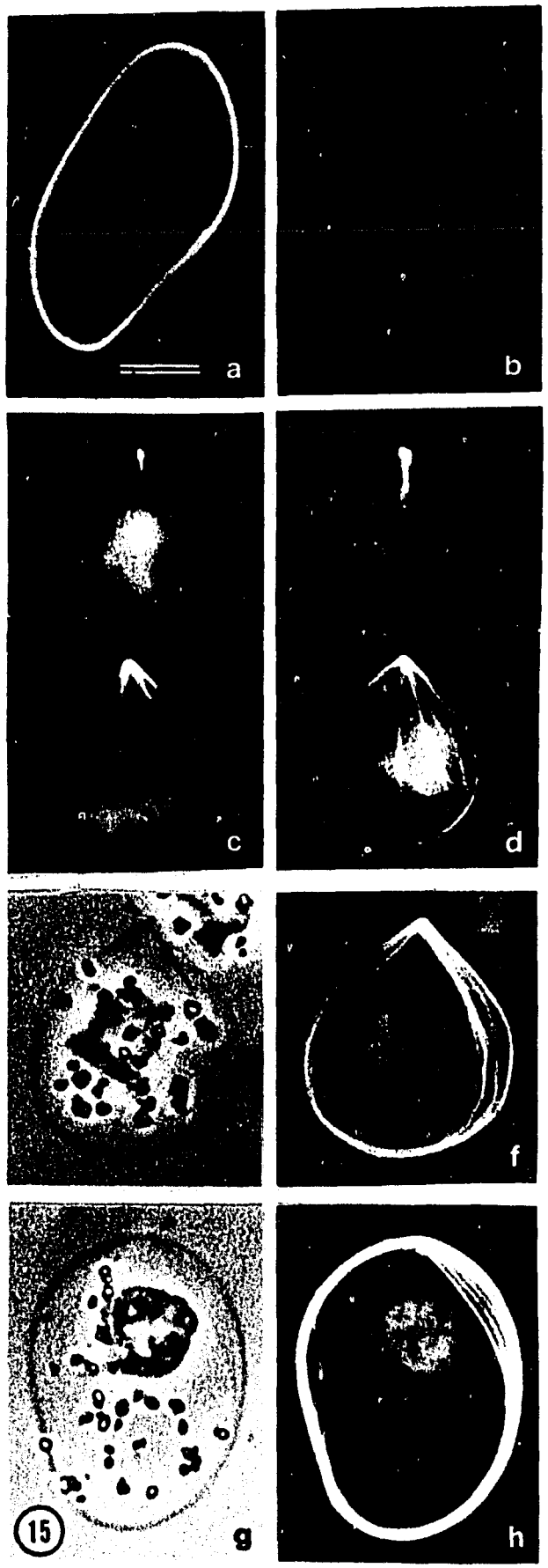

a
b
c
d
f
15
g
h

Figure 16. Anadara ovalis MB reassembly monitored by indirect immunofluorescence microscopy with anti-tubulin. Erythrocyte MBs were disassembled at 0°C and subsequently rewarmed to 20°C for 1h. Anti-tubulin immunofluorescence of nucleated cytoskeletons prepared after 2 min (a) and 10 min (c) of rewarming show MTs radiating from a single point (arrowhead) creating a pole-like appearance. The corresponding phase micrographs (b and d) show centrioles at this focal point (arrows). At 1h (e), reassembly of a complete MB is seen (arrowhead). N=nucleus. (X 2400).

16

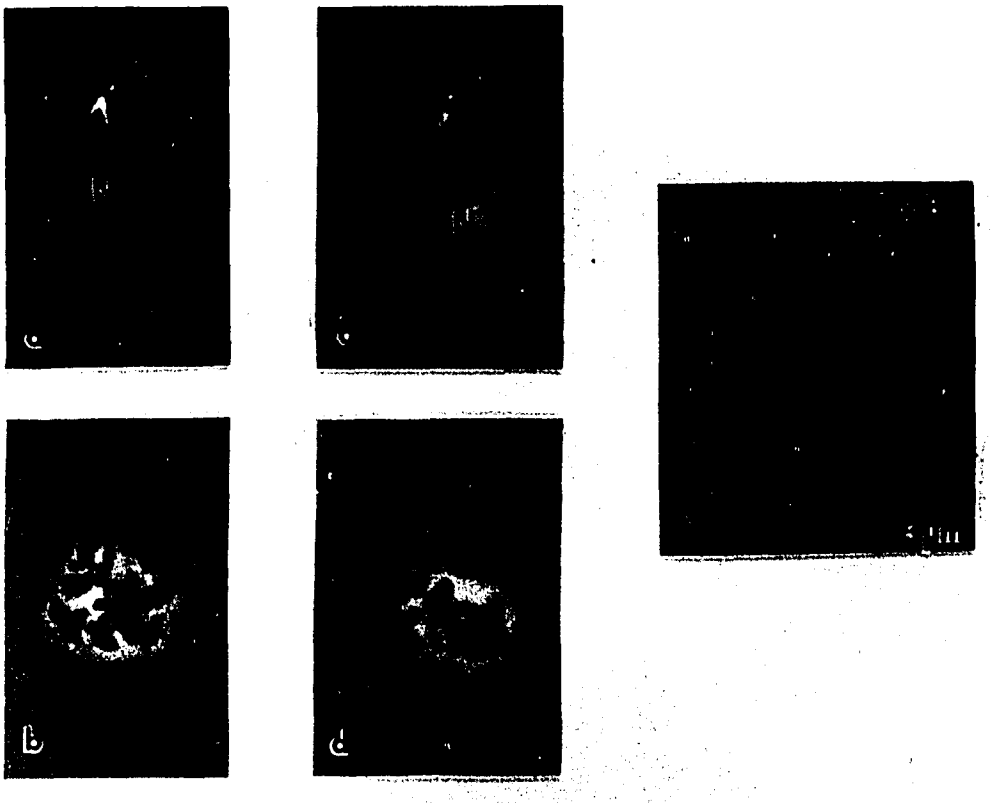
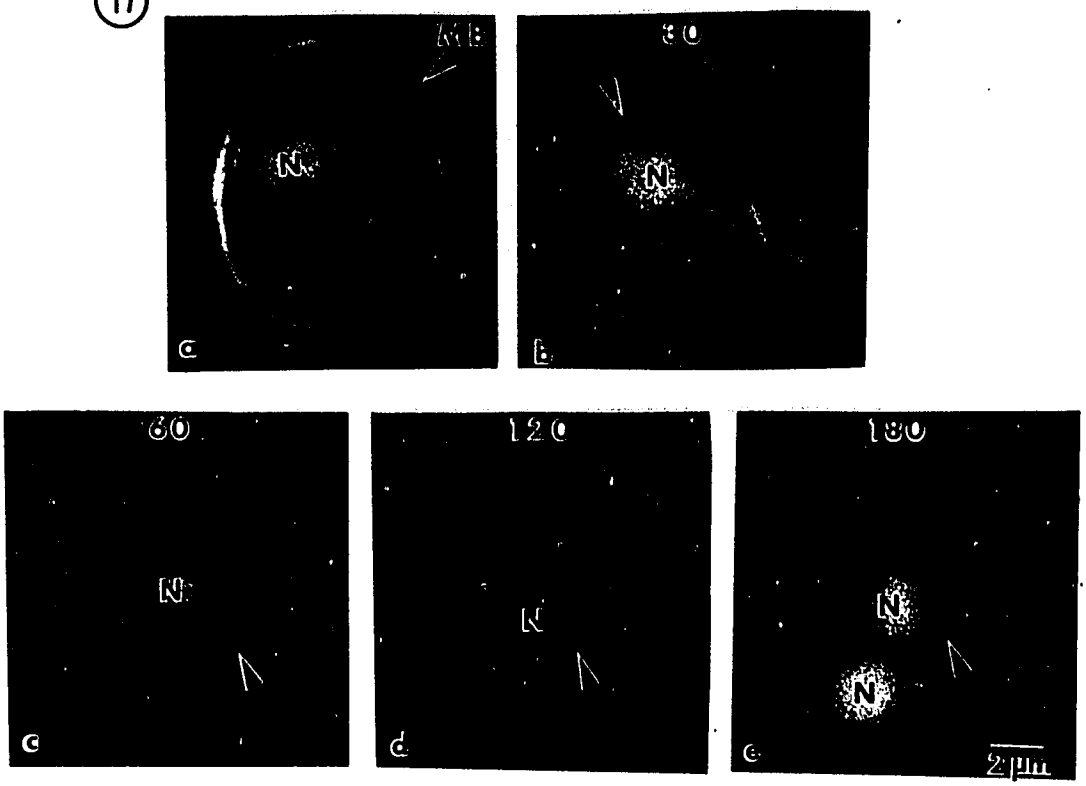


Figure 17. Anadara ovalis MB disassembly monitored by indirect immunofluorescence microscopy with anti-tubulin. a) Control erythrocyte at room temperature contains an intact MB (arrowhead). Progressive, uniform thinning of the MB (arroehead) is seen when cells are incubated at 0°C for 30 min (b), 60 min (c) and 120 min (d). At 180 min (e) no MB is seen. The arrowhead identifies a short, thin MT bundle that may be a remnant of the MB. N=nucleus. (X 2920).

(17)



LOCALIZATION OF ERYTHROCYTE CYTOSKELETAL PROTEINS

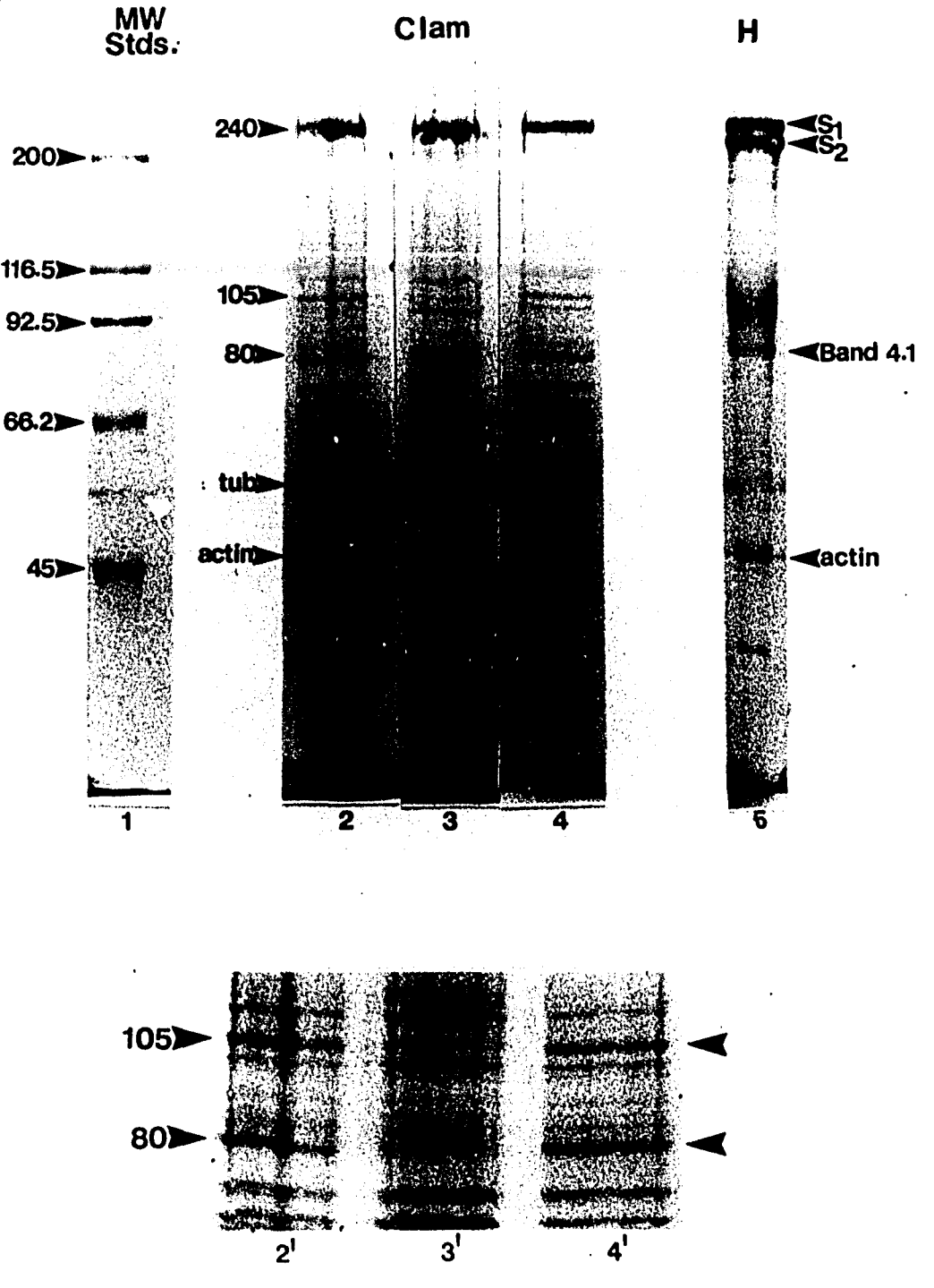
Changes in Erythrocyte Cytoskeletal Composition with Temperature-Cycling

As previously reported (Nemhauser, Joseph-Silverstein and Cohen, 1983) and demonstrated above, the MB of the blood clam erythrocyte is cold labile, disassembling at 0°C. However, whole mount and thin section electron microscopy of blood clam erythrocyte cytoskeletons following this treatment showed that the SAC is not a cold-labile structure (Nemhauser, 1980). Therefore, a tentative localization of proteins to the MB or SAC can be made by comparing the cytoskeletal composition of cells containing MBs at room temperature with those lacking MBs at 0°C.

Protein profiles of nucleated cytoskeletons prepared by Triton extraction of cells with normal and reassembled MBs were obtained by SDS-polyacrylamide gel electrophoresis and compared to those without MBs at 0°C (Fig.18). The most obvious difference was a great diminution of the tubulin doublet in the cytoskeletons lacking MBs. A minor band comigrating with human erythrocyte band 4.1 (78,000Mr) as well as one migrating at approximately 105,000Mr were conspicuous by their diminution in low temperature preparations as well. The presence of the protein with an

Figure 18. Comparison of cytoskeletal protein profiles from 20°C, 0°C and rewarmed Noetia ponderosa erythrocytes. Nucleated cytoskeleton preparations were analyzed by 7.5% SDS-PAGE. Lane 1) high molecular weight standard proteins. Lane 2) Cytoskeletons from cells at 20°C contain major bands of Mr 240K, Mr 56K (tubulin) and Mr 43K (actin). Lane 3). Cytoskeletons from cells incubated at 0°C have diminished amounts of tubulin as well as the 2 bands of Mr 80K and 105K, while the Mr 240K and 43K bands remain constant. Lane 4) Tubulin and the 80K and 105K bands reappear in cytoskeletons from cells rewarmed for 1h (after initial incubation at 0°C to disassemble the MB) . Lanes 2', 3' and 4' are enlargements of the gel regions containing the Mr 80K and 105K bands (arrowheads). Lane 5) Human erythrocyte ghost proteins.

(18)


MW Stds.
Clam
H
240
200
116.5
105
92.5
80
66.2
tub
actin
45
S1
S2
Band 4.1
actin
1
2
3
4
5
105
80
2'
3'
4'

apparent molecular weight of 240,000 and actin (Mr43,000) were not affected by temperature-cycling of the erythrocytes, tentatively localizing them to the SAC and not the MB

Immunofluorescence Localization of Cytoskeletal Proteins

To localize the proteins identified by Western blotting within the "blood clam" cytoskeleton, indirect immunofluorescence was carried out using the same antibodies (Table II). Nucleated cytoskeletons containing MBs as verified by phase contrast microscopy, were studied using the polyclonal anti-actin antibody. When viewed by fluorescence microscopy, a pattern of diffuse fluorescence throughout the cytoskeleton was seen (Fig.19a and b). Anti-actin antibody did not bind to the MB. As described previously, monoclonal anti-tubulin antibody bound only to the MB (Fig.19c and d) and no specific fluorescence was visible in cytoskeletons from cells lacking MBs.

When blood clam erythrocyte cytoskeletons were incubated in anti-"α-spectrin" antibody raised against chicken erythrocyte "α-spectrin", a very weak, diffuse fluorescence of the SAC was observed (Fig.20).

Figure 19. Localization of actin and tubulin in nucleated cytoskeletons by indirect immunofluorescence microscopy. Anti-chicken actin binds to the surface associated cytoskeleton (SAC) but not to the MB (a) (arrowhead). The MB can be seen in the corresponding phase micrograph (b). Anti-yeast tubulin binds to the MB only (c). The MB is present in the corresponding phase micrograph (d). Nuclear fluorescence is non-specific and is due to binding of the fluorescein conjugated anti-mouse or anti-rat antibodies (e and f). Nuclear fluorescence was helpful in locating cytoskeletons on the slides. N=nucleus. Phase contrast and fluorescence microscopy (X 2920).

(19)

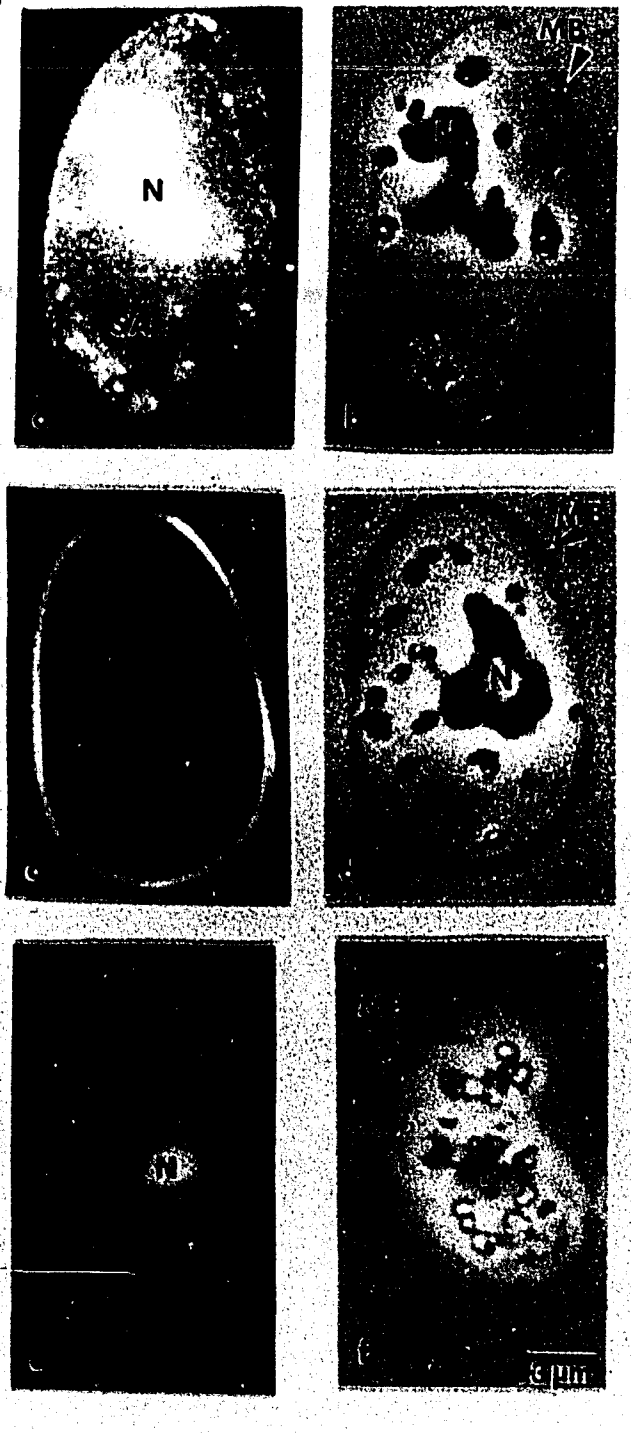
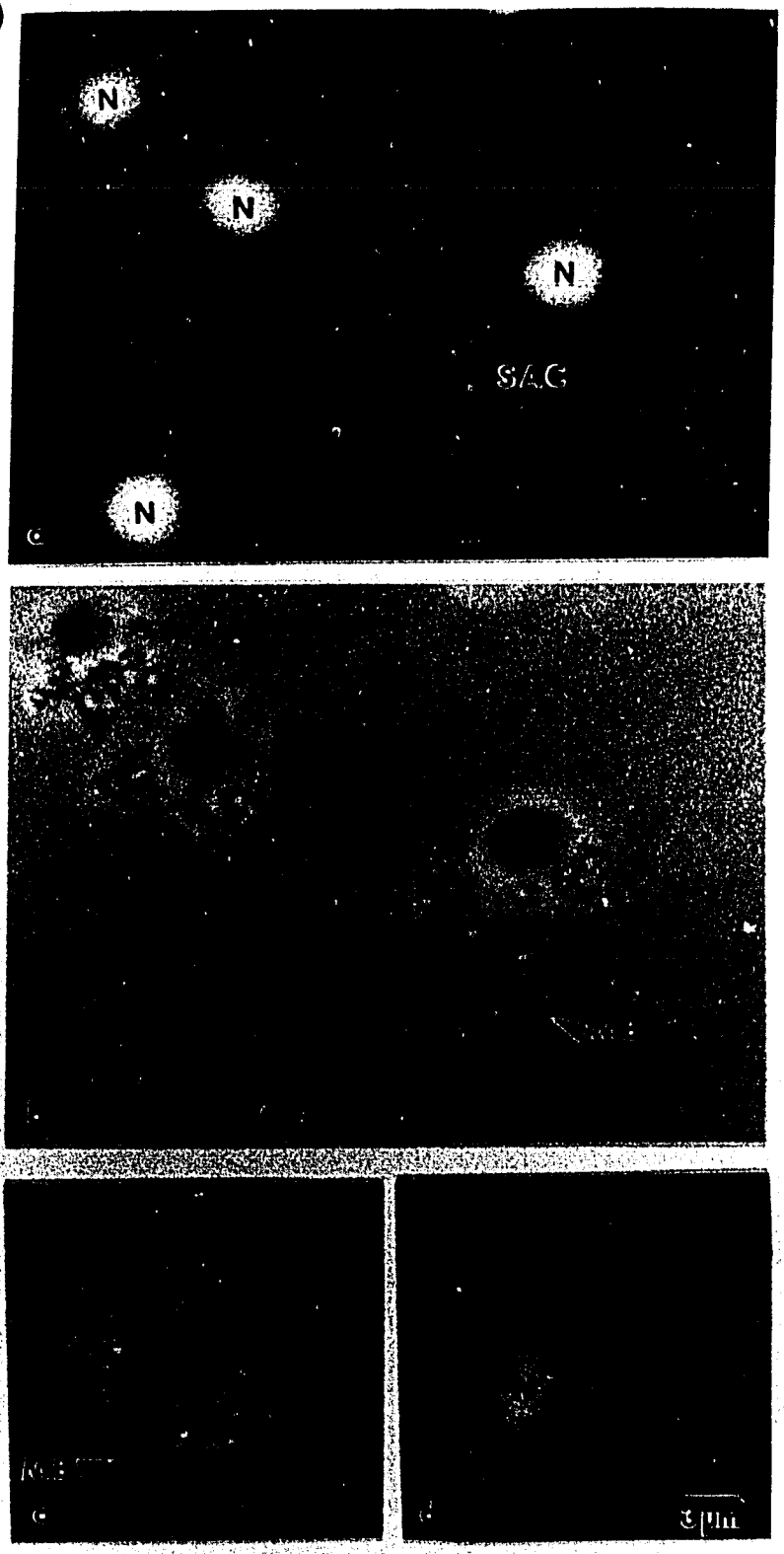


Figure 20. Anti-chicken erythrocyte "α-spectrin" binding to nucleated cytoskeletons shown by indirect immunofluorescence microscopy. Anti-chicken erythrocyte "α-spectrin" binds weakly to the SAC but not to the MB (a). The MB can be seen in the corresponding phase micrograph (b). Nuclear fluorescence is non-specific and due to binding of the fluorescein conjugated anti-rabbit antibody (c and d). N=nucleus. Phase contrast and fluorescence microscopy (X 2280).

(20)



The fluorescence pattern obtained with an anti-human ankyrin antibody was striking in that the MB, SAC and centrioles were all highly fluorescent (Fig.21a and b). The anti-MAP 2 antibody also bound to both the MB and SAC, but in this case, the fluorescence was weak (Fig.21c and d).

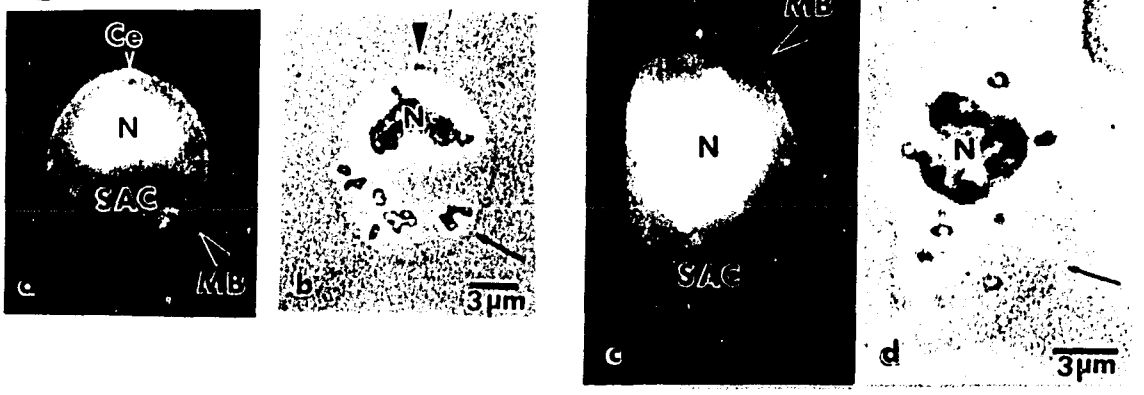
In all experiments in which nuclear fluorescence was seen, it was demonstrated to be due to the fluorescein-conjugated second antibody, as shown in experiments in which the cytoskeletons were incubated with fluorescein-conjugated second antibody only (Fig.19e and f;20c and d).

Immunocytochemistry was also done with the anti-vimentin antibody which did not bind "blood clam" erythrocyte cytoskeletal proteins in the Western blotting system described above, to determine if this antibody would bind to glutaraldehyde-fixed proteins as opposed to SDS-denatured proteins. When eythrocyte cytoskeletons were prepared under IF-stabilizing conditions and were incubated with anti-vimentin antibody, the fluorescence pattern did not differ from that obtained with non-immune serum (Fig.22b and d). Chicken erythrocyte cytoskeletons prepared under IF-stabilizing conditions and incubated with anti-vimentin antibody showed a positive reaction, with the expected filamentous staining pattern, demonstrating functionality of the antibody (Fig.22a).

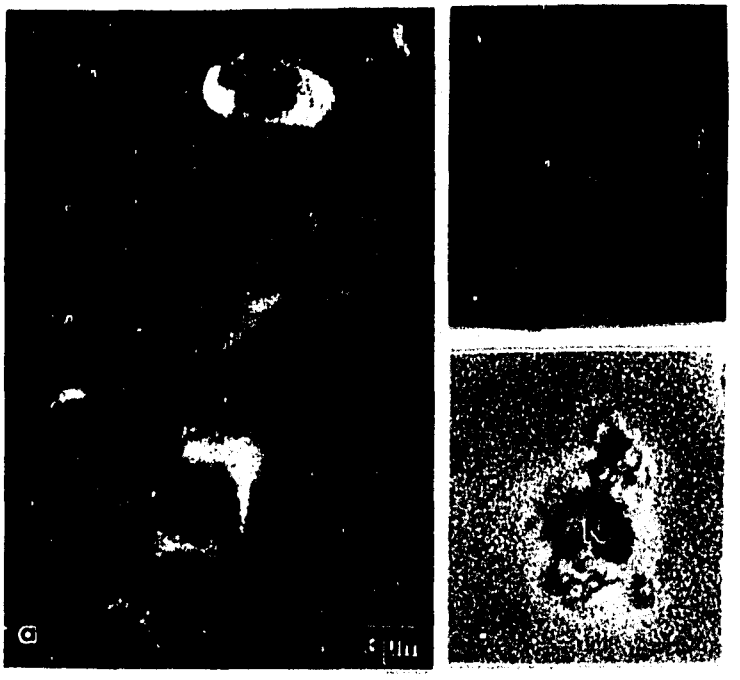
Figure 21. Anti-human ankyrin and anti-hog brain MAP2 binding to nucleated cytoskeletons shown by indirect immunofluorescence microscopy. Anti-ankyrin binds to the MB, SAC and centrioles (Ce)(a). The centrioles (arrowhead) and MB (arrow) can be seen in the corresponding phase micrograph (x 2010) (b). Anti-MAP2 binds weakly to the MB and SAC (c). The MB (arrow) can be seen in the corresponding phase micrograph (N=nucleus) (d). (X 2560)

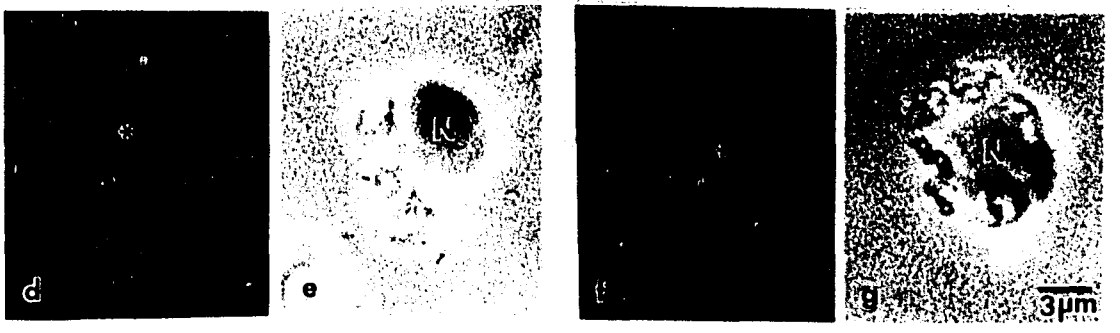
Figure 22. Anti-chicken vimentin binding to nucleated cytoskeletons prepared under intermediate filament stabilizing conditions. a) Chicken erythrocyte cytoskeletons prepared under IF stabilizing conditions contain a network of fibers binding anti-vimentin as shown by indirect immunofluorescence microscopy (X 2400). No binding of the antibody to clam erythrocyte cytoskeletons is seen (b and c). Neither nonimmune IgG (d and e) nor fluorescein conjugated anti-rabbit IgG (f and g) binds to the cytoskeletons. Asterisk denotes nuclear region of cytoskeletons. N=nucleus. (X 2400)

(21)



(22)





PREPARATION OF CELLS WITH MBs AT 0°C

Taxol-stabilized MBs

Cells incubated with taxol at 0°C for 3 hrs contained MBs that appeared to be stabilized in essentially normal condition with little or no assembly of other non-MB MTs (Fig.23a), while cells incubated at 0°C in DMSO alone contained no MBs (Fig.23b).

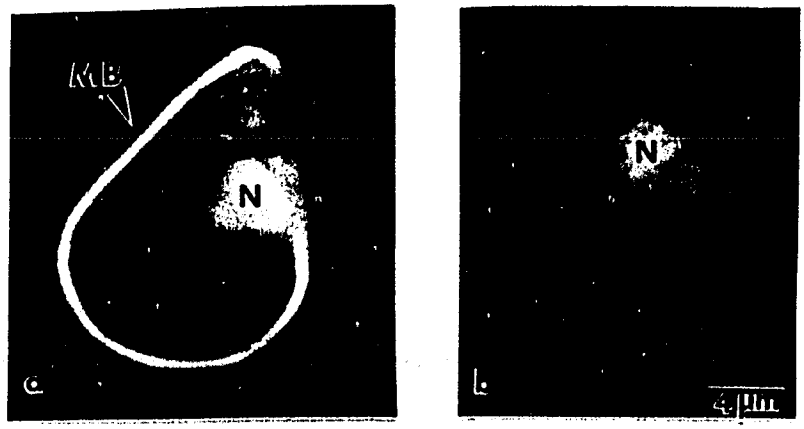
Taxol-induced MBs

It has been reported that taxol induced MT polymerization *in vitro* at 0°C (Thompson et al., 81; Hamel et al.,81) and that it overcame the inhibitory effect of nocodazole (deBrabander et al., 81). We thus studied the effect of taxol on "blood clam" erythrocytes incubated at 0°C following MB disassembly, and found that taxol induced MT polymerization in these cells. Anti-tubulin immunofluorescence demonstrated that not only did MTs reassemble at 0°C in cells incubated with 10ug/ml taxol, but the MB was reformed within 21 hours following the initial addition of taxol (Fig.24g and h). The optimum concentration of taxol necessary to induce MB reassembly within 21 hours was found to be 10μg/ml. "Blood clam" erythrocytes were incubated with varying concentrations of taxol and MT

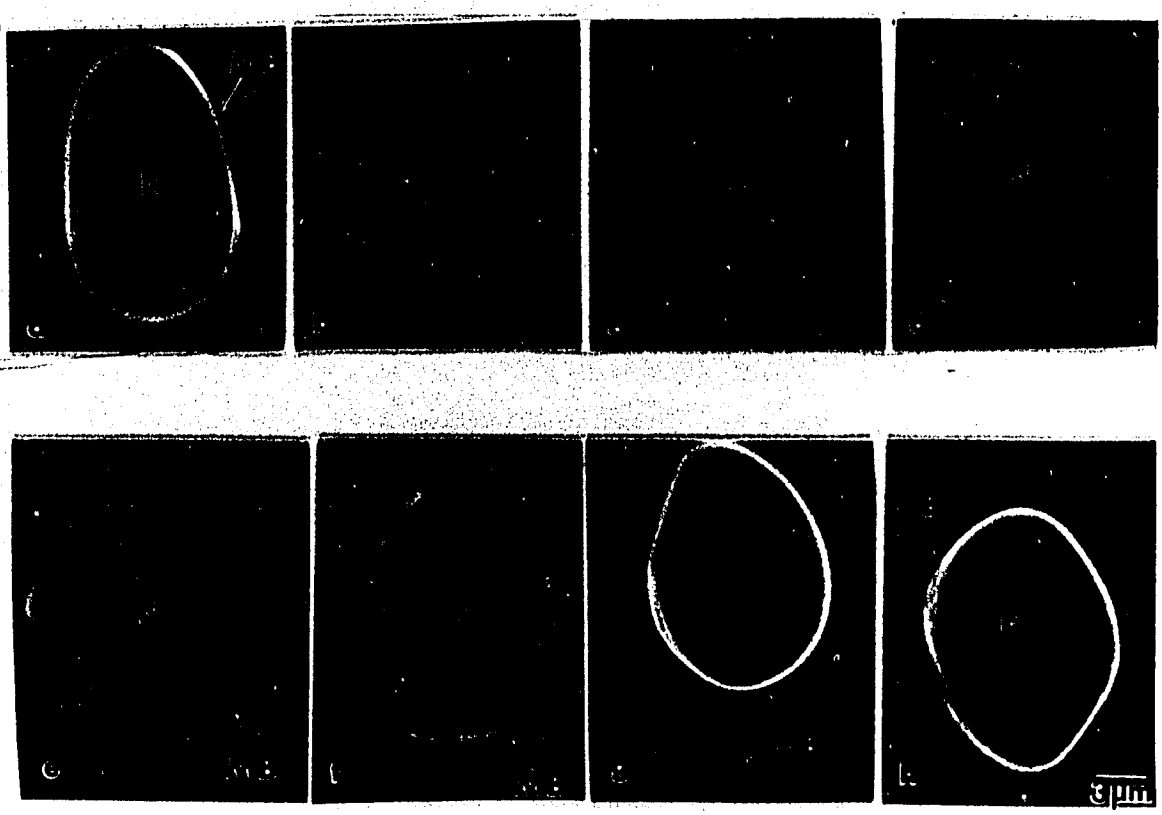
Figure 23. Taxol stabilization of MBs at 0°C as shown by indirect immunofluorescence microscopy with anti-tubulin. a) Following incubation at 0°C in the presence of 10 μg/ml taxol the MB remains intact. The MB is partially obscured by the nucleus. b) The MB disassembles in cells incubated at 0°C in the absence of taxol. (N=nucleus). (X 2400)

Figure 24. Effect of taxol concentration on MB reassembly at 0°C as shown by indirect immunofluorescence microscopy with anti-tubulin. Following 0°C incubation to disassemble the MB, cells were incubated in taxol at 0°C for 21 h. a) A control cell at RT contains a normal MB. b) After 3h at 0°C no MB is present. c) and d) When cells were incubated in 1 μg/ml taxol no MB reassembles at 21h. e) and f) When cells were incubated in 5μg/ml taxol MTs reassemble into a recognizable MB in which the MTs are not well-bundled. The double arrowheads identify MTs which are not yet incorporated into the MB. g) and h) When cells were incubated in 10μg/ml taxol, normal appearing MBs with some cytoplasmic MTs are present. (N=nucleus) (X 2200)

(23)



(24)



reassembly monitored by anti-tubulin immunofluorescence microscopy. In the presence of 5ug/ml taxol, MBs were reformed by 21 hours (Fig.24e and f). However these MBs were less tightly bundled than those incubated in 10ug/ml taxol for the same period of time (Fig.24g and h). A taxol concentration of 1 ug/ml was not sufficient to induce MB reassembly in erythrocytes at 0°C; no MBs were present by 21 hours, or at most a few short MTs could be seen (Fig.24c and d). When cells were incubated in 20μg/ml taxol, 21 hours was still required for reassembly of a normal-appearing MB.

A kinetic study of MB reassembly at 0°C in the presence of 10ug/ml taxol was carried out using immunofluorescence microscopy to monitor reassembly. By 2 hours after the addition of taxol, single small "asters" formed in the cells (Fig.25b), while by 4 hours, MT elongation resulted in cytoskeletons containing a pole-like configuration of MTs resembling a half-spindle (Fig.25c). By 21 hours at 0°C, continuous elliptical MBs had reassembled, often with some additional cytoplasmic MTs present (Fig.25d). The small "asters" which formed after 2 hours in taxol were examined in thin section and the MTs were seen to be radiating from the centrioles (Fig.26). Cross-sections through cytoskeletons from cells incubated for 21 hours in taxol (Fig.27) revealed that the MB contained fewer MTs and was

Figure 25. Taxol-induced MB reassembly at 0°C. Cells incubated at 0°C for 3h contained no MBs (a). Taxol was then added. After 2h in the presence of taxol small "asters" formed (b; arrow), and after 4h the MTs had lengthened so as to give the appearance of a pointed pole (c). By 21h complete elliptical MBs had reassembled at 0°C. Nuclear fluorescence was due to non-specific binding of the second antibody. (X 2000).

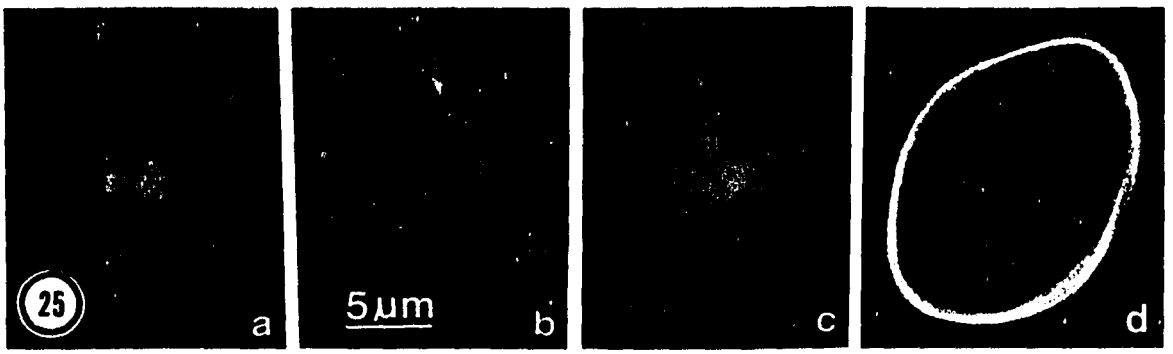

25
a
5µm
b
c
d

Figure 26. Centriole associated MT reassembly at 0°C in the presence of 10μg/ml taxol. Treatment of erythrocytes at 0°C to disassemble the MB was followed by incubation in taxol for 2h. An aster-like configuration of MTs (arrowheads) associated with a centriole (Ce) is seen. Arrowheads identify MTs radiating from the centriole. (Hb=hemoglobin, N=nucleus) TEM (X 20,300; insert X 25,000).

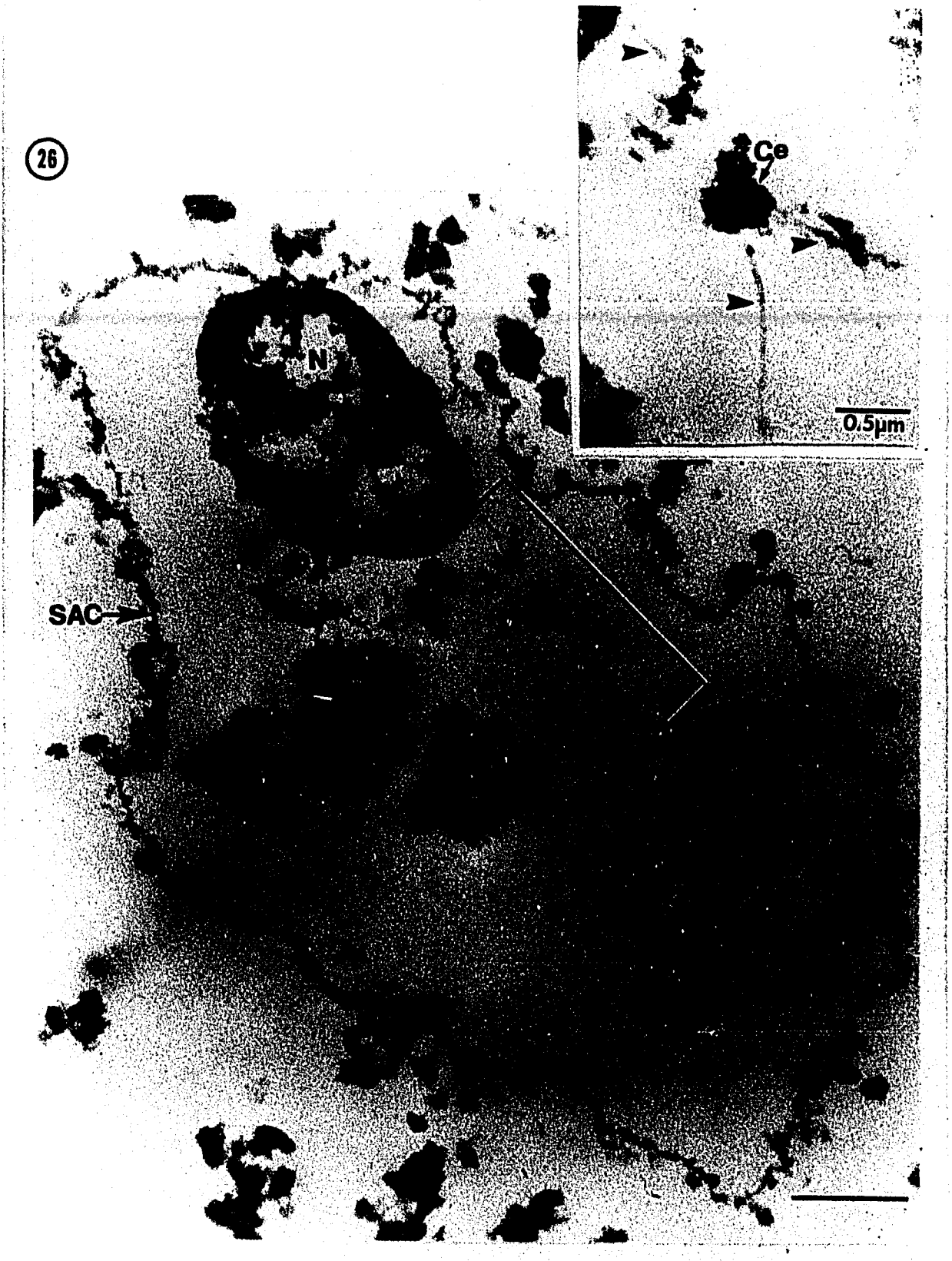
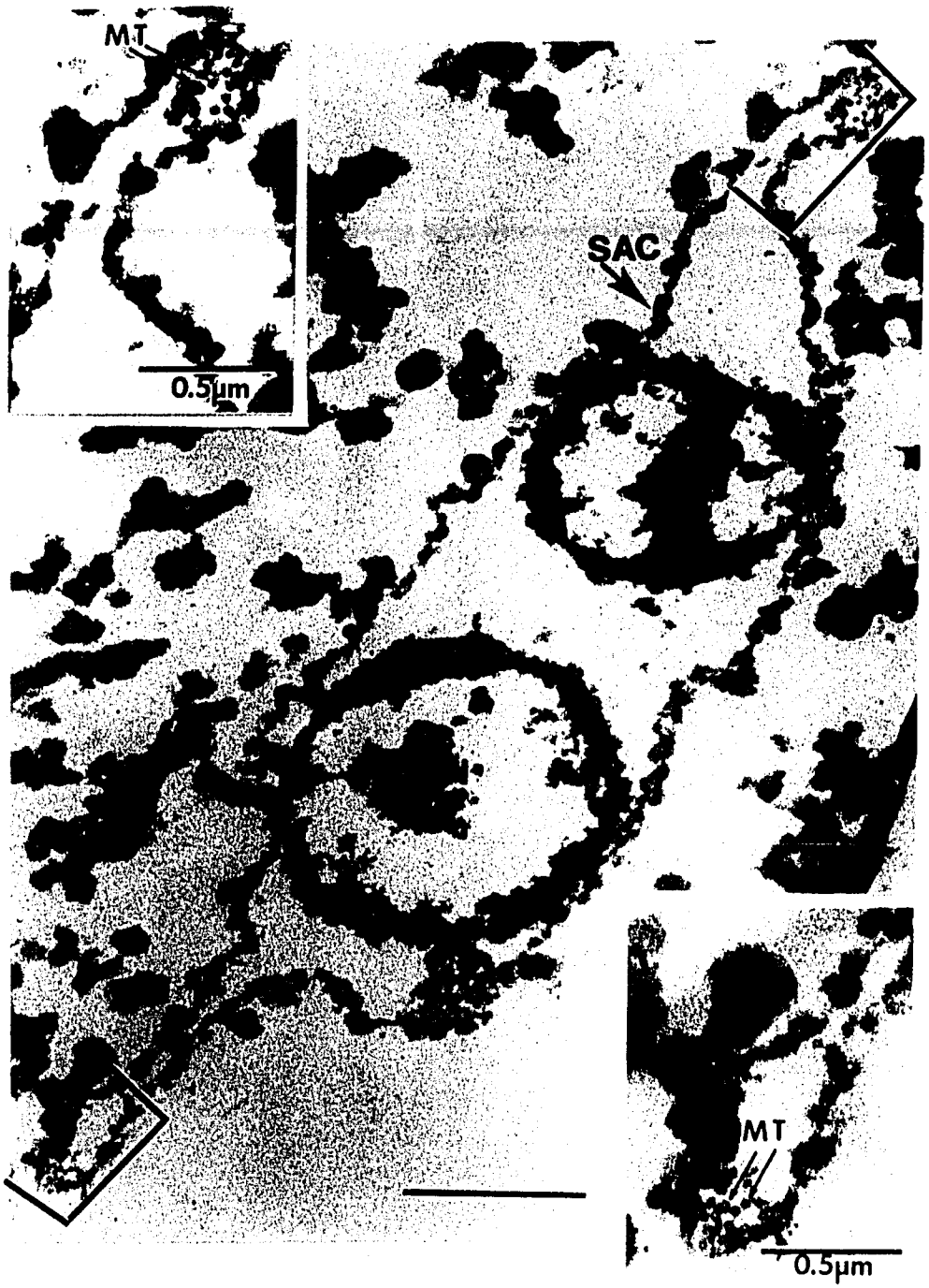

26
N
SAC
Ce
0.5μm

Figure 27. Reassembly of the MB at 0°C in the presence of 10μg/ml taxol. Treatment of erythrocytes at 0°C to disassemble the MB was followed by incubation in taxol for 21h. As seen in cross-section, the SAC is stretched over an intact, although thinner than normal MB. (N=nucleus, Hb=hemoglobin) TEM (X 24,500; insert X 37,500).

(27)



less tightly bundled than the MBs of cells incubated at room temperature described by Nemhauser, Joseph-Silverstein and Cohen (1983).

PREPARATION OF CELLS WITHOUT MBs AT ROOM TEMPERATURE

Cohen et al (1982) reported that dogfish erythrocyte MBs were inhibited from reassembling at room temperature following 0°C disassembly by the drug colchicine. To test whether reassembly of "blood clam" erythrocytes could be blocked by MT inhibitors, cells were incubated in 10ug/ml nocodazole or 0.1mM colchicine for one hour prior to the 0°C incubation as well as during low temperature incubation and during subsequent rewarming. As shown in Fig.28, when cells were rewarmed for one hour in the presence of nocodazole, MB reassembly was inhibited, while cells lacking any drug but containing DMSO as a control, contained normal reassembled MBs. The same result was obtained with colchicine.

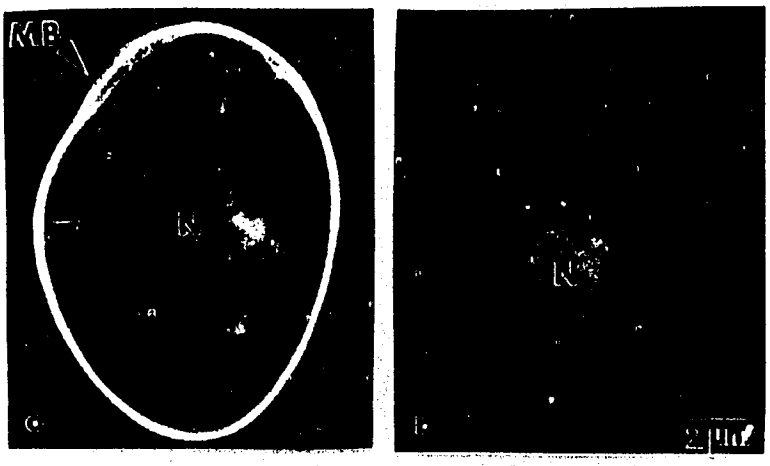
IDENTIFICATION OF PROTEINS ASSOCIATED WITH THE MB

Cytoskeletal Composition of Cells With and Without MBs

Comparison of the cytoskeletal composition of cells at 0°C and at room temperature by gel electrophoresis revealed that two minor proteins with apparent molecular weights of 80,000 and 105,000 temperature-cycled with MB tubulin. To identify them as MB associated proteins, it was first necessary to demonstrate that they were not simply cold-labile cytoskeletal proteins unrelated to the MB. This was done by preparing cells with and without MBs at the same temperature and comparing their cytoskeletal profiles by

Figure 28. Colchicine inhibition of MB reassembly at 20°C following low temperature treatment, as shown by indirect immunofluorescence microscopy with anti-tubulin. a) When cells were rewarmed following 0°C incubation to disassemble the MB, a normal MB was formed. b) When colchicine was present throughout 0°C treatment and subsequent rewarming no MTs are seen. (N=nucleus) (X 3470).

(28)



SDS-PAGE.

Cells with and without MBs at 0°C

As described above, cells incubated with taxol at 0°C for 3 hrs contained MBs that appeared to be stabilized in essentially normal condition while cells incubated at 0°C for 3 hrs in DMSO alone contained no MBs.The cytoskeletal proteins from these cells were separated by SDS-PAGE and compared. Three differences in protein profile were observed as shown in Fig.29. Tubulin, the 105,000Mr protein and the 80,000Mr protein were present in cytoskeletons from cells that contained MBs, while they were either missing or present in decreased amounts in cytoskeletons from cells that lacked MBs.

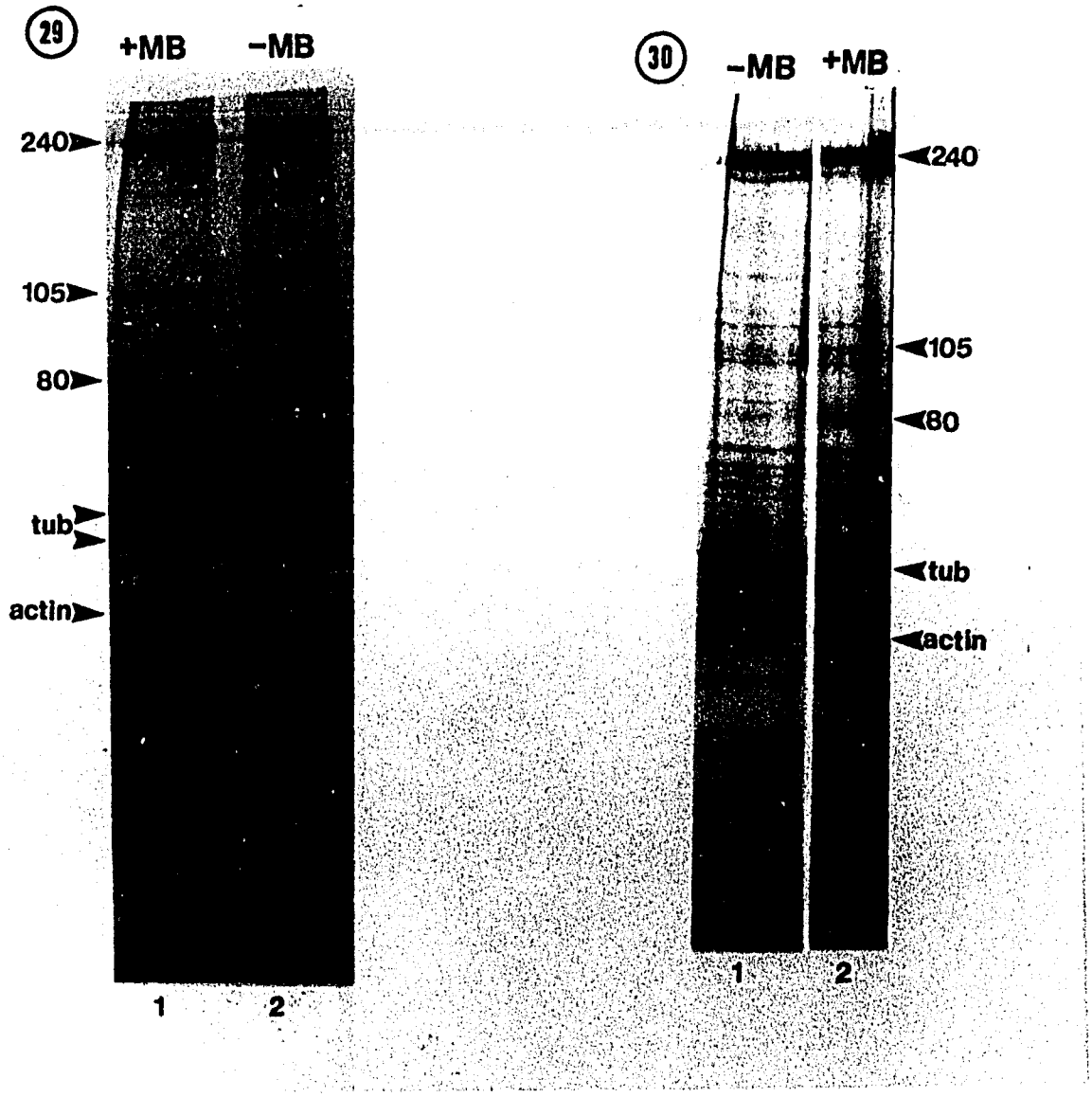
In addition, as described above, cells at 0°C were induced to reassemble MBs by incubation in taxol for 21 hrs. The cytoskeletal composition of these erythrocytes was also studied by SDS-PAGE (Fig.30). Tubulin, the Mr80,000 protein, and the Mr105,000 protein were present in decreased amounts in cytoskeletal samples from control cells that lacked reassembled MBs, when compared with cytoskeletal samples from cells that contained reassembled MBs and significant amounts of these three proteins.

Cells with and without MBs at Room Temperature

SDS-PAGE analysis was carried out on nucleated cytoskeletons prepared from cells with and without MBs under

Figure 29. Comparison of the protein profiles of nucleated cytoskeletons from cells with and without taxol stabilized MBs at 0°C. Cytoskeletal composition was analyzed by 7.5% SDS-PAGE. Lane 1) Cytoskeletons from cells with taxol stabilized MBs (+MB) contain clam 240, tubulin, and actin as major components. Mr 80K and Mr 105K are also present. Lane 2) Cytoskeletons from cells lacking MBs (-MB) contain clam 240 and actin as major components, but less tubulin, Mr 80K and Mr 105K.

Figure 30. Comparison of the protein profiles of nucleated cytoskeletons from cells with and without taxol-induced MBs at 0°C. Cytoskeletal composition was analyzed by 7.5% SDS-PAGE. Lane 1) Cytoskeletons from cells lacking MBs (-MB) contain clam 240 and actin as major components, but little or no tubulin, Mr 80K and Mr 105K. Lane 2) Cytoskeletons from cells with taxol-induced MBs (+MB) contain clam 240, tubulin and actin as major components. Mr 80K and Mr 105K are also present.

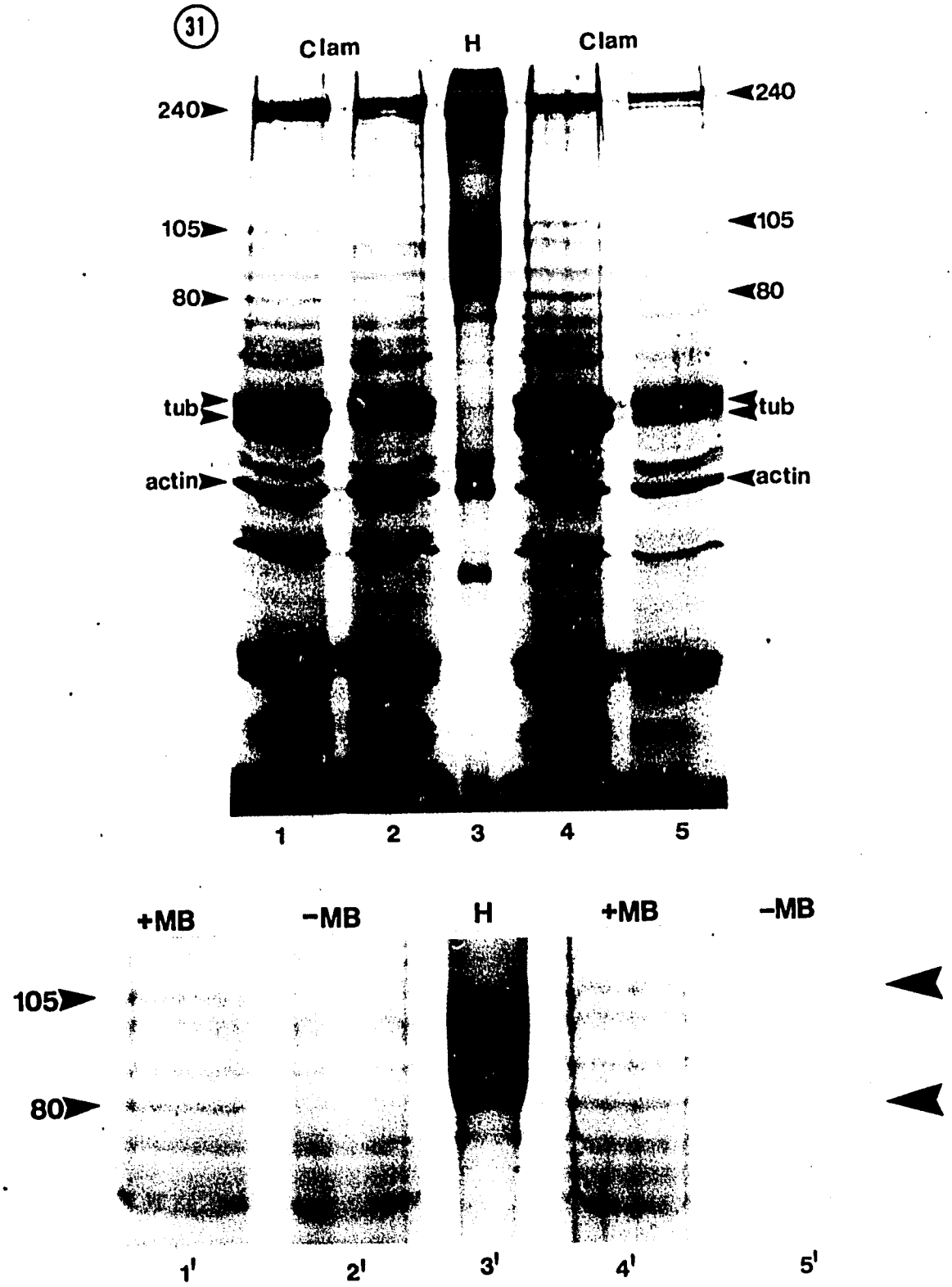

29
+MB
−MB
240
105
80
tub
actin
1
2
30
−MB
+MB
240
105
80
tub
actin
1
2

MT-stabilizing conditions. When the protein composition of these 2 cell types was compared, it was obvious that tubulin, as well as the Mr80,000 and Mr105,000, proteins were diminished in the samples which had been incubated with either colchicine or nocodazole and therefore lacked MBs, while all 3 proteins were present in cytoskeletal samples containing MBs (Fig.31).

MB Disassembly in a Brij-lysed System and Analysis of the Disassembly Products

To provide further evidence for the association of the Mr80,000 and Mr105,000 proteins with the MB, a system was devised to analyze the products of MB disassembly. Erythrocytes were extracted in the detergent Triton X-100 following MB disassembly and the soluble components analyzed by SDS-PAGE. This procedure, however, was not successful for several reasons. Gel electrophoresis in the presence of Triton did not effect normal separation of proteins and furthermore, cytoskeletons prepared by Triton extraction of cells could not be resuspended following pelleting in the clinical centrifuge, making it impossible to remove the detergent. Also membrane proteins extracted by the detergent and cytoplasmic proteins such as hemoglobin could not be removed from this system prior to low temperature

Figure 31. Comparison of the protein profiles of nucleated cytoskeletons from cells with or without MBs prapared at 20°C. Colchicine or nocodazole were added to erythrocyte suspensions prior to 0°C incubation and subsequent rewarming to block MB reassembly. Protein profiles were analyzed by 7.5% SDS-PAGE. Lanes 1 and 4) Cytoskeletons from cells with MBs contain clam 240, tubulin and actin as major components, as well as Mr 105 and Mr 80. Lanes 2 and 5) Cytoskeletons from cells treated with colchicine (2) or nocodazole (5) and therefore lacking MBs contain clam 240 and actin as major components but little or no tubulin, Mr 105 or Mr 80. Lane 3) Human erythrocyte ghost protein profile. Lanes 1'-5' are enlargements of the region of the gels containing the 80K and 105K bands.


31
Clam
H
Clam
240
105
80
tub
actin
1
2
3
4
5
+MB
−MB
H
+MB
−MB
105
80
1'
2'
3'
4'
5'

incubation. This made analysis of the disassembly products extremely complicated. Therefore, it was necessary to develop a procedure in which detergent-solubilized and cytoplasmic material could be removed prior to MB disassembly so that the medium in which the extracted cells were incubated contained only the proteins released by MB disassembly during the 0°C treatment.

Deery et al. (1983) reported that disassembly of cytoplasmic MTs occurred in Brij-lysed cells. We found that when "blood clam" erythrocytes were extracted in 0.6% Brij-58 under MT-stabilizing conditions, hemoglobin was released into the supernate and the cytoskeletons could be washed and resuspended repeatedly. When "blood clam" erythrocyte cytoskeletons were incubated in a small volume of LyM wash for 3 hours at 0°C, MB disassembly occurred as verified by immunofluorescence microscopy using anti-tubulin antibody (Fig.32). SDS-PAGE of the supernate from the cytoskeleton preparation following MB disassembly, revealed that tubulin escaped from the extracted cells, since it appeared as the major component of the supernate (Fig.33). A densitometric scan of the gel showed that 78% of the total protein present in this supernate was tubulin (Fig.33). The Mr 80,000 protein and Mr 105,000 protein made up an additional 12% of the total protein, while actin, and proteins with apparent molecular weights of greater than 300,000, Mr270,000, Mr240,000, Mr220,000 and Mr60,000 made up the remaining 10%

Figure 32. Disassembly of the MB in Brij-extracted cells as viewed by indirect immunofluorescence microscopy with anti-tubulin. a) and b) Brij-extracted cells at 20°C contain an intact MB. c) and d) Brij-extracted cells incubated at 0°C for 3h contain no MB. (N=nucleus) Phase/fluorescence pairs (x 2400).

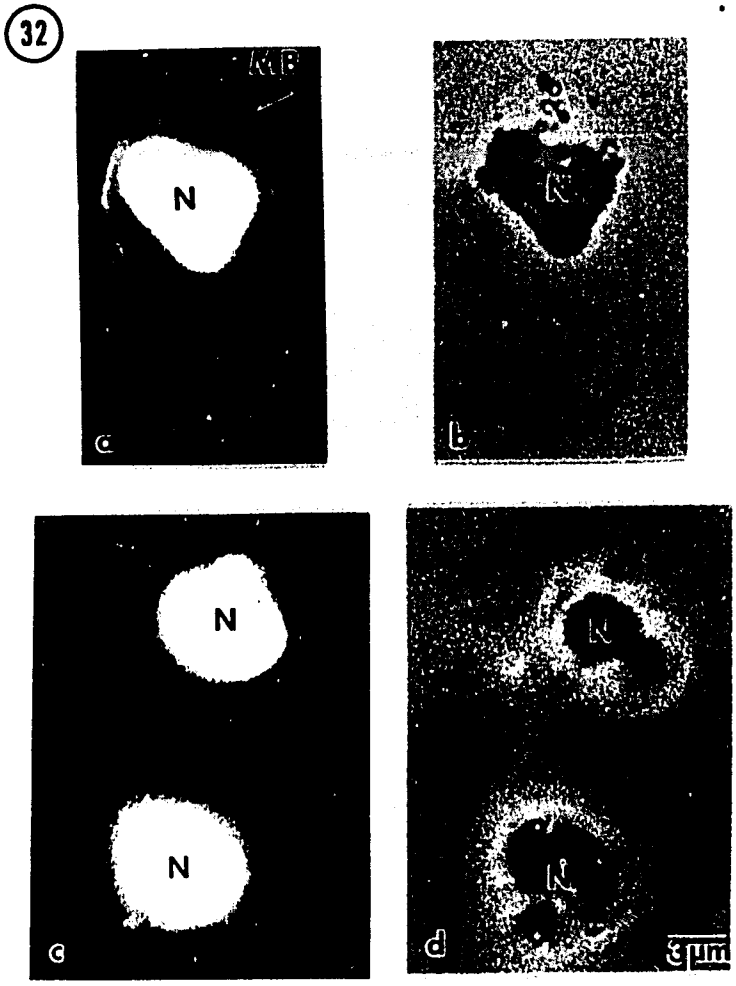
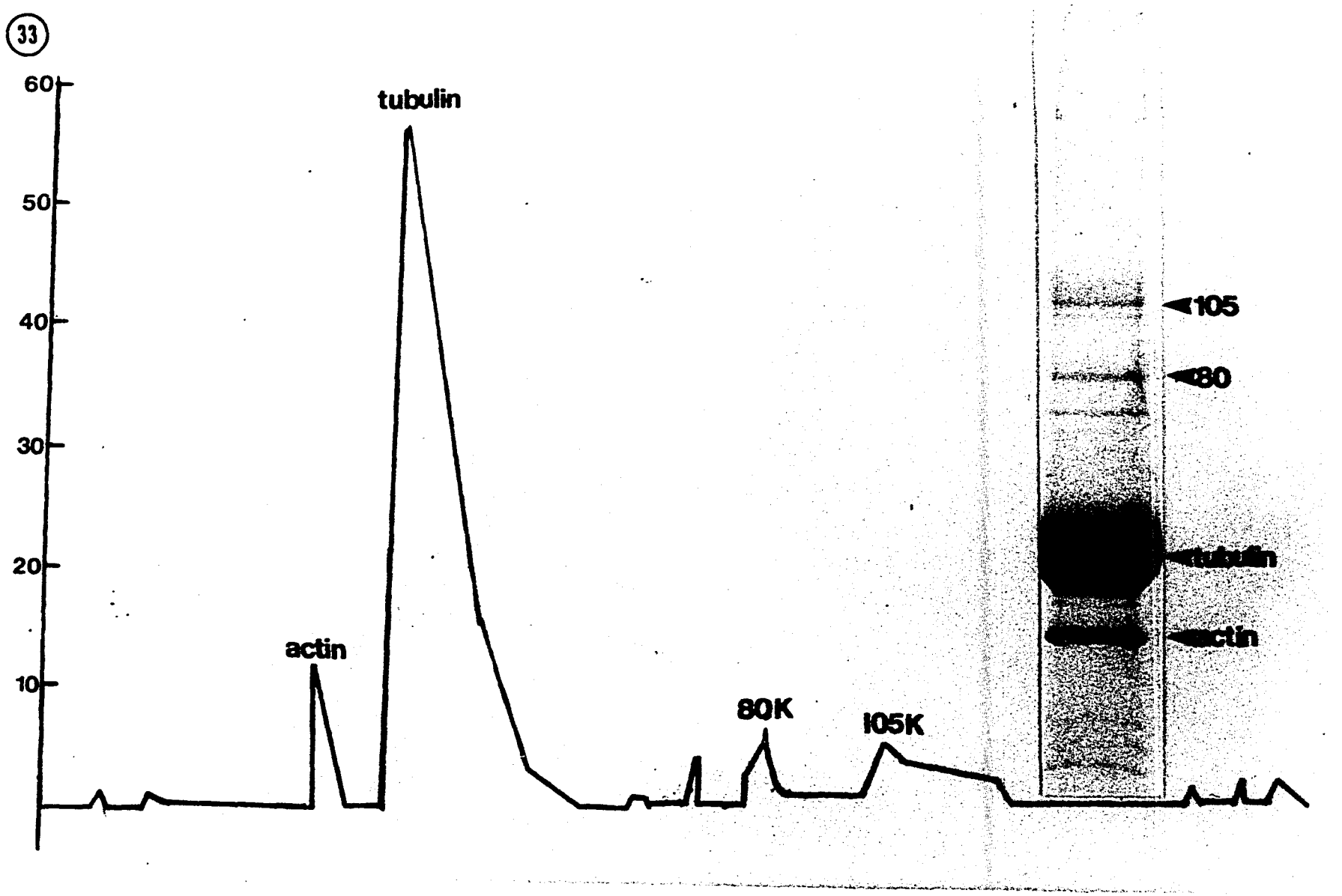

32
KB
N
a
b
N
N
c
N
d
3μm

Figure 33. MB disassembly in Brij-extracted cells; analysis of the disassembly products. Brij-extracted cells were incubated for 3h at 0°C. Following centrifugation to remove the cytoskeletons, the supernatant was concentrated and analyzed by SDS-PAGE. The major proteins present were tubulin, actin, Mr105 and Mr80 with smaller amounts of proteins migrating at 320K, 270K, 240K, 220K and 60K. Densitometric analysis of the gel shows that tubulin makes up 78% of the total protein.

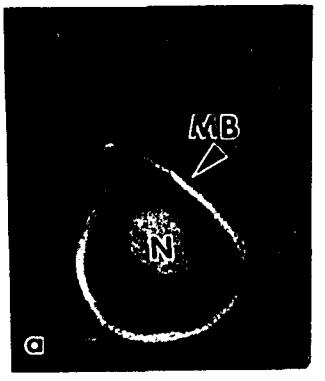

33
60
50
40
30
20
10
tubulin
actin
80K
105K
105
80
tubulin
actin

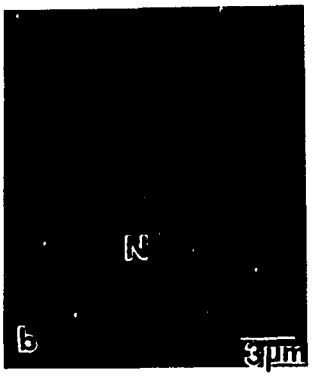
of the total protein. Since it was demonstrated earlier that actin is not a component of the MB, it was apparent that the appearance of actin in the supernate was an artifact of the Brij-lysed system and probably due to extraction of actin during $0^{o}C$ incubation from some other site in the cytoskeleton.

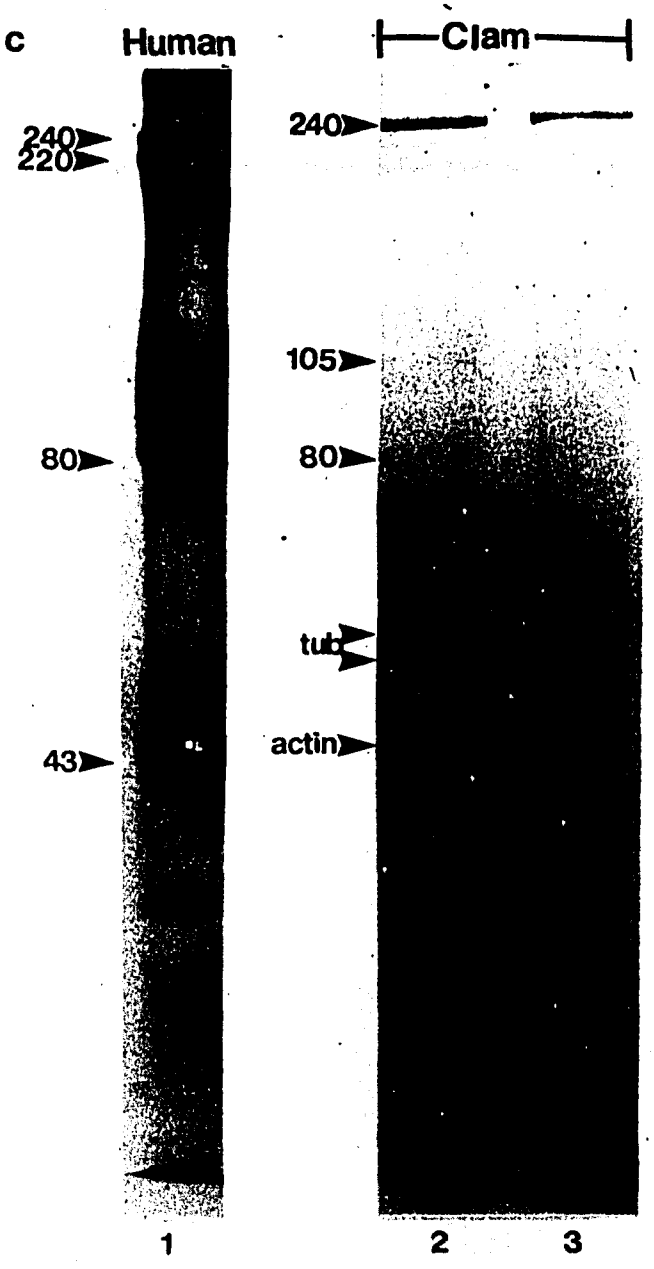
The addition of GTP to concentrated supernatant samples followed by incubation at $37^{0}C$ for 1 hr did not induce polymerization of MTs which could be recognized by electron microscopy of negatively stained material. We were interested in determining whether cytoskeletons with and without MBs could be produced using this <u>in vitro</u> system. When we incubated Brij-extracted cells at $0^{o}C$ in the presence of 10μg/ml taxol, the MB was stabilized (Fig.34a). When the cytoskeletal components from this preparation were compared to those of extracted cells incubated without taxol by SDS-PAGE, we found that tubulin, the Mr80,000 and the Mr105,000 proteins were retained in the cytoskeletons incubated in the presence of taxol, but were lacking in the cytoskeletons incubated in the absence of taxol (Fig.34c).

Figure 34. Comparison of cytoskeletal protein profiles of Brij-extracted cells incubated at 0°C in the presence or absence of taxol. Cells extracted with Brij-58 and incubated for 3h at 0°C in the presence of taxol contain MBs (a) while those incubated in the absence of taxol lack MBs (b) as viewed by indirect immunofluorescence microscopy with anti-tubulin (N=nucleus) (X 2160). The nucleated cytoskeletons were analyzed by 7.5% SDS-PAGE (c). Lane 1) Human erythrocyte ghosts. Lane 2) Cytoskeltons incubated with taxol contain clam 240, actin, and tubulin as major components. Lane 3) Cytoskeletons incubated in the absence of taxol contain clam 240 and actin, but little tubulin.

(34)







THE ERYTHROCYTE CYTOSKELETON AND CELL MORPHOLOGY

Maintenance of Cell Shape Under Static Conditions

Chicken (Behnke; Barrett and Dawson) and dogfish (Cohen et al.,1982;Joseph-Silverstein et al.,1984) erythrocytes maintain their flattened elliptical shape in the absence of the MB as long as they are not exposed to any external forces. When "blood clam" erythrocytes without MBs are viewed under static conditions, they too remain flattened and elliptical.

Human erythrocyte ghosts prepared by hypotonic lysis retain the shape of the intact cells from which they were prepared (Yu et al., 1973). We were interested in determining whether "blood clam" erythrocyte ghosts prepared by hypotonic lysis behaved in a similar manner, and if so, which cytoskeletal components of the ghosts might be responsible for this property. As shown earlier, in Fig.10, nucleated ghosts prepared by osmotic lysis of cells with MBs retained the flattened elliptical shape of the cell. When cells incubated at $0^{o}C$ in the presence of taxol to stabilize the MB were extracted in LyM containing 0.6% Brij-58, the cytoskeletons appeared flattened, elliptical, and smooth in contour (Fig.35a). Those incubated in the absence of taxol and therefore lacking MBs, although flattened did not retain a smooth contour (Fig.35b). Triton extraction of erythrocytes resulted in cytoskeletons which were smooth and elliptical when MBs were stabilized by taxol at $0^{o}C$,

(Fig.35c), but produced cytoskeletons which had collapsed inward upon the nucleus when no taxol was present at 0°C and therefore MBs were lacking (Fig.35d).

The Effect of Mechanical Stress on Cells With and Without MBs

Cells Prepared at Room Temperature

To investigate whether MBs function to maintain normal cell shape in "blood clam" erythrocytes under conditions of mechanical stress, we subjected cells with and without MBs to fluxing in glass capillary tubes. As shown in Table III, no more than 2% of the erythrocytes with MBs folded or buckled, while at least 20% of the erythrocytes without MBs folded or buckled under the same conditions. The shapes of such cells are seen to advantage in the scanning electron microscope (Fig.36).

Cells Prepared at 0°C

Since some of the previous data bearing upon MB function had been obtained by observation of cells at low temperature, we studied the effects of mechanical stress on cells with and without MBs at 0°C. Cells with and without MBs (as described above) were subjected to fluxing in glass capillary tubes with the result shown in Fig.37. Cells with MBs were generally normal in shape while those without MBs were deformed. As shown in Table IV (taxol-stabilized MBs), no more than 18% of the erythrocytes with MBs folded or buckled while at least 33% of the erythrocytes without MBs folded or buckled under the same conditions.

Figure 35. Cytoskeletal morphology of Brij and Triton extracted cells with and without MBs. Cells were incubated at 0°C in the presence or absence of taxol and then extracted with Brij-58 (a and b) or Triton X-100 (c and d). Cytoskeletons containing MBs were flattened and elliptical and smooth in contour after either Brij (a) or Triton (c) extraction. Cells lacking MBs were spread but no longer smooth in contour after Brij extraction (b). Triton-extracted cells lacking MBs (d) collapsed inward upon the nucleus and the SAC was no longer obvious. (N=nucleus) (Phase contrast X 2120).

35

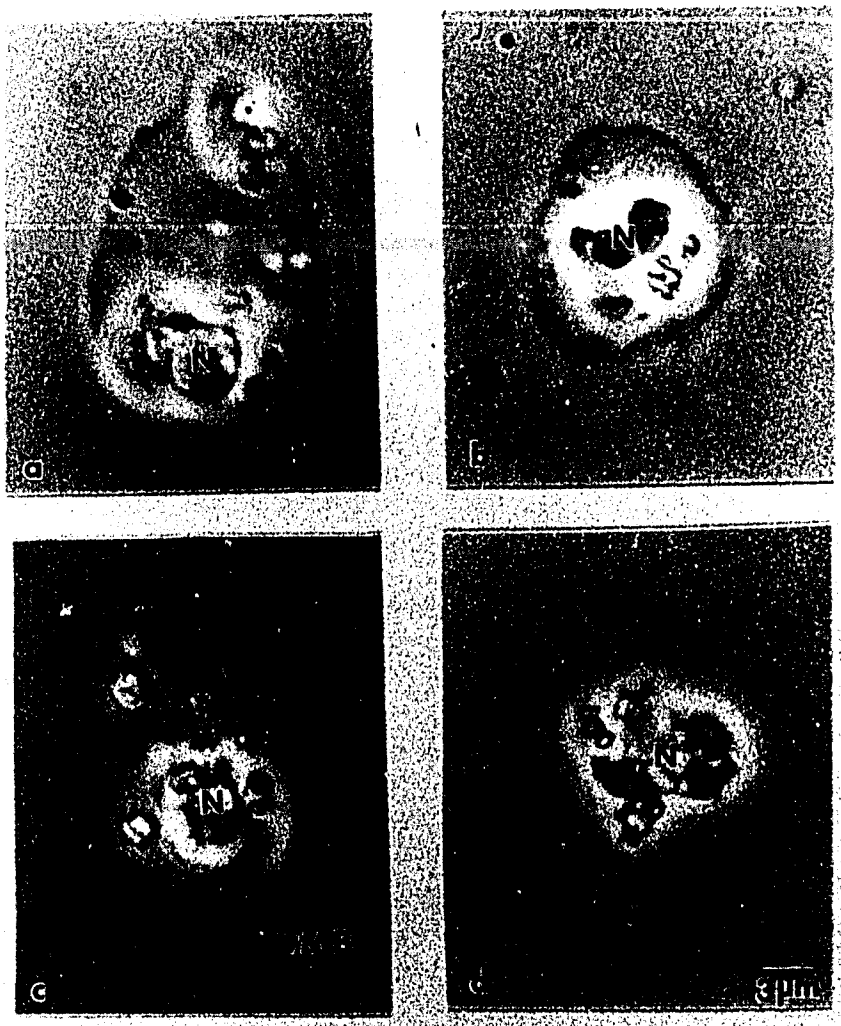


TABLE III

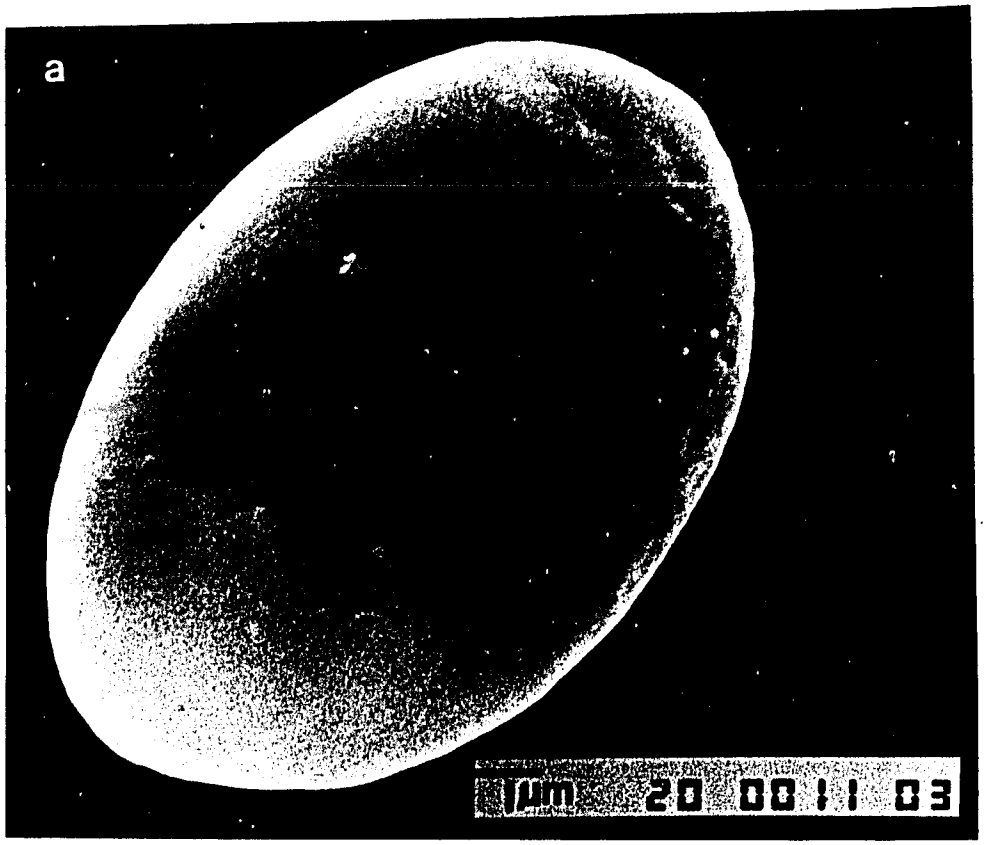
EFFECT OF MECHANICAL STRESS ON CELLS WITH AND WITHOUT MBs

Treatment	Presence of MB	%Deformed Cells exp. I	exp. II
+ colchicine	-	46	20
+ nocodazole	-	47	20
- nocodazole	+	2	2

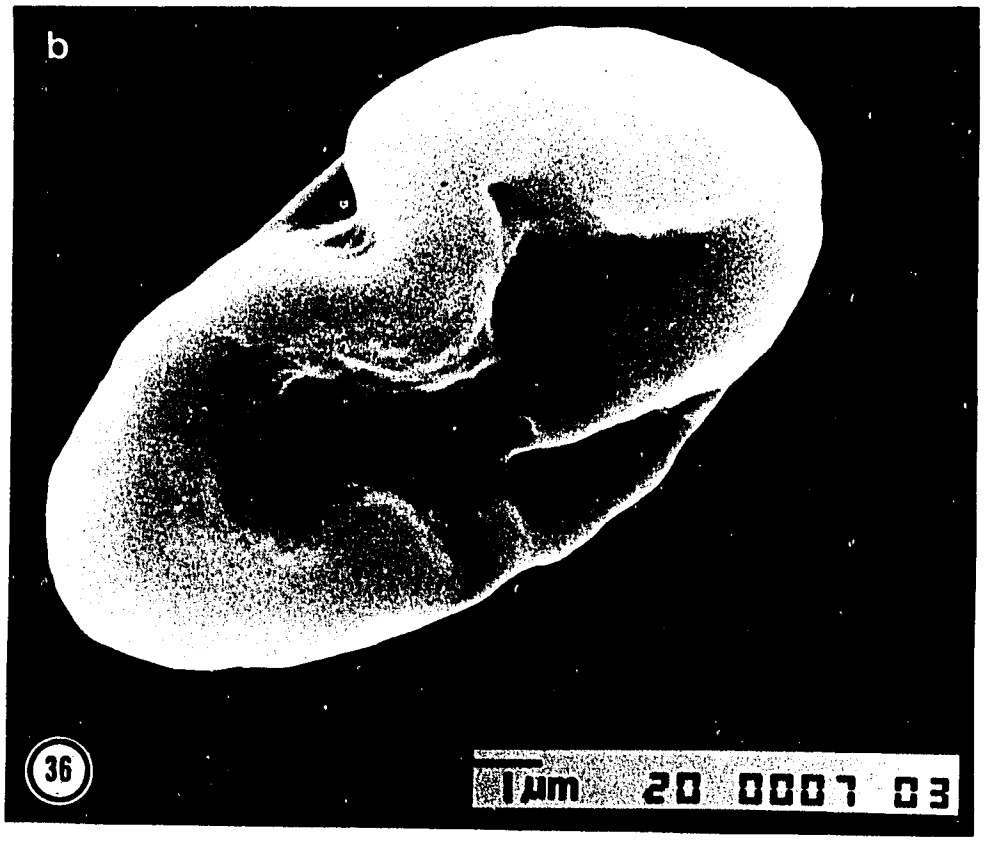
Cells were fluxed 10 times in 10 μl capillary tubes and fixed as described in Materials and Methods. A minimum of 100 cells was counted per experimental protocol.

Figure 36. Effect of mechanical stress (fluxing through capillary tubes) as seen in representative cells with (a) and without (b) MBs. Cells without MBs were prepared by colchicine inhibition of MB reassembly at 20°C following disassembly at 0°C; for cells with MBs, colchicine was omitted. Cells with MBs are normal in appearance, while many cells without MBs are buckled. Scanning electron microscopy X (8000).

+MB



-MB



Cells with and without 21 hour taxol-reassembled MBs at 0°C were also subjected to fluxing in glass capillary tubes with the results shown in Table IV (taxol-induced MBs). As in previous experiments, cells with MBs were generally normal in shape (not more than 28% of erythrocytes deformed) while most of the erythrocytes without MBs were folded or buckled (85% or more).

Demonstration of "Blood Clam" Erythrocyte Deformability

Previous observations on dogfish erythrocytes suggested that MBs may function in the response of vertebrate erythrocytes to deformation (Joseph-Silverstein et al.,1984). To verify that blood clam erythrocytes were normally deformable, we observed cells in flow under coverslips (Fig.38). Cells were seen to be highly deformable in contact with each other, and to recover more normal contours upon loss of contact.

Figure 37. Effect of mechanical stress (fluxing through capillary tubes) on cells with (a) and without (b) MBs. Cells containing MBs at 20°C were prepared by taxol stabilization; for cells without MBs, taxol was omitted. Cells with MBs are normal in appearance, while many cells without MBs are buckled. Phase contrast microscopy (X 1000).

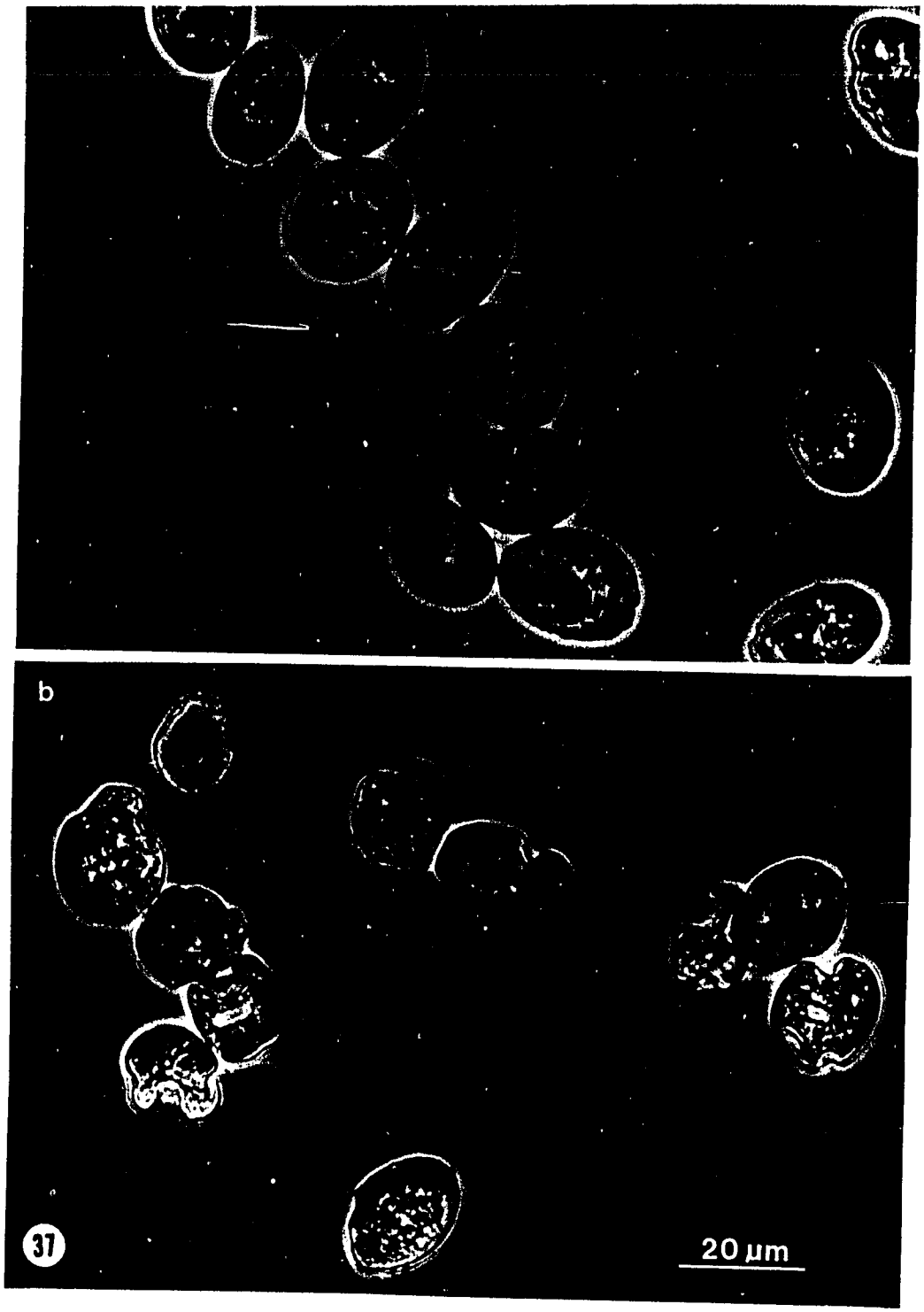

b
37
20 µm

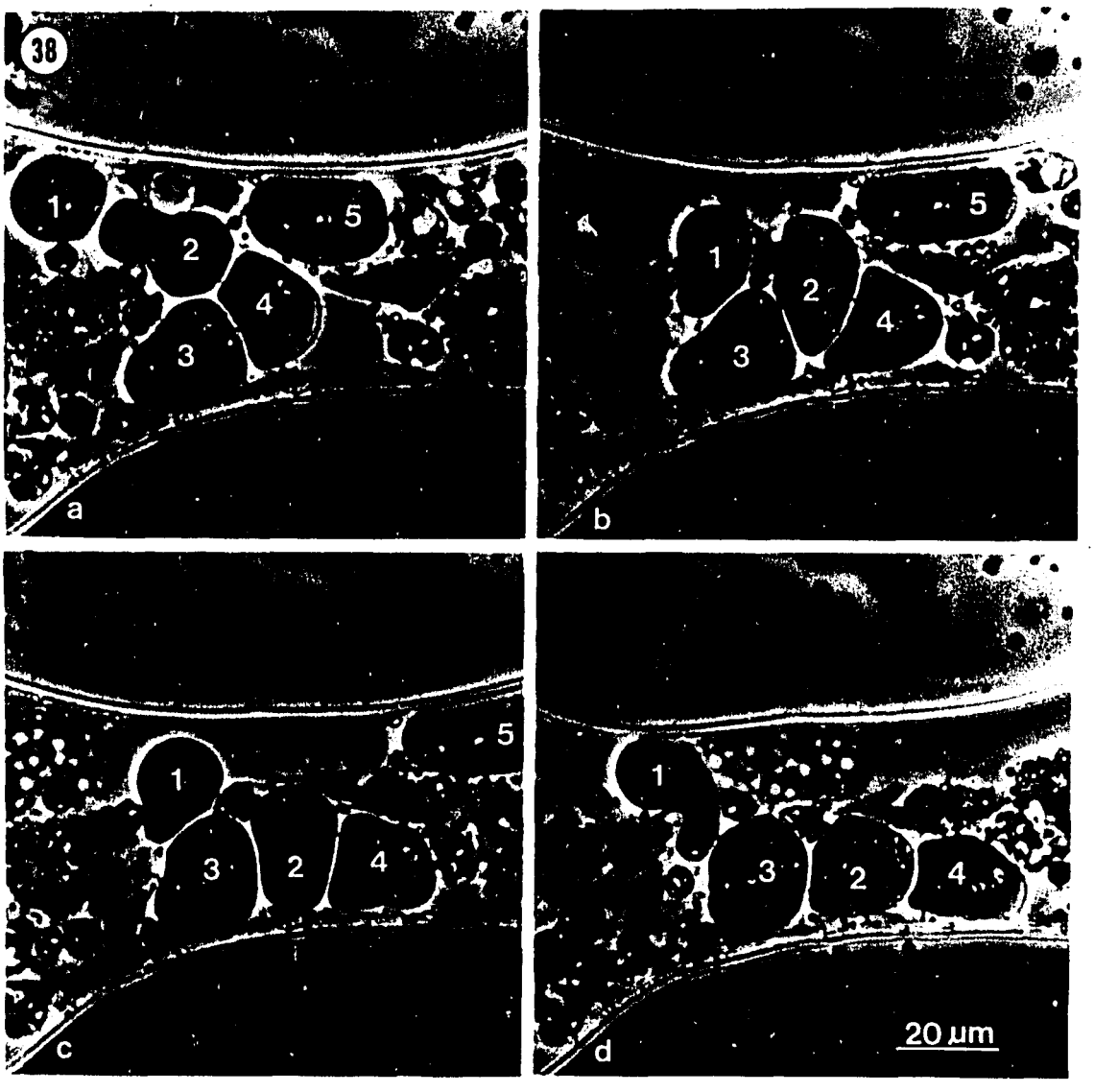
TABLE IV

EFFECT OF MECHANICAL STRESS ON CELLS WITH AND WITHOUT MBs

Experiment	Treatment	Presence of MB	%Deformed Cells
TAXOL-STABILIZED MBs			
1	+ taxol	+	18
	- taxol	-	71
2	+ taxol	+	13
	- taxol	-	33
TAXOL-INDUCED MBs			
1	+ taxol	+	28
	- taxol	-	85
2	+ taxol	+	21
	- taxol	-	89

Cell were fluxed 10 times in 10μl capillary tubes and fixed as described in Materials and Methods. A minimum of 100 cells was counted per experimental treatment.

Figure 38. Deformability of normal blood clam erythrocytes observed in flow under the coverslip. Direction of flow is from left to right, with time sequence (a) to (d). Note extreme deformation of cell #2 as it is squeezes between cells #3 and #4. Cell #1, which starts with nearly normal shape, is deformed in contact with cell #3. Cell #5 moves out of the field in the sequence. Phase contrast (X 750).


38
1
2
3
4
5
a
1
2
3
4
5
b
1
3
2
4
5
c
1
3
2
4
d
20 µm

Abnormal Erythrocyte Morphology in Anadara ovalis

Induction of abnormal morphology

When blood removed from Anadara ovalis was not diluted immediately into MBL sea water, the erythrocytes lost their flattened elliptical shape and became amorphous or "potato-shaped" (Fig.39). Immediate dilution of blood at least 100-fold in MBL sea water was usually effective in blocking this morphological conversion in over 80% of the cells. Greater than 90% of the cells had normal morphology when a 200-fold dilution in sea water was used. Therefore, it seemed likely that the fluid portion of the blood contained some factor responsible for the change in erythrocyte morphology. To test this possibility, normal cells prepared by dilution in sea water were incubated in the undiluted supernatant prepared from cells with abnormal shape as described in the Methods section. Populations initially containing a minimum of 80% normal erythrocytes (mean+SE=85+2) were incubated in this supernatant for a minimum of 15 minutes and were then examined by phase contrast microscopy. The mean number (+SE) of normal shaped cells following this incubation was 8.3+4.This was statistically significant, $p<0.001$ using the Student's t test.

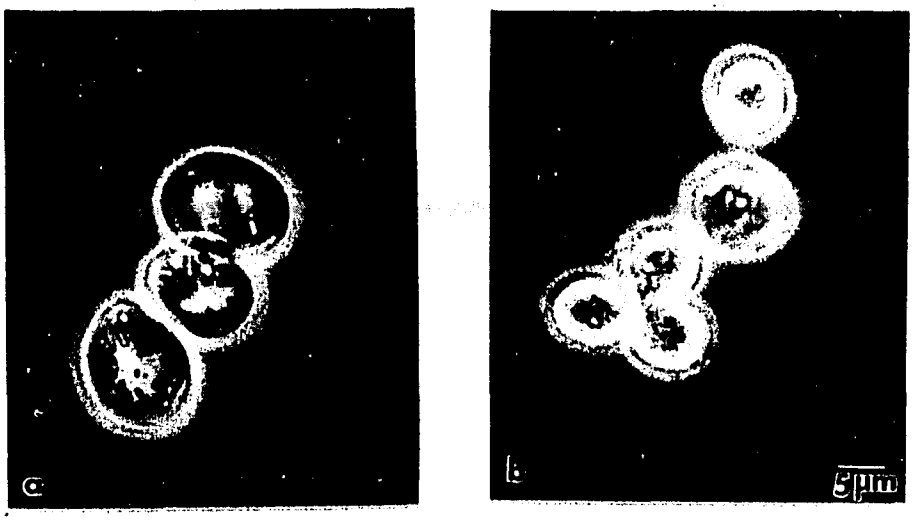
MT Configuration in Erythrocytes with Abnormal Morphology

The MT configuration in erythrocytes of normal shape was compared to that in erythrocytes with abnormal morphology,

Figure 39. Normal and abnormal morphology of Anadara ovalis erythrocytes. a) Normal flattened elliptical erythrocytes prepared by diluting cells 100X in MBL formula sea water. b) Erythrocytes prepared without dilution have abnormal amorphous morphology. Phase contrast microscopy (X 1150).

Figure 40. Microtubule configuration of Anadara ovalis erythrocytes having abnormal morphology as viewed by indirect immunofluorescence microscopy with anti-tubulin. An intact MB is present in an erythrocyte with normal morphology (a). Cytoskeleton preparations from cells with abnormal morpholgy have a recognizable MB containing some unbundled microtubules (double arrowheads) (b and c), tangled MTs with a "ball of yarn" appearance (b) and a thickened abnormally sized MB (c). (N=nucleus) (X 2080).

39



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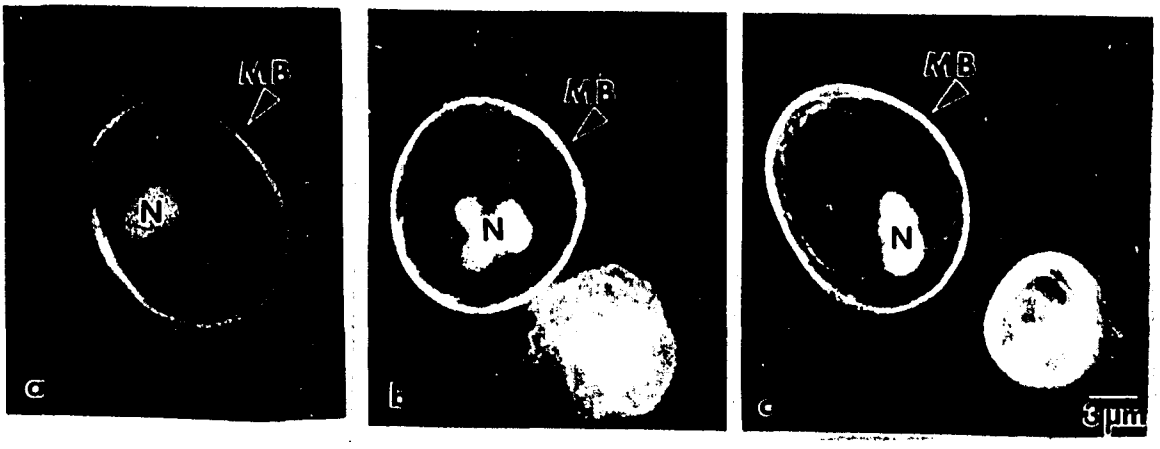


using immunocytochemistry with anti-tubulin antibody. The MBs in the abnormally shaped cells were less tightly bundled and MT loops were present in the cytoplasm. In some cytoskeletons the level of disorganization was such that no recognizable MB was present and the MTs were tangled in such a manner as to resemble a ball of yarn (Fig.40).

MT configuration was also studied by thin section electron microscopy. In thin sections through cytoskeletons prepared from normal cells in which the MB is present in cross-section, the MB appeared as two tightly bundled clusters of MTs at opposite ends of the cytoskeleton, with the SAC stretched over it (Fig.41). In contrast, thin sections through cytoskeletons prepared from cells with abnormal morphology, contained MBs in which MT clusters were less tightly bundled, being spread over a larger area, and, although positioned near the periphery of the cytoskeleton, the SAC did not appear to be stretched across this somewhat disorganized MB. In some cases, MTs in both longitudinal and cross-section could be seen internal to the position of the MB in a single section (Fig.42).

Figure 41. Thin section through an Anadara ovalis erythrocyte cytoskeleton having normal morphology. In a cross section through the MB the SAC appears stretched over the MB. The MTs are present in 2 discrete areas at opposite poles of the cytoskeleton. (TEM X 16,700). Inset shows a higher magnification of the cross-sectioned MB. (N=nucleus) (X 30,000).

(41)

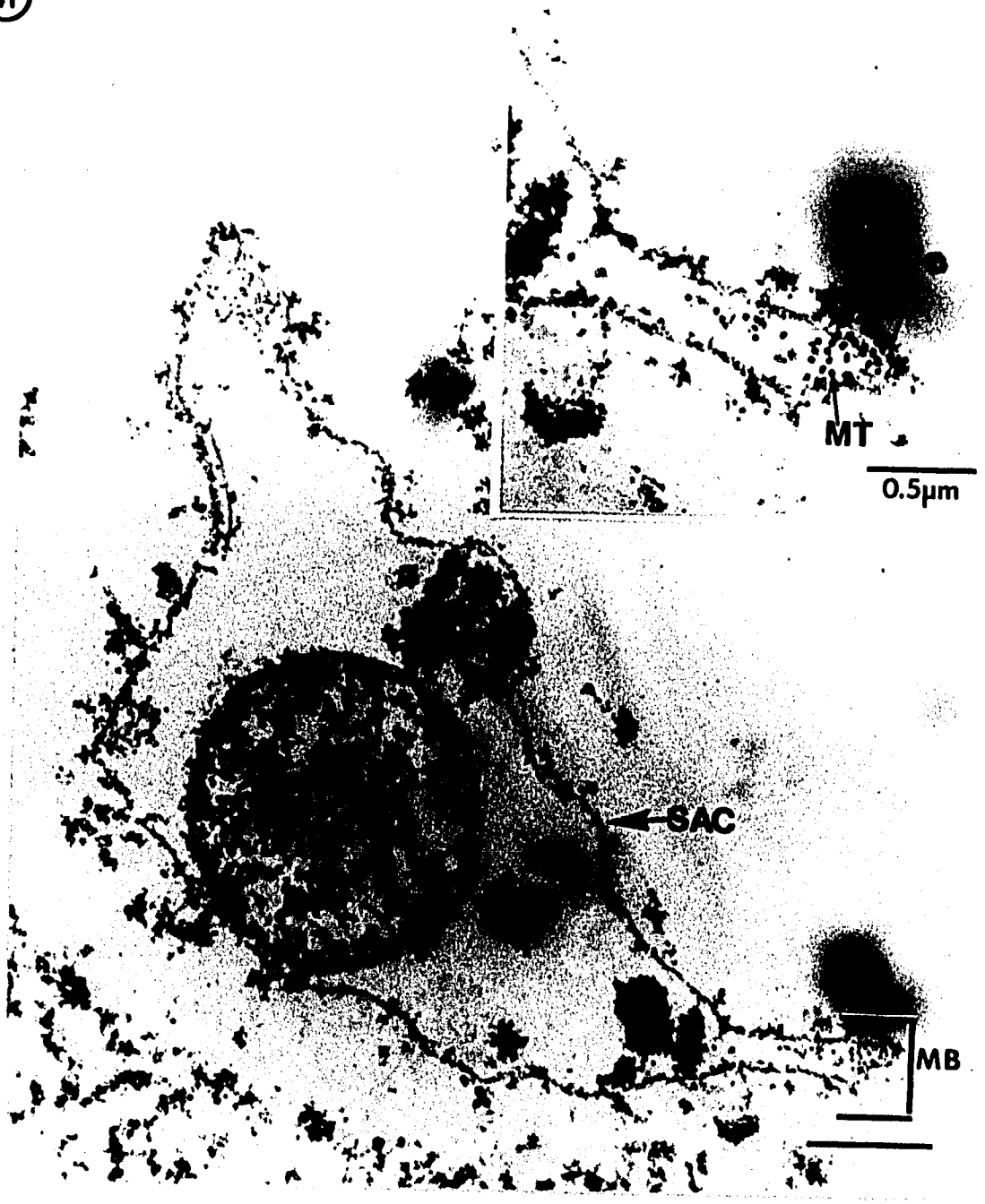
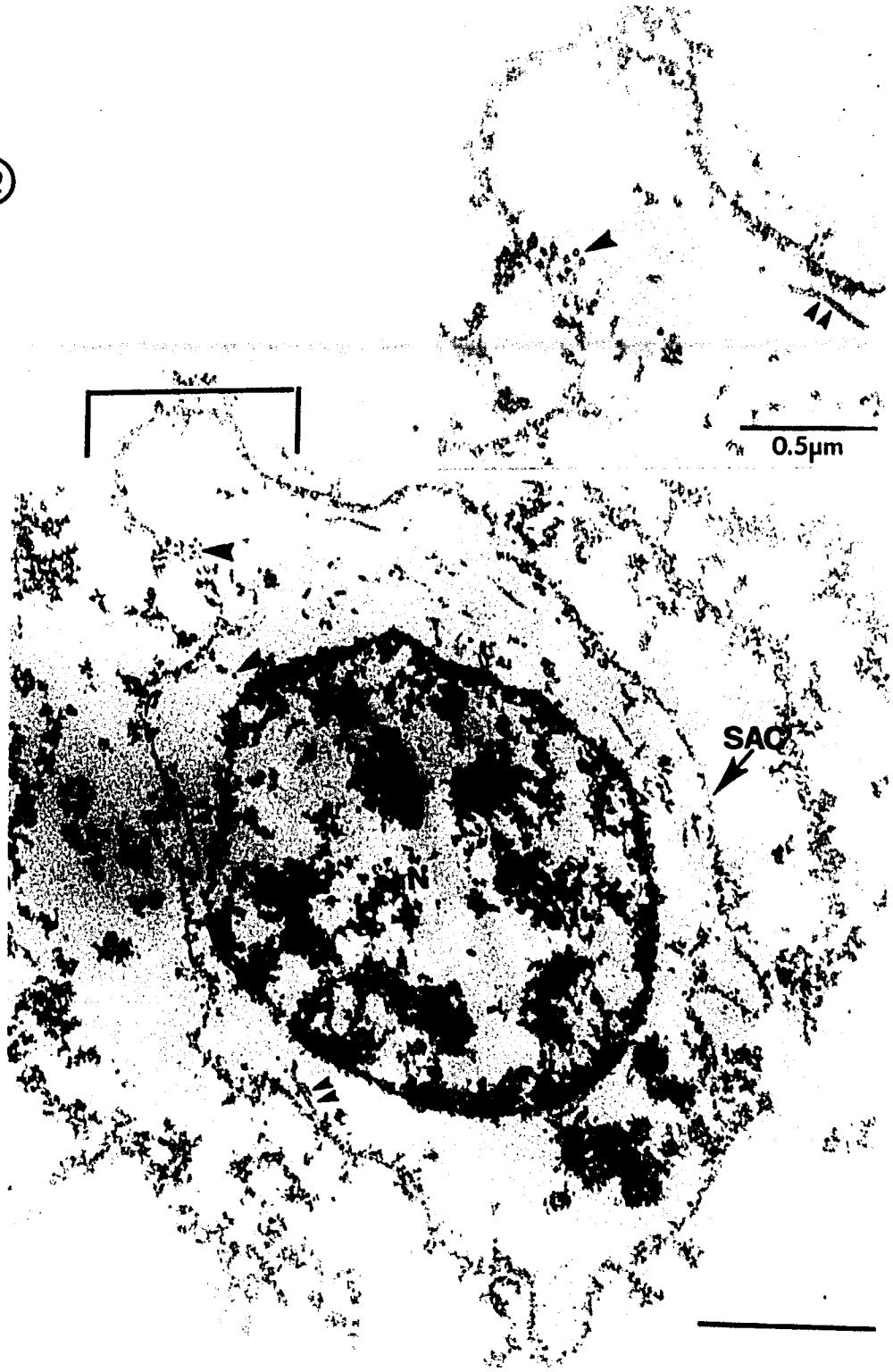


Figure 42. Thin section through an Anadara ovalis erythrocyte cytoskeleton having abnormal morphology. In this section, MTs are present throughout the cytoskeleton rather than in discrete regions. MTs are present in both cross (large single arrowheads) and longitudinal sections (small double arrowheads). (N=nucleus) (TEM X 26000).

(42)



MB Reassembly in Erythrocytes with Abnormal Morphology

Erythrocytes were temperature-cycled in the same manner as described previously for normal cells. MT reassembly was monitored by immunofluorescence microscopy with monoclonal anti-tubulin antibody. In most cases, a normal MB was not reassembled, but rather a tangled web of MTs was formed (Fig.43). Although during early stages of reassembly short MTs were often focused on a single site, rather than being located at the periphery of the cytoskeleton, with MTs emanating from it in such a manner as to give the structure a "pole"-like appearance as is the case in normal rewarming erythrocytes, the site of MT assembly was located nearer the center of the cytoskeleton.

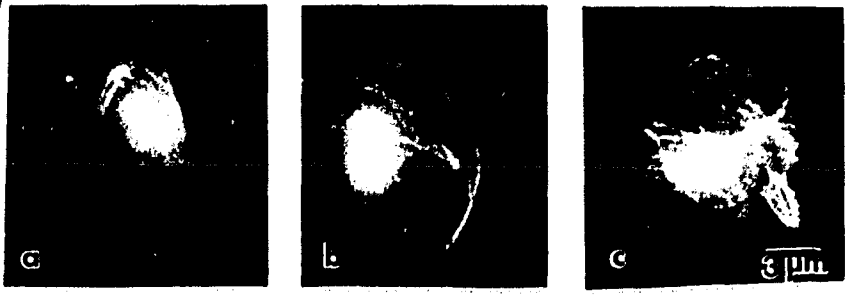
Relationship Between MB Disorganization and Abnormal Cellular Morphology

To determine whether a causal relationship existed between MB disorganization and the induction of abnormal morphology, the active supernatant from abnormal unwashed cells was tested on initially morphologically normal cells with and without MBs. Such cells were prepared at room temperature by temperature-cycling in the presence or absence of 0.1mM colchicine. It was reported by Nemhauser et al (1983) that as many as 25% of the cytoskeletons of Anadara ovalis contain a few MTs following $0^{o}C$ incubation for 4 hours. We now found that a similar percentage of

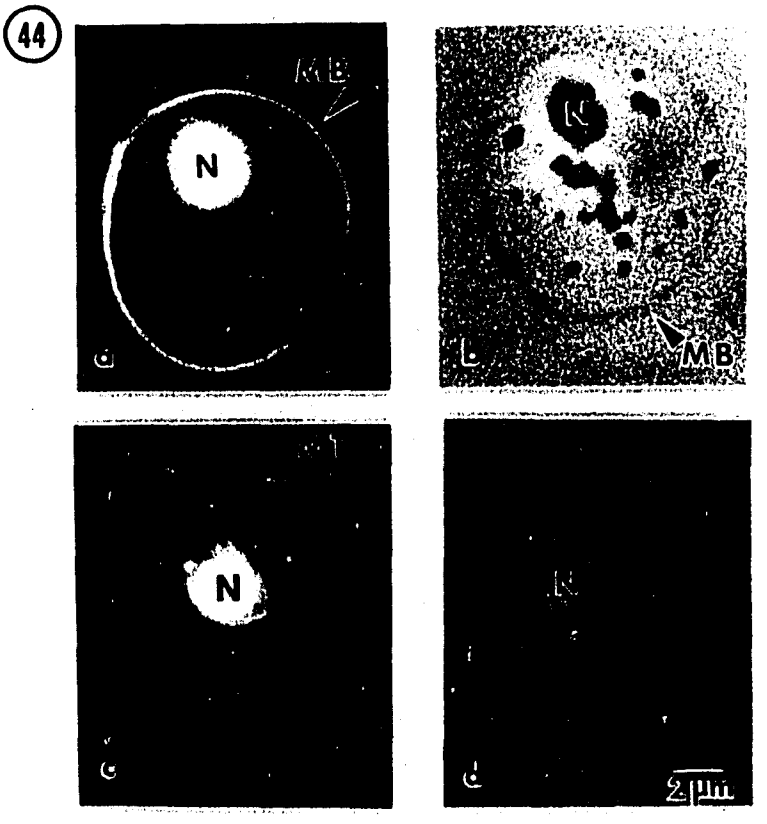
Figure 43. MT reassembly in Anadara ovalis erythrocytes having abnormal morphology as viewed by indirect immunofluorescence microscopy with anti-tubulin. Cells having abnormal morphology were incubated at 0°C for 3h and then rewarmed at 20°C. a) Early in the reassembly sequence (5 min) MTs radiate from a perinuclear site. b) and c) Unlike reassembly in normal cells, MTs do not focus on a single site (10 min, b) and do not bundle into a normal MB (45 min, c). (X 2080).

Figure 44. Inhibition of MB reassembly in Anadara ovalis erythrocytes by colchicine as viewed by immunofluorescence microscopy with anti-tubulin. Cells were incubated at 0°C and subsequently rewarmed to 20°C in the presence or absence of colchicine. a) and b) In the absence of colchicine a normal MB is reassembled. c) and d) In the presence of colchicine no MBs are reassembled and short MTs are present. (N=nucleus) Phase contrast/fluorescence pairs (X 2800).

43



44



cytoskeletons from Anadara ovalis cells incubated in colchicine contained MTs following rewarming. However, in no cases were normal-appearing MBs present. MB remnants such as a few short cytoplasmic MTs (Fig.44) or very thin MBs probably consisting of only a few MTs were seen in these cytoskeletons.

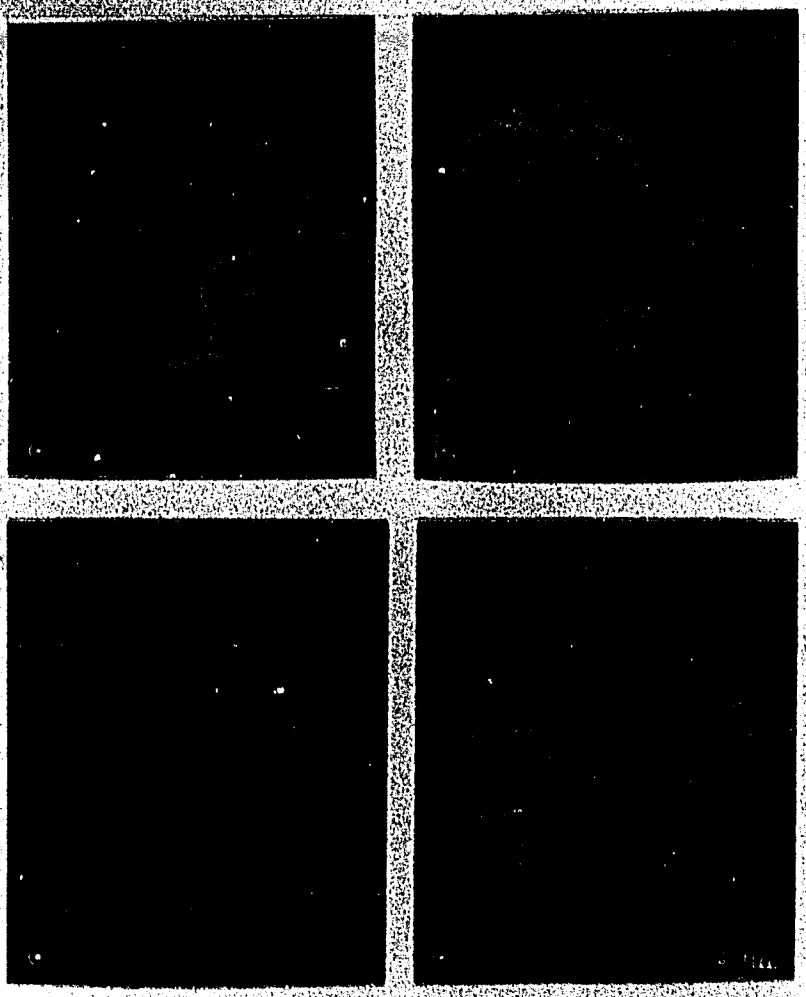
Populations of cells containing or lacking MBs were incubated in the supernatant (containing colchicine where appropriate) prepared from unwashed, abnormal cells for at least 20 minutes to test whether cells lacking MBs developed an abnormal morphology. Both cells that did not contain MBs and cells with normal MBs assumed an abnormal morphology when incubated in the supernatant (Fig.45). Approximately equal numbers of abnormal cells were present in populations of cells both with (14%) and without (17%) MBs. This difference was not statistically significant; $p>.5$.

The Effect of Dihydrocytochalasin B and Cytochalasin D on Cell Morphology

To determine whether erythrocyte shape could be perturbed by incubation in drugs which are known to disrupt actin filaments (Atlas et al., 1978), cells were incubated in either 10ug/ml dihydrocytochalasin B or cytochalasin D for 3 hours. Following this treatment, erythrocytes maintained their normal morphology as verified by phase contrast microscopy (Fig.46a-c). When cytoskeletons from

Figure 45. Induction of abnormal morphology in Anadara ovalis erythrocytes with and without MBs. Normal cells with and without MBs were prepared with colchicine (as described in text) then incubated in supernatant from unwashed abnormal erythrocytes to induce morphological changes. Cells with MBs were normal in morphology prior to the addition of supernatant (a) but abnormal after supernatant was added (b). Similarly cells lacking MBs had normal morphology prior to the addition of supernatant (c) and abnormal morphology after supernatant was added (d). (X 1150).

45



these cells were incubated with polyclonal anti-actin to determine whether the drugs elicited any changes in cytoskeletal structure, no differences were seen between erythrocytes incubated with the drugs and those incubated with DMSO as a control (Fig.46d-f). MBs were intact in all cases as verified by phase contrast microscopy (Fig.46g-i).

Agents Tested for Their Usefulness in Isolating MBs

As shown in Table V, none of the substances tested here were useful in isolating intact MBs. Only free nuclei were visible by phase contrast microscopy when erythrocytes were extracted in LyM containing either 0.5M KCl or 0.1-0.5U/ml elastase. The same was true for cells extracted in LyM in the absence of protease inhibitors. Extraction in the presence of 0.5mg/ml heparin or o.5-4mg/ml DNase failed to remove the SAC.

Cytoskeletons prepared by extraction in LyM containing ATP were monitored by indirect immunofluorescence microscopy using anti-actin and anti-tubulin antibodies. When extracted in the presence of 1-10mM ATP the SAC appeared intact as reflected by anti-actin binding to it (Fig.47f and g), while at 10mM ATP the MB appeared to be unravelling with free MT ends visible (Fig.47b-d). At 20mM ATP the SAC was often absent or appeared to be extracted as shown by anti-actin binding to it (Fig.47h), while MBs unravelled so that only long tangled MTs binding the anti-tubulin antibody remained (Fig.47e).

Figure 46. The effect of dihydrocytochalasin B and cytochalasin D on Noetia ponderosa erythrocytes and their cytoskeletons. Cells were incubated for 3h in the drug and were then viewed by phase contrast microscopy or extracted in Triton X-100 and prepared for anti-actin immunofluorescence. Control cells had normal morphology (a), showed a diffuse fluorescence pattern when incubated with anti-actin (d), and had a normal MB (g). Cells incubated in either dihydrocytochalasin B (b) or cytochalasin D (c) had normal morphology, contained normal appearing SACs (e and f respectively), and normal MBs (h and i respectively). (N=nucleus); a,b,c (X 1500). d,e,f,g.h.i (X 2280).

46

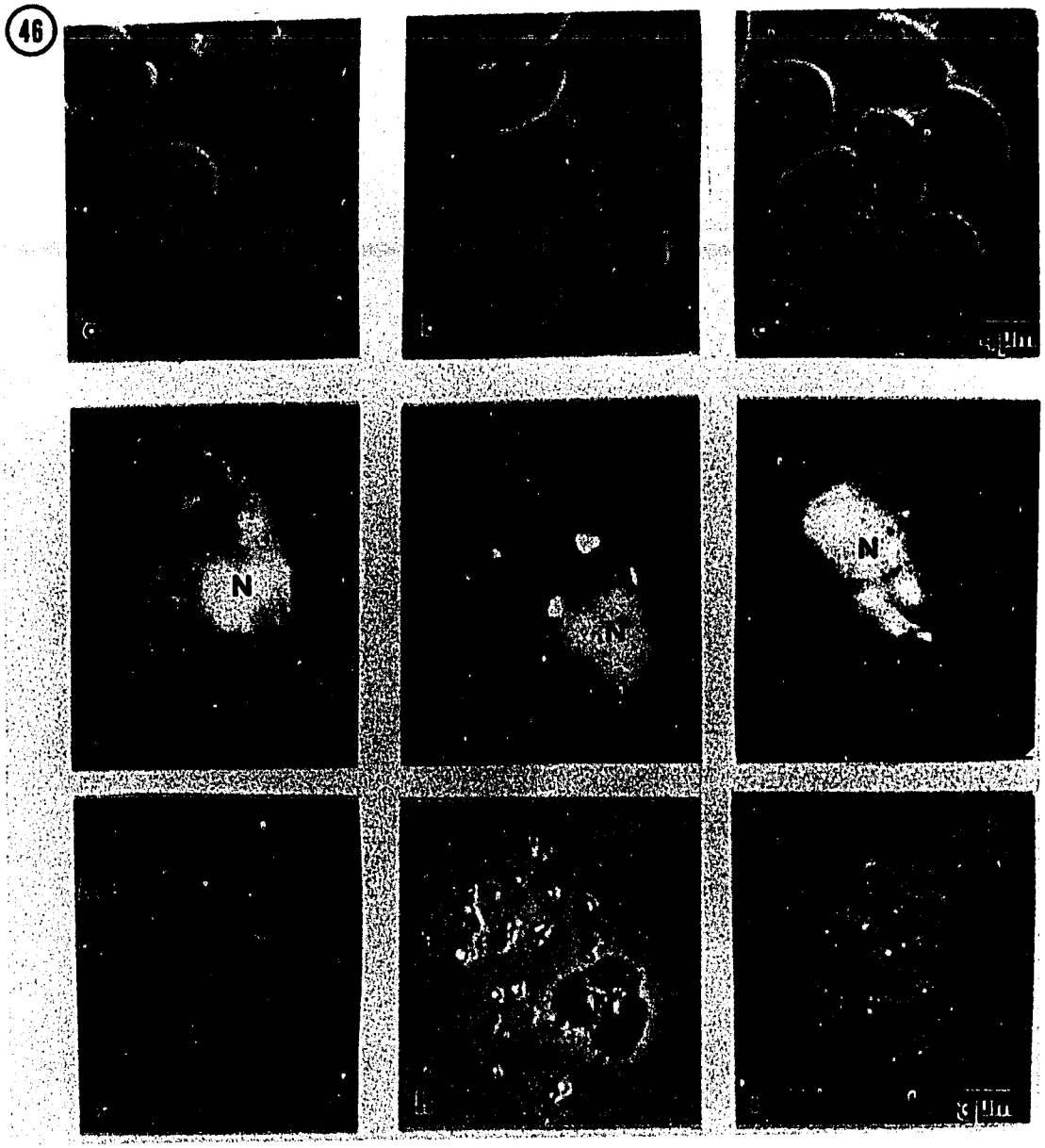


TABLE V

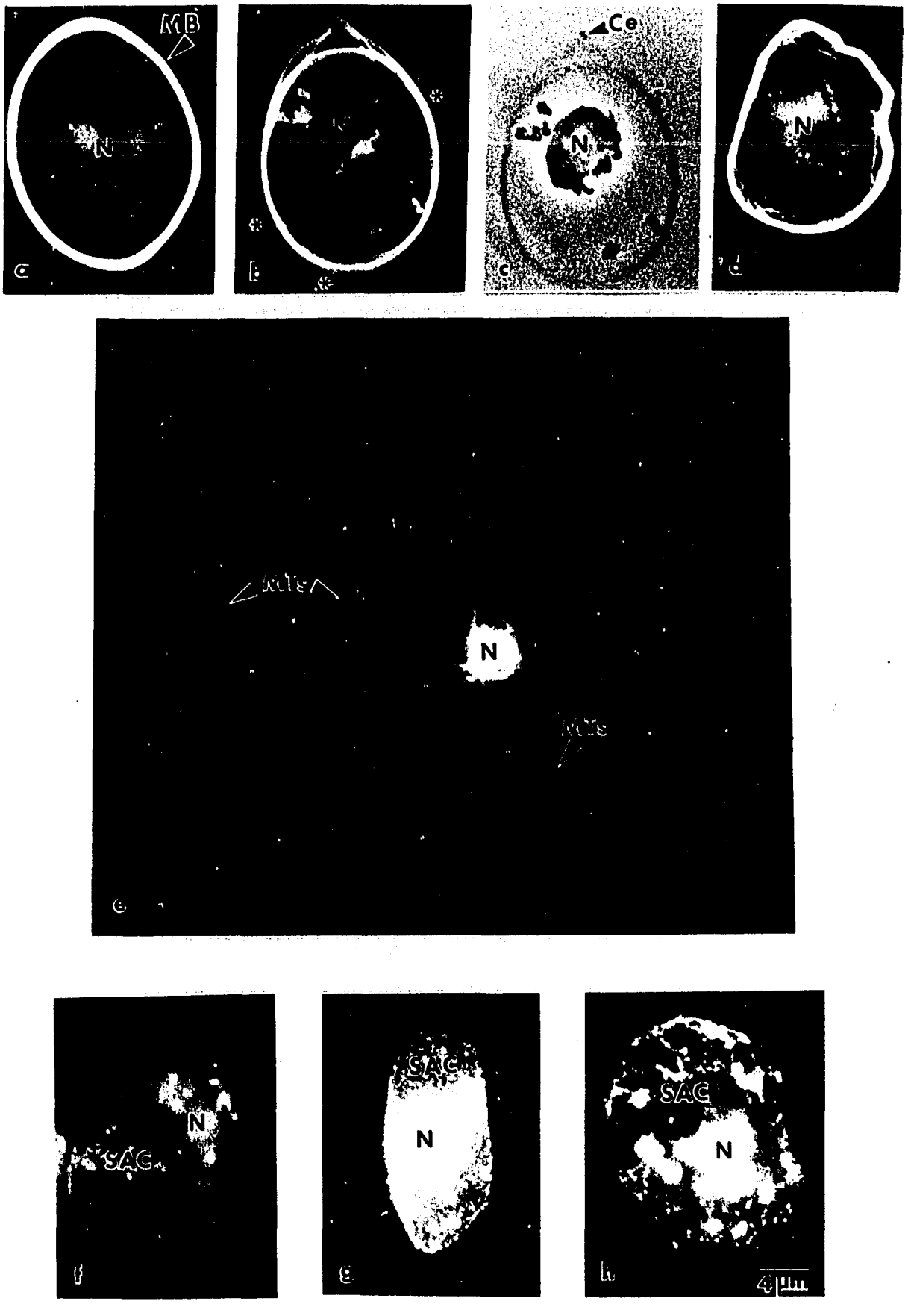
METHODS ATTEMPTED TO ISOLATE MARGINAL BANDS

Lysis Medium	Observed Effect
LyM* plus	
ATP 1-10 mM	No isolation of MB
ATP 20 mM	SAC partially extracted; MB unravelled
KCl 0.5M	Only free nuclei seen
Heparin 0.5 mg/ml	Only free nuclei seen
Elastase 0.1-0.5 U/ml	Only free nuclei seen
DNAse 0.5-4.2 mg/ml	No isolation of MB
LyM minus	
TAME/PMSF	Only free nuclei seen

*LyM=100mM PIPES, 1mM $MgCl_2$, 5mM EGTA, 10mM TAME, 0.1 PMSF, pH 6.8; MB=marginal band; SAC=surface-associated cytoskeleton.

Figure 47. The effect of ATP on Noetia ponderosa erythrocyte cytoskeletons as viewed by indirect immunofluorescence microscopy with anti-tubulin or anti-actin. Cells were extracted in Triton X-100 in the presence of varying concentrations of ATP and immediately prepared for immunofluorescence microscopy. A control cell has a normal MB (a) and a normal SAC which fluoresces diffusely with anti-actin (f). MBs in cells incubated in 10mM ATP often contain free MT ends (*) and a centriole (Ce) containing point (b and c) as well as wavy MTs (d). At 20 mM ATP, MBs unwound, producing long tangled MTs (e). Cytoskeleton prepared in the presence of 10 mM ATP has a normal SAC as viewed with anti-actin immunofluorescence (g). At 20 mM ATP anti-actin fluorescence becomes less regular and more fibrillar in appearance (h). (N=nucleus) (x1920).

47



DISCUSSION

MAJOR PROTEIN COMPONENTS OF THE 'BLOOD CLAM' ERYTHROCYTE CYTOSKELETON

The cytoskeletons of all erythrocytes, both vertebrate and invertebrate, studied thus far contain a cell surface-associated cytoskeleton (SAC) underlying the plasma membrane (Hainfeld et al., 1977:human; Cohen, 1978:Rana pipiens, Amphiuma, Notophthalmus viridescens; Cohen et al., 1980:molluscs; Nemhauser et al., 1980:Sipunculan; Cohen et al., 1982b: dogfish). In the mature, circulating erythrocytes of mammals, the SAC is the sole cytoskeletal component, responsible for cell shape and deformability (Sheetz, 1983). In all other vertebrate and several invertebrate erythrocytes studied, however, MBs of MTs located in the plane of cell flattening comprise a second cytoskeletal element (Meves, 1911; Fawcett, 1959; Maser et al., 1964; Barclay, 1966; Sekhon et al., 1969; Behnke, 1970; Cohen, 1978; Nemhauser et al., 1980; Cohen et al., 1980; Cohen et al., 1982,1982b). Although the structural similarities of erythrocytes from such phylogenetically diverse organisms as birds, elasmobranch fishes, and bivalve molluscs is striking, the work presented here provides evidence for the existence of biochemical differences within the cytoskeletons of vertebrate and invertebrate red blood cells.

Clam 240,000

Analysis by SDS-PAGE of the protein composition of nucleated cytoskeletons from "blood clam" erythrocytes, prepared by Triton extraction, revealed at least one major difference between the vertebrate and invertebrate erythrocyte cytoskeleton. Whereas the gel profiles of cytoskeletons of both mammalian and non-mammalian vertebrate erythrocytes contain a doublet migrating at Mr240,000 and Mr220,000 (α and β spectrin) with a 1:1 stoichiometry (Blanchet,1974; Steck, 1984; Cohen at al.,1982b; Bartelt et al., 1984) the "blood clam" erythrocyte cytoskeletal profile contained a single major band at Mr240,000 (Fig.8). The relative amount of this protein in "blood clam" erythrocytes based on coomassie staining of gels, did not diminish with $0^{o}C$ incubation and MB disassembly (Fig.18). Since the SAC is reported to be cold-stable (Nemhauser et al., 1982), the clam Mr240,000 protein was tentatively localized to it. Further evidence for this localization was shown by immunofluorescence microscopy of Triton-extracted cells utilizing an anti-chicken "α-spectrin" antibody which had reacted weakly with clam Mr240,000 on a nitrocellulose blot. Diffuse fluorescence of the SAC was seen with this antibody (Fig.20).

Clam Mr240,000 appears to belong to a class of proteins referred to as spectrin-like, which includes human erythrocyte spectrin, fodrin, and intestinal brush border TW260/240. That clam Mr240,000 is a member of this class is

based on the observations that clam Mr240,000 comigrated with human erythrocyte α-spectrin, bound chicken erythrocyte " α-spectrin" antibody and localized to the SAC along with actin. Anti-human spectrin antibody that was available to us however, did not react with clam Mr240,000 in a Western blot. Interestingly, Pinder et al. (1978) reported that erythrocytes from another marine invertebrate, Terebella lapidaria contained a 270,000Mr protein which cross-reacted with human erythrocyte spectrin. It is possible that common epitopes exist in the "blood clam" erythrocyte Mr240,000 and human erythrocyte spectrin which were not recognized by the antibody provided to us.

Differences also exist between clam Mr240,000 and non-mammalian vertebrate erythrocyte spectrin. Like clam Mr240,000, Bartelt et al. (1982) reported that an anti-human spectrin antibody did not bind to dogfish erythrocyte spectrin (fodrin). The non-mammalian vertebrate spectrin also differed from the human protein in the ability of the upper band of the dogfish spectrin doublet to bind calmodulin (Bartelt et al., 1982; Bartelt et al., 1984), a ubiquitous calcium-binding protein (Cheung, 1980). Clam Mr240,000 did not bind calmodulin under these conditions (Bartelt, personal communication). Chicken erythrocyte "α-spectrin", which as shown in the present study cross-reacted immunologically with clam Mr240,000, did however, bind calmodulin (Bartelt et al., 1982,1984). This ability to bind

calmodulin is a general feature of the higher molecular weight subunit of the non erythroid protein doublet, fodrin (Glenney et al., 1983), a spectrin-like protein consisting of Mr240,000 and Mr235,000 subunits (Glenney et al., 1982).

Based on the observations that clam Mr240,000 exists alone rather than in a 1:1 stoichiometry with a 220,000 protein, did not bind human spectrin antibody, did not bind calmodulin but did cross-react with chicken erythrocyte "α-spectrin", it was apparent to us that we could identify this protein as neither spectrin nor fodrin, but could only place it into the class of spectrin-like proteins as discussed above. Peptide mapping may provide a means of further identifying this protein and its relationship to fodrin and spectrin. It has been hypothesized that spectrin diverged from fodrin as erythrocytes evolved to the present mammalian type lacking the nucleus and all cytoskeletal components save the SAC (Bartelt et al., 1984). It is tempting to speculate that clam Mr240,000 is a progenitor of both proteins, with the calmodulin-binding property of fodrin being a later occurrence. Recently, Pollard (1984) has isolated an actin- binding protein (GP-260) from Acanthameba which migrates as a single 260,000Mr polypeptide on SDS polyacrylamide gels. This actin-binding protein cross-reacts with human erythrocyte β-spectrin. Antibodies prepared against GP-260 react weakly with an Mr260,000 protein in scallop membranes as well as in some vertebrate cell types. The presence of such a high molecular weight protein

migrating as a single band on SDS-polyacrylamide gels in invertebrate and protozoan cells, supports the idea of a single polypeptide as a progenitor of the spectrin-like heterodimers.

Actin

Gel analysis of clam erythrocyte cytoskeletons also revealed a 43,000Mr protein which we identified as actin based on antibody binding with both monoclonal and polyclonal anti-actin antibodies on a nitrocellulose blot (Figs.11 and 12). Immunofluorescence microscopy using the polyclonal anti-actin antibody localized the actin to the SAC (Fig.19). That the MB did not contain actin was verified by the fact that when the MB could be seen by phase contrast microscopy, it did not bind the anti-actin antibody. Our contention that actin is a SAC protein was further strengthened by the observation that the relative amounts of this protein did not diminish with 0°C incubation and MB disassembly (Fig.18).

This is consistent with the report of Cohen et al. (1982) who found that isolated dogfish MBs, when analyzed by SDS-PAGE, did not contain actin. Monaco, Saliestri and Bertolini (1982) however, found that both isolated MBs and the peripheral regions of circulating erythrocytes in tissue sections bound an anti-actin antibody obtained from the serum of a chronic hepatitis patient and that gel profiles of isolated MBs contain actin. The result obtained with

sectioned material which they report,could be interpreted as binding of the antibody to the SAC in cross-section, and does not necessarily refute the results presented here, but may only be due to a difference in interpretation of the micrographs. It is more difficult to reconcile their data on anti-actin binding to the isolated MB with the localization of actin to the SAC reported here. One can not rule out the possibility that actin is an integral component of amphibian MBs but not "blood clam" MBs. Recently, troponin T,a protein associated with thin filaments in skeletal muscle, has been localized to the MB of an amphibian erythrocyte (Lim et al., 1984). In addition, the association of MTs and actin via MAPs has been demonstrated in vitro and short filaments with the diameter of actin filaments have been seen in HeLa cell mitotic spindles (Pollard et al., 1984). "Blood clam" erythrocyte MBs are cold-labile (Nemhauser, Silverstein and Cohen, 1983) while the MBs of amphibians are cold stable (Behnke,1970a). Such differences in MB properties could be due to the presence of different proteins with which the MB associates. However, an alternative explanation for the binding of anti-actin to isolated MBs from amphibian erythrocytes and the presence of actin in polyacrylamide gel profiles of these MBs is the incomplete solubilization of an actin-containing SAC which remains associated with the MB. It was reported by Granger et al. (1982a), that MTs often remain associated with membrane fragments prepared by sonication of chicken erythrocytes, and therefore with the

cytoskeleton underlying the plasma membrane. If the SAC and MB do form a stable complex, disassociation of one from the other may be difficult to effect in regions of contact between them. Consistent with this idea is a recent report by Fach et al. (1984) that bovine brain MTs isolated in a glycerol-containing medium, have fodrin and actin associated with them prior to temperature-cycling. However, these two proteins do not temperature cycle with the tubulin, making them unlikely candidates for integral MT components.

The state of actin polymerization in "blood clam" erythrocytes was not elucidated in this study. Human erythrocyte actin is thought to exist in an oligomeric form based on its ability to nucleate polymerization of G-actin (Brenner et al., 1980). The diffuse pattern of actin antibody binding to "blood clam" erythrocyte cytoskeletons suggests that long F-actin filaments are not present, however this does not rule out the presence of short oligomers which may not be resolved with the light microscope.

Clam 85-120,000

Band 3, the anion channel transmembrane protein of the human erythrocyte is also involved in linking the cytoskeleton to the plasma membrane (C. Cohen, 1983). The treatment of human erythrocyte membranes with Triton X-100 is reported to extract 60% of this protein from the cell (Sheetz, 1979). When human erythrocyte ghost proteins are

analyzed by SDS-PAGE, band 3 migrates as a diffuse band with an average molecular weight of 95,000. The identification of band 3 in non-mammalian vertebrate erythrocytes was based on the presence of a diffuse band of similar although not identical molecular weight upon SDS-PAGE analysis of anucleate ghosts (Cohen et al., 1982b). Consistent with this identification is the observation that little or none of this protein was present in dogfish anucleate cytoskeletons prepared by Triton extraction (Bartelt, 1982). The "blood clam" erythrocyte anucleate ghost also contained a diffuse band with a similar molecular weight to human band 3 which was removed upon Triton extraction of the ghosts (Fig.9). Based upon its migration pattern and solubility in Triton we tentatively identified this protein as band 3. Recent reports based on the observations that antibodies raised against human band 3 cross-reacted with proteins in non-erythroid cells (Kay et al., 1983; Drenckhahm et al., 1984) suggests that this protein is conserved in nature.

An interesting correlation was observed between the presence or absence of clam band 3 and the shape of the erythrocyte cytoskeletons prepared from cells lacking MBs. Anucleate ghosts and Brij-extracted erythrocytes, both of which contain band 3, retained a flattened shape, similar but not identical to that of the intact erythrocyte in the absence of the MB. However, when erythrocytes lacking MBs were extracted with Triton, the cytoskeletons which contained little or no band 3 when analyzed by SDS-PAGE,

collapsed inward on the nucleus (Fig.35). When an MB was present, cytoskeletons were flat and elliptical in contour. A similar result was reported by Bartelt (1982) for dogfish erythrocytes. We hypothesize that the extraction of band 3 releases the SAC from the plasma membrane, so that the scaffolding necessary to keep the SAC well spread in the absence of the MB is no longer intact. This hypothesis is consistent with the identification of band 3 in the human erythrocyte as the integral membrane protein linked to the underlying cytoskeleton via ankyrin and 4.1 (C.Cohen,1983).

Ankyrin Cross-Reactivity

Ankyrin, or band 2.1 is a 215,000Mr protein which contains binding sites for spectrin and band 3 in the human erythrocyte (Bennett, 1980). Proteins cross-reacting with human erythrocyte ankyrin have been found in both HeLa cells and cultured fibroblasts. Immunofluorescence microscopy using anti-ankyrin antibody revealed a binding pattern similar to that for tubulin (Bennett, 1981;1982b). In brain extracts, MAP 1 was found to cross-react with antibodies prepared against human erythrocyte ankyrin. Therefore, it has been hypothesized that ankyrin evolved from a MAP (Bennett, 1982b). When "blood clam" erythrocyte cytoskeletons were probed with anti-ankyrin antibody, there was a reactive band at Mr200,000, as well as 2 bands with apparent molecular weights of greater than 300,000 (Fig.13). Immunofluorescence microscopy of Triton-extracted cells with

this antibody showed that the antibody bound to both the MB and SAC (Fig.21). Binding to the SAC is consistent with the localization of ankyrin in the mammalian erythrocyte. Binding to the MB is puzzling since it is not likely that these 2 high molecular weight proteins are MB MAPs which cross-link adjacent MTs. Two proteins with similar molecular weights are present in "blood clam" anucleate ghosts which lack MBs and tubulin (Fig.9). However, these high molecular weight proteins could be involved in binding the MB to the SAC. This possibility will be discussed in more detail in the section on MAPs.

Clam 80,000

Band 4.1 is a 78,000Mr protein present in mammalian red blood cells which mediates the connection between spectrin and actin (C. Cohen, 1983). Granger et al. (1984) reported that avian erythrocytes contain an analogous protein, based on peptide mapping, which is composed of multiple variants, differentially phosphorylated with apparent molecular weights varying from 87,000 to 175,000. Bartelt (1982) reported that only trace amounts (less than 1% of the entire cytoskeletal protein) of a protein comigrating with Band 4.1 was present in dogfish erythrocytes. In the "blood clam" erythrocyte a protein comigrating with human erythrocyte band 4.1 (80,000Mr) was identified here as an MB MAP based on the fact that its presence was dependent on the presence of an intact MB. It is unlikely that this protein is a 4.1 analog since one would expect such a protein to localize to

the SAC rather than to the MB. A solid phase radioimmunoassay done by Spiegel (personal communication,1983) revealed no specific binding of anti-human 4.1 antibody to "blood clam" erythrocyte cytoskeletons immobilized in plastic wells.

Intermediate Filaments

The term intermediate filament is a general categorization for 5 distinct types of 10nm filaments which exhibit cell or tissue specificity (Lazarides, 1982). The major IF protein reported to be present in chicken erythrocytes is vimentin, which has been found in many cells in culture and in mesenchymal cells, of which erythrocytes are one type. A filamentous pattern surrounding the nucleus and extending into the cytoplasm has been seen in chicken erythrocyte cytoskeletons incubated with anti-vimentin antibody (Granger et al., 1982). It has been hypothesized, based on the distribution of filaments, that they are responsible for anchoring the nucleus within the erythrocyte (Virtanen, 1979).

To test for the presence of IFs, "blood clam" erythrocyte cytoskeletons were prepared by extraction in a Triton-containing medium of sufficient ionic strength to retain IFs in the Triton-insoluble fraction (Granger et al., 1982a,b), and incubated with the same anti-vimentin antibody that reacted in a filamentous pattern with chicken erythrocytes. No specific antibody binding was apparent

(Fig.22), and furthermore, no reactivity was seen when these cytoskeletal proteins were transferred to nitrocellulose and probed with the anti-vimentin antibody (Fig.14). However, this negative result does not conclusively demonstrate the absence of IFs in "blood clam" erythrocytes. In the course of these experiments we observed that chicken erythrocytes lysed in the IF-stabilizing medium could not be enucleated by fluxing through a 22g needle affixed to a 10 ml syringe, suggesting that the IFs are anchoring the nucleus within the cytoskeleton. The "blood clam" erythrocyte cytoskeletons prepared in the same medium, however, were easily enucleated. This observation is consistent with the hypothesis that the nucleus of the "blood clam" erythrocyte is not held in place by IFs. Cohen (1978) proposed a model for the non-mammalian erythrocyte cytoskeleton in which the nucleus is kept in place by the tension of the SAC upon it. In such a model, nucleus-anchoring IFs are not a necessary component of the cytoskeleton.

Marginal Band Microtubules

In 1959, the ultrastructure of the MB was first described by Fawcett. However, it was not until 1982 that Cohen et al. isolated MBs from dogfish erythrocytes and analyzed MB components biochemically. SDS-PAGE analysis of these dogfish MBs revealed only tubulin and a minor protein component at Mr35,000 (Cohen et al., 1982a). The tubulin, which often focused into 4 distinct bands, was identified by its comigration with bovine brain tubulin. MT protein

isolated from avian erythrocytes and purified by an *in vitro* assembly procedure similar to that used for brain tubulin, was found to consist of 95% tubulin with small amounts of "spectrin" and the low molecular weight MAPs called the tau proteins (Murphy et al., 1983a,b). Murphy et al., also reported that the β tubulin subunit (the slower migrating subunit on SDS-polyacrylamide gels) of erythrocyte MBs was different from that of brain MT protein based on peptide maps and isoelectric focusing. The question of whether the anomalous β tubulin is present in MBs of all species is an interesting one and could be approached using specific antibodies prepared against this protein.

In this study we identified clam erythrocyte tubulin using a monoclonal antibody raised against yeast tubulin. Immunofluorescence microscopy of clam erythrocyte cytoskeletons using this monoclonal antibody revealed selective binding to the MB (Fig.19). This antibody reacted only with the a single 60,000Mr protein on a Western blot of blood clam cytoskeletal proteins (Fig.11). Tubulin cycled with the MB during its disassembly at $0^{o}C$ and subsequent reassembly at room temperature (Fig.18), further demonstrating that tubulin was a major component of the MB. In addition, two minor proteins of Mr105,000 and Mr80,000 also cycled with the tubulin. These may be MB MAPs.

Marginal Band Associated Proteins

The presence of cross-bridges between adjacent MTs in the

MB has been demonstrated by thin section electron microscopy of dogfish erythrocyte cytoskeletons (Cohen et al., 1982b), "blood clam" erythrocyte cytoskeletons (Nemhauser, Joseph-Silverstein and Cohen, 1983), and Limulus amebocytes (Nemhauser et al., 1980). The presence of cross-bridges or bundling proteins, the composition of which has yet to be fully elucidated, is consistent with the observation that the MB remains intact upon isolation (Bertolini et al., 1976; Cohen et al., 1982b). Sloboda et al. (1980) demonstrated the binding of a MAP2 polyclonal antibody to the MB of amphibian and chicken erythrocytes by immunofluorescence microscopy, and Centonze et al. (1984) localized the binding of this antibody to the cross-bridges present between adjacent MTs of an amphibian MB by immunoelectron microscopy. However, no one has yet co-isolated MAP2 with MB tubulin. As described previously, Murphy et al. (1983a,b) isolated MT protein from chicken erythrocytes and found only small amounts of the tau proteins associated with it, while Cohen et al.(1982a) isolated intact MBs from dogfish erythrocytes and found a small amount of a 35,000 Mr protein associated with the tubulin. Monaco et al. (1982) reported that salamander erythrocyte MBs isolated by deoxycholate contain a glycoprotein (as determined by the NaB^3H_4 method) with an apparent molecular weight of 90,000. Based on the observation that dense peritubular material visible in thin sections through erythrocyte ghosts could be removed by

neuraminidase (a carbohydrate removing enzyme) resulting in a decreased distance between MB MTs and the appearance of incomplete C-microtubules, they have hypothesized that this 90,000Mr protein is a component of the peritubular "cement" which bridges MTs to one another and imparts stability to the MB.

That MAPs other than MAP1, MAP2 or the tau proteins could be associated with the MTs of an MB is consistent with the recent identification of multiple kinds of MAPs in various cell types. Many of these proteins have been identified as MAPs based on the fact that they co-cycle with tubulin during cycles of polymerization and depolymerization of MT proteins. For example, Bulinski et al. (1979,1980) reported the presence of a MAP with an apparent molecular weight of 210,000 in HeLa cells, while Olmsted et al. (1981) reported that two proteins with apparent molecular weights of 215,000 and 71,000 as well as proteins with apparent molecular weights of 59,000 and 58,000 were present when purified MT protein from neuroblastoma cells was polymerized in vitro.

A different approach to the problem of the identification of MAPs was taken by Solomon and his coworkers (1979) who reasoned that implicit in the definition of the term MAP was the idea that these proteins should be released, along with tubulin, under depolymerizing conditions. They devised a system in which cytoskeletons

from cells with intact MTs and those from cells in which the MTs had been depolymerized using colchicine, were extracted with Ca^{++}. Extracts from cytoskeletons which had contained intact MTs were then compared to those from cytoskeletons in which the MTs had been initially depolymerized. In this way a 150,000Mr protein associated with MTs in the mitotic spindle of mammalian cells (Zieve et al., 1982), a 69,000Mr and a 220,000Mr protein associated with cytoplasmic MTs in a hamster cell line, and a 55,000Mr and a 80,000Mr protein associated with cellular MTs in mouse neuroblastoma cells were identified (Duerr et al., 1981).

We have followed reasoning similar to that of Solomon to identify MAPs in the "blood clam" erythrocyte MB. We devised conditions in which cells with and without MBs could be made at the same temperature, reasoning that MAPs acting as cross-bridges would be solubilized upon MB disassembly and therefore be missing, along with tubulin, from the Triton-insoluble residue when the cells were extracted. We always compared cytoskeletons at the same temperature to avoid misidentification of cold-labile proteins as MAPs. Nocodazole and colchicine blocked MB reassembly following $0^{o}C$-induced disassembly (Fig.28), while taxol blocked $0^{o}C$-induced disassembly (Fig.23) and initiated MB reassembly at $0^{o}C$ (Fig.25). We thus prepared cells with and without MBs at the same temperature, and identified two proteins, Mr80,000 and Mr105,000, whose presence in the Triton-insoluble residue was dependent on the presence of an intact MB. We

also showed that these two proteins were released from cytoskeletons into the soluble fraction when MBs were disassembled *in vitro*. In this system Brij-extracted cells were incubated at 0°C to disassemble the MB. In these experiments 78% of the total protein was tubulin and 12% the Mr80,000 and Mr105,000 proteins. The remaining 10% of the total protein present consisted of actin, clam Mr240,000 and proteins with apparent molecular weights of greater than 300,000, Mr270,000, Mr220,000 and Mr60,000. Two independent lines of evidence demonstrated that actin was not MB-associated: a) anti-actin antibody did not bind to the MB and b) actin did not temperature-cycle with the tubulin. The proteins with apparent molecular weights of greater than 300,000, Mr270,000, Mr220,000 and Mr60,000 may be MAPs which are normally present in such small quantities in cytoskeletal preparations that cycling with the MB is not obvious by SDS-PAGE analysis. The presence of clam Mr240,000 in the soluble fraction may be due to its extraction from the SAC. Human erythrocyte spectrin is extracted at low ionic strength in the presence of calcium chelators (Marchesi, 1979). The medium in which the "blood clam" erythrocyte cytoskeletons are incubated is of low ionic strength and contains EGTA. MAPs with similar molecular weight to the Mr80,000 and Mr105,000 proteins seen here have been identified in other cell types (Duerr et al., 1981; Olmsted, 1981; Vallee et al., 1983). However, neither Murphy

et al. (1983a,b) nor Cohen et al. (1982a) detected proteins with these molecular weights in cytoskeletal preparations from vertebrate erythrocyte MBs. Preparation of antibodies which would recognize the Mr80,000 and Mr105,000 proteins in intact cytoskeletons would provide a means of verifying their identification as MAPs by immunofluorescence localization as well as studying their distribution in MBs from other species.

As described above, an antibody prepared against hog brain MAP2 recognized a protein with an apparent molecular weight of 240,000 as well as two bands with apparent molecular weights similar to tubulin in the clam erythrocyte cytoskeleton (Fig.14). When immunocytochemistry was carried out on these cytoskeletons using this anti-MAP2 antibody, weak fluorescence of the MB and SAC was observed (Fig.21). Davis et al. (1982) reported that MAP2 was immunologically related to human erythrocyte α-spectrin based on cross-reactivity with an anti-α-spectrin antibody. It is thus possible that the MAP2 antibody used in this study recognizes an epitope in clam Mr240,000, explaining fluorescence of the SAC.

The binding of anti-MAP2 to two bands which comigrate with tubulin was unexpected since this antibody did not bind to bovine brain tubulin in the immunoblot system used here. There are several alternative explanations for this result. 1) This antibody is binding nonspecifically to clam tubulin. However this is unlikely since it only binds to these two

proteins and Mr240,000 in a complex mixture of proteins from anucleate ghosts. 2.) The antibody is binding to low molecular weight breakdown products of MAP2 with apparent molecular weights similar to those of tubulin, or low molecular weight MAPs that share an epitope with MAP2. Neither has been described in the literature. 3.) MAP2 and clam tubulin contain a shared epitope. There is no evidence suggesting that MAP2 and tubulin are structurally similar. However, they can share a function. For example, MAP2 binds to the surface of MTs as does tubulin under certain experimental conditions and in some specialized MT systems. The C-microtubule of the flagellum outer doublet pair (subfiber B) binds to the adjacent complete MT (subfiber A). Experimentally, one can promote the addition of exogenous bovine brain tubulin to the surface of cellular MTs in a "hook-like"conformation (Heidemann et al., 1980). The MAP2 antibody used here may recognize a binding site for MTs in both MAP2 and clam tubulin. Erythrocyte clam tubulin may be structurally different from vertebrate nonerythroid tubulin and therefore may contain binding domains not found in brain tubulin. Murphy et al. (1983b) reported that chicken erythrocyte β tubulin is different from nonerythroid β tubulin.

That no binding of MAP2 to high molecular weight proteins occurred in our Western blotting system is not a conclusive demonstration that such proteins do not exist.

The presence of bands greater than 300,000Mr in our cytoskeletal preparations is variable, and when present, they contribute little to the total protein of our sample. The proteins greater than 300,000Mr seen in "blood clam" cytoskeletal preparations are unlikely to be MAPs cross-bridging adjacent MTs, since they are seen in samples of anucleate ghosts in which tubulin is absent. However, it has been hypothesized that proteins could be present in the periphery of the cell which determine the path of the MB (Granger et al., 1982b; Swan et al., 1984) and which if associated with the SAC would not necessarily cycle with the MB. Solomon (1984) has tentatively identified MAP2 as a component of the chicken erythrocyte cytoskeletons and has hypothesized that it is such an "informational" protein since it is present in the cytoskeleton at both 0°C (in the absence of a MB) and at 37°C (when an MB is present). As discussed earlier, however, Centonze et al. (1984) has localized anti-MAP2 binding to the cross-bridges between adjacent MTs of the MB in an amphibian erythrocyte. It is obvious that the role of MAP2 in MBs is still to be determined.

THE REGULATION OF MB REASSEMBLY

The mechanism of reassembly of a normal appearing functional MB in the original plane of cell flattening following low temperature treatment of erythrocytes has yet to be fully elucidated. Nemhauser, Joseph-Silverstein, and Cohen (1983) reported that MB reassembly in the "blood clam"

erythrocyte was initiated by a centriole pair and associated material. However, Barclay (1966) reported that MB reassembly in chicken erythrocytes involved the reorganization of randomly oriented reassembled MTs into a single plane. A similar pattern of organization was seen with the reassembly of dogfish erythrocyte MBs (Joseph-Silverstein, unpublished observations) In contrast, Miller et al. (1984) reported that the MB in the chicken erythrocyte reassembles spontaneously at the periphery of the cell with no more than two MTs obvious prior to the appearance of a complete circumferential structure. No obvious MTOCs were present in the chicken erythrocytes.

The "blood clam" erythrocyte is the only MB system studied thus far in which reassembly is initiated at a pair of centrioles. Immunofluorescence microscopy with anti-tubulin antibody was used to monitor MB reassembly in both Noetia ponderosa and Anadara ovalis to verify the electron microscopic observations of Nemhauser et al. (1983). Larger numbers of cells could be sampled and the entire cytoskeleton, rather than only a single plane through it could be viewed with this method. The results of this study verified the observation that MB reassembly in the "blood clam" erythrocyte was initiated at the centrioles with early intermediates resembling a half-spindle (Fig.15c and d; 16a and c). Large numbers of cytoskeletons did not contain continuous MBs until 20 minutes of rewarming. Even these

continuous MBs contained many MT profiles which were not yet bundled together (Fig.15e-h), in contrast to the report of Miller et al. (1984) described above for chicken MB reassembly. These observations suggested that bundling of MTs to form an MB is not concurrent with initial MT polymerization and elongation, but rather occurs at a later stage when MTs elongate to reach the pole of the cell opposite the centrioles, where one could hypothesize that MTs begin to "zip" together with bundling working towards the centrioles.

That centriole positioning may provide the information necessary for the spatial organization of the MB, was one of several hypotheses put forth by Nemhauser, Joseph-Silverstein and Cohen (1983) to explain MB reassembly. However, this would not account for reassembly of a normal MB in systems lacking centrioles. An alternative hypothesis originally put forth by Granger et al. (1982a), invoked the presence of a track or groove in the inner plasma membrane surface which would direct the reformation of the MB. This was based on his observations of membrane patches prepared by hypotonic lysis and sonication of chicken erythrocytes. When present following sonication, MB MTs often remained associated with these patches, while when absent, track-like structures were seen on the inner surface of the membrane, reflecting the position of the MTs. Miller et al. (1984) observed MB reassembly in intact chicken erythrocytes and similarly hypothesized that the determinants for reassembly

of a normal MB were present in the periphery of the cell. Swan et al. (1984) incubated avian erythrocyte cytoskeletons lacking MBs with exogenous brain tubulin to determine whether the remaining cytoskeleton contained the necessary information for MB reassembly. They found that in 35% of the cytoskeletons smooth continuous MBs were reassembled, while short peripheral MTs were reassembled in an additional 40% of the cytoskeletons. The peripheral location of the MTs suggested to Swan et al. that determinants for the spatial positioning of MB MTs are present in cytoskeletons lacking endogenous tubulin.

A similar hypothesis was put forth by Murray et al. (1984) based on his observations of a nonerythroid system. Membrane fragments from the algae Distigma proteus were isolated and the complex MT arrays on their inner surface solubilized. With the addition of exogenous tubulin, reassembly of MT arrays with similar structure to those normally present occurred, suggesting that MT-binding sites in the plasma membrane guided reassembly. In Purkinje cell dendrites, a high molecular weight MAP associated with the growing dendritic tip and underlying the plasma membrane, is present prior to the appearance of MTs, suggesting that this protein acts as a framework for dendritic MTs (Bernhardt et al., 1982). Therefore, the presence of determinants underlying the plasma membrane which act as organizers of MT pattern has been suggested for cell types from a diversity

of species.

The study of MT reassembly in neuronal cells has led to another interesting possible mechanism for MT reassembly. Heidemann et al. (1984) depolymerized axonal MTs by cold treatment and then studied the organization and polarity of MTs reassembled by incubation at 37°C. A population of short cold- stable MTs was present in these axons. Since the reassembled MTs were of the same polarity as the original MTs, they hypothesized that these cold-stable MTs acted as nucleating centers and therefore organizers for MT reassembly.

In heliozoan axonemes, which are made up of a complicated 12-sectored double spiral MT pattern, the term "self-linkage" has been used to describe the assembly of such a pattern (Jones et al., 1981). Jones et al. found that axoneme MTs radiate randomly from MTOCs during early stages of reassembly following 0°C incubation of the heliozoan, Echinosphaerium, suggesting that although necessary for initiating reassembly, the MTOCs do not organize the double spiral pattern. Fine strands or bridges were present between MTs during pattern arrangement suggesting that adjacent MTs sufficiently close to one another, "zipped" together by cross-linking.

Cultured interphase cells usually contain a single perinuclear MTOC from which cytoplasmic MTs radiate (Brinkley et al., 1981). To study the role of the MTOC in the spatial organization of cytoplasmic MT arrays, Karsenti

et al. (1984) prepared cytoplasts (enucleated cell fragments) from cells in which the MTs had been depolymerized by nocodazole. Reassembly of MTs (by removal of the drug) in cytoplasts containing or lacking centrosomes was studied by immunofluorescence microscopy using anti-centrosome and anti-tubulin antibodies. In the presence of centrosomes, cytoplasts from confluent cells contained MTs which were focused on these MTOCs as well as some peripheral MTs, while in the absence of centrosomes, MTs with a mostly peripheral distribution were present. Therefore, centrosomes influence MT distribution in cultured cells and act as MT organizers (Karsenti et al., 1984).

Other experimentally produced cell fragments have been used to study the spatial organization of MT assembly. Gray et al. (1982) experimentally induced the assembly of peripheral MTs in synaptosomes, which are vesicles formed by homogenization of nerve endings. Filamentous projections were reported to be present between adjacent synaptosome MTs, although no MAP2 was present (Burgoyne et al., 1983). The mechanism of MT assembly in synaptosomes has yet to be elucidated. One would not expect peripheral determinants to be present in experimentally produced vesicles and MTOCs have not been found as yet to be associated with these MTs.

It is obvious from the above discussion of MT assembly and reassembly in various cell types and under varying experimental conditions, that no unifying theme for the

mechanism of spatial organization of MTs exists as yet. It is possible that different cell types employ different mechanisms for MT assembly and reassembly. In cultured cells, interphase MT arrays must give way to mitotic spindles during the cell cycle while the MB or the cortical MTs of a protozoan are present throughout the cell cycle. Such differences in lability may necessitate the existence of different MT organizers. It is also possible that more than one of the hypothetical spatial organizers described here are utilized by a single MT array. The "blood clam" MB, with its centrioles, cross-bridges, and peripheral location may help to elucidate this problem. Although we did not directly address this problem here, the experiments in which MBs were induced to reassemble in the presence of taxol at $0^{o}C$ and those on MB reassembly in cells with abnormal morphology, may be useful in further examination of this problem.

When we incubated "blood clam" erythrocytes at VF-20!o_{C} (following MB disassembly) in the presence of taxol, a normal appearing MB was formed (Fig.25d and 27). In contrast, atypical MT arrays which are not associated with an MTOC are formed when nonerythroid cells are incubated at room temperature in the presence of taxol (de Brabander et al., 1981; Manfredi et al., 1982). de Brabander et al. (1981) reported that when cultured cells were preincubated with nocodazole prior to the addition of taxol, an MT network radiating from the MTOC and resembling a somewhat

normal network could be observed. This experiment was analogous to ours in that MT reassembly was induced in cells lacking MTs. These results suggested to us that the information necessary for MB reassembly was present in the cell even at low temperature. That taxol-induced MT reassembly was initiated at the centrioles in an "astral" configuration, rather than the normal "pointed" configuration did not seem to affect the final appearance of the MB, suggesting that although the centrioles function to initiate MB reassembly at 0°C, they may not provide the information for the spatial organization of the MB. However, the presence of "informational" proteins in the SAC or plasma membrane could account for normal MB reassembly. If such informational MB-binding proteins do indeed exist, they could be MB associated proteins which do not cycle with the MB.

The reassembly of MTs in erythrocytes of the "blood clam" Anadara ovalis which have an abnormal amorphous morphology, was studied to determine whether normal MBs could reassemble in these cells. If peripheral determinants or "tracks" in the SAC are responsible for normal MB reassembly, then an amorphous cell containing an irregular SAC may reassemble MTs in an abnormal configuration. These erythrocytes sometimes contained recognizable but loosely bundled MBs with additional looping MTs in the cytoplasm (Fig.40b and c). However, when incubation at 0°C to

disassemble the MTs was followed by reassembly at 18-20°C recognizable MBs were rarely encountered. Rather, tangled loops of MTs were present, often resembling a ball of yarn (Fig.43). We have shown that MT configuration is not responsible for the abnormal shape of these erythrocytes since normal erythrocytes lacking MBs undergo morphological transformation in the presence of supernatant from abnormally shaped cells. Therefore, the MB is not the primary effector of the erythrocyte's change in shape from flattened ellipse to amorphous mass. We hypothesize that the SAC is the cytoskeletal component initially responding to the factor in the fluid phase of the blood to bring about the shape change and that the change in the SAC also results in a loss of MB organizing information. Based on earlier observations that removal of the SAC caused MB circularization (Cohen, 1978; Cohen et al., 1982; Bertolini et al., 1976) and the observation by Joseph-Silverstein et al. (1984) that pointed dogfish erythrocytes with correspondingly pointed cytoskeletons contained pointed MBs, it is obvious that the function of the MB and SAC are intimately tied together.

This failure of normal MB reassembly in the abnormally shaped Anadara ovalis erythrocytes is consistent with the possibility that a continuous track within the SAC which is responsible for normal MB reassembly is lost. Alternatively, the molecular basis for the initial disorganization of the MB in response to the supernatant factor may be a loss of

cross-bridging proteins, including those that may bridge the MTs to the SAC. Although the observations on Anadara ovalis erythrocytes could be interpreted in a way consistent with the concept of an informational track, we have no direct evidence for a track in either of the "blood clam" species. The approach used here for the identification of MB MAPs was based on the assumption that such proteins would cycle with the MB and therefore this approach would not identify non-cycling MB associated proteins. Experiments similar to those of Swan and Solomon (1984) in which MT reassembly was induced in a detergent-extracted cell may help to elucidate this problem. It would be interesting to extract the cells under varying conditions such as different detergents and different ionic strengths and determine which conditions promote reassembly of a recognizable MB. In this way one may eventually be able to identify the "informational" proteins if, indeed, they do exist.

MB FUNCTION

Our ability to prepare cells with and without MBs under otherwise similar conditions allowed us to study the function of the erythrocyte MB in addition to the MB associated proteins discussed above.

The MB is generally believed to function in erythrocyte morphogenesis. The evidence most often cited for this role is the temporal correlation of MB appearance with the spherical to discoidal transformation in cell shape (Barrett

et al., 1972; Small et al., 1972; Barrett et al., 1974; Yamamoto et al., 1975). In addition, in the camel family MBs can be found in erythroblasts taken from the bone marrow, but are not usually present in circulating mature cells (Goniakowska-Witalinska et al., 1977; Cohen et al., 1979).

When platelet MBs are disassembled at 0°C the cells lose their disc shape and assume a spherical or irregular morphology. The normal flattened shape is regained, however, upon reassembly. Occasionally, platelet MBs will reassemble abnormally into a rod rather than a continuous MB, and in this case the platelet takes on an abnormal spindle-like morphology (Behnke, 1970). In contrast, disassembly of the erythrocyte MB does not lead to a loss of the normal flattened elliptical shape in mature cells. Taken together with the observation that the number of MTs in the MB decreases in the erythrocyte over time with no effect on normal cell shape (Small et al., 1972; Barrett et al., 1974; Yamamoto et al., 1975), these data have led many investigators to conclude that the MB does not play a continuing role in mature erythrocytes.

The fibrous and flexible nature of the erythrocyte MB observed with the light microscope suggested to Meves in 1911 that MBs might function in erythrocytes to maintain cell shape in response to mechanical forces. Meves wrote, "It is well known that the red blood corpuscle can change its shape passively due to a mechanical influence, be it within the body or without; however, it assumes its original

shape as soon as the force ceases. This is made possible by the elasticity inherent in the marginal ring which allows it to return to its natural state..." (Meves, 1911). Fawcett raised the same issue in later work (Fawcett et al., 1964). We have reported evidence for such a function in dogfish erythrocytes (Joseph-Silverstein et al., 1984). When cells prepared with and without MBs were compared for their response to the mechanical stress of fluxing through capillary tubes, we observed that a significant number of those without MBs tended to be deformed following the treatment, while those with MBs typically remained normal. Thus we hypothesized that the MB in mature, circulating erythrocytes functions in one of two ways: 1.) the MB resists deformation of the erythrocyte during flow 2.) the MB resists deformation up to a certain force above which the erythrocyte becomes deformed, but rapidly recovers its normal shape due to the flexible nature of the MB.

Here we provide evidence that such a role for MBs in mature cells is universal. The erythrocytes of "blood clams" respond similarly to those of vertebrates, with extensive folding and buckling observed in cells lacking MBs when mechanical stress in applied (Fig.36 and 37). As in the case of dogfish erythrocytes (Joseph-Silverstein et al., 1984), this general result was not dependent on the treatment used to prepare cells with and without MBs. It was apparent in cells with taxol-stabilized MBs, taxol-reassembled MBs, and

MBs reassembled after rewarming. However, we did observe increased numbers of deformed erythrocytes in control (MB-containing) samples when taxol was used to reassemble MBs at $0^{o}C$, as compared with the other methods used to prepare cells containing MBs. This may be due to a lower MT number or lesser degree of bundling in MBs reassembled under these unusual conditions. However even in this experiment, the frequency of deformation in preparations of erythrocytes lacking MBs was at least three times that of the controls.

We have in addition, demonstrated here the deformable nature of normal (untreated) "blood clam" erythrocytes (Fig.38). The question was raised above as to whether the MB functions in initial cellular resistance to deformation, recovery from deformation, or both. This is not resolved in our experiments. However, the demonstration of clam erythrocyte deformability, taken together with the results of the mechanical stress experiments and previous work on the effects of mechanical stress on vertebrate erythrocytes (Joseph-Silverstein et al., 1984), one can hypothesize that the MB functions to resist deformation up to some threshold value above which the cell becomes deformed, and subsequently promotes rapid recovery of normal cell shape. Preparation of erythrocytes with and without MBs and their visualization during flow would provide evidence for this hypothesis. One would expect to see cellular deformation in both cases, but more rapid recovery of cells containing MBs. One could also measure deformability with an ecktacytometer

to determine whether cells with and without MBs were equally deformable. Our hypothesis suggests that cells with MBs may resist small forces while cells lacking MBs would be deformed at those same forces.

The work of White et al. (1984) on platelets with and without MBs is consistent with this hypothesis. When he compared the responses of platelets to partial aspiration into micropipettes, those with MBs were more resistant to deformation, but once deformed, rapidly rapidly recovered their normal discoidal shape.

The simplest mechanism which would account for such continuing MB function is one based on redistribution of strain within a flexible MB. An inward directed force would initially meet resistance and then deformation of the MB would occur. Upon removal of the force, the MB would spring outward, restoring cell shape. The same mechanism would account for linearization of broken MBs (Bertolini et al., 1976; Cohen, 1978; Joseph-Silverstein et al., 1984) and for generation of pointed erythrocytes, a phenomenon described in detail for amphibian erythrocytes by Meves (1911) in attempting to explain erythrocyte deformability.

This mechanical function as proposed for MBs in mature erythrocytes would be independent of the cellular mechanism of reassembly. In dogfish erythrocytes and in mammalian platelets centrioles do not appear to participate in experimentally induced reassembly (Behnke, 1967; Behnke,

1970; Joseph-Silverstein, unpublished observation), while in "blood clam" erythrocytes centrioles act as organizing centers for MB reassembly (Nemhauser et al., 1983).

Thus, through the development of a system to prepare erythrocytes with and without MBs under otherwise similar conditions, we have been able to elucidate an ongoing function for the MB, a structure previously thought to be involved only in cellular morphogenesis.

APPENDIX

Abbreviations

IFs - intermediate filaments

LyM - lysing medium containing 100 mM PIPES, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM TAME, 0.1 mM PMSF and Triton X-100

LyM wash - lysing medium lacking Triton X-100

MAP - microtubule-associated protein

MB - marginal band

PMSF - phenylmethylsulfonyl fluoride, a protease inhibitor

SAC - surface-associated cytoskeleton

TAME - tosyl arginine methyl ester, a protease inhibitor

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