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Marginal band protein components and microtubule bundling

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MARGINAL BAND PROTEIN COMPONENTS AND MICROTUBULE BUNDLING

by

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Abstract

MARGINAL BAND PROTEIN COMPONENTS AND MICROTUBULE BUNDLING

by

Ivelisse Sánchez

Advisor: Professor William D. Cohen

The marginal band (MB) is a mechanically functional continuous bundle of microtubules (MTs) found in non-mammalian vertebrate erythrocytes. The mechanical properties of MBs may be determined by proteins involved in MT stability and interactions at the interface between MB and membrane skeleton (MS). To identify proteins in the MB, the structure and protein composition of isolated MBs were examined. A new MB isolation method was developed which avoids use of proteases, based on the differential solubilization of the membrane skeleton (MS) by detergents.

Dogfish erythrocyte cytoskeletons and isolated MBs contained proteins in the 50-67kD range located along the length of MBs which exhibited reactivity with mammalian brain tau antibodies as shown by immunoblotting and immunofluorescence. Two dimensional SDS-PAGE of cytoskeleton and isolated MB proteins revealed in addition to tubulin (pI ~5.3), proteins of pI ~6.8 as would be expected for brain tau. Isolated chicken MBs also contained tau as shown by anti-mammalian brain tau immunofluorescence.

Spectrin, F-actin, and a 290kD protein immunologically related to syncolin were minor components of isolated MBs, comprising MS remnant patches which may represent a specialized region of MB/MS interface.

Rewarming of low temperature extracts of dogfish cytoskeletons induced MT assembly and the formation of MT bundles with mechanical properties reminiscent of MBs, as visualized by video enhanced light microscopy and TEM (negative staining). The cold-labile fraction of cytoskeletons and assembled MT bundles consisted of tubulin plus proteins of the tau family as shown by immunoblotting. Inclusion of antibodies against brain tau during temperature-induced MT assembly inhibited tubulin polymerization and thus bundling. Immunoprecipitation of MB tau prior to MT assembly had the same effect.

Dogfish isolated MBs and assembled MT bundles were unbundled by exposure to either subtilisin or high salt. Heat stable proteins obtained from salt-extracts of MT bundles exhibited bundling activity when mixed with tubulin prior to temperature-induced reassembly. However, no bundling activity was observed with pre-assembled MTs.

The results indicate that tau is an intrinsic component of isolated MBs and assembled MT bundles, and that it plays a role in MT bundling.

FOREWORD

Of all the sections found in this thesis this is probably the most difficult for me to write. Expressing my feelings at the end of my graduate education gives a sense of finality, for at least this part of my life. Compiling data which you have obtained through many hours of work is strenuous, but very satisfying. It is amazing how little you can really put down on paper. There is no way to account for all the personal knowledge you acquire during the course of your doctoral studies. In order to come to this point its important to learn how to conquer feelings of inability day by day as you pursue at least a glimpse of answers to questions about nature and its secrets.

I have obtained a deep appreciation for nature and how cellular processes are exquisitely fine tuned, so much so, that their intricacies excite minds all over the world. The realization of such complexity increases my curiosity and challenges me to ask more questions and think of ways by which they may be answered. In a sense, graduate studies have awaken my child-like curiosity which gets lost by simple memorization and blind acceptance of concepts which seem alien to our reality.

At this time when education is almost unaffordable and our ability to dream is affected by so many factors, I feel we are obliged to keep students eyes open to the future. To encourage them to dream, to create, and to question the

world around them: this makes life an enjoyable challenge.

I have been fortunate to have people around me who encouraged me to pursue my goals. For this I'm grateful. I have been blessed with parents whom I both love and respect. Their strength and integrity are an inspiration to me. Their caring always motivated me to continue even when my confidence lacked. The support, love, and prayers of my family and friends have brought me this far. To them this thesis is dedicated.

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The completion of this thesis would not have been possible without the input of the special people who have touched my life in the last years. I welcome the opportunity to thank them for their assistance and support.

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I thank the faculty of the Biology department of Hunter College, in particular to Professors Peter Lipke and Katherine Lyser, for their input and interest in my graduate studies. To my fellow students and especially to the students in my lab, I thank you for your friendship and contribution to the research presented here and preparation

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INTRODUCTION

Erythrocytes are highly specialized cells which carry and deliver oxygen throughout the body of vertebrate organisms. The functional efficiency of these cells depends on their ability to withstand mechanical stress during flow. In this respect, the location and interaction of the erythrocyte cytoskeletal proteins are essential in determining the mechanical properties and thereby proper functioning of the cell. Unlike mammalian erythrocytes, those of non-mammalian vertebrates such as the smooth dogfish (Mustelus canis) and chicken (Gallus domesticus) are nucleated and contain marginal bands (MBs) of microtubules (MTs). They also have a protein network composed mainly of actin and spectrin (actually α fodrin and ß spectrin), the membrane skeleton (MS). This protein network is believed to be very similar in mammalian and non-mammalian erythrocytes, differing mostly in component isoforms.

THE MEMBRANE SKELETON

The MS protein interactions play an important role in the maintenance of cell shape (Lazarides and Woods, 1989). The α spectrin of the non-mammalian erythrocyte MS is closely related to brain α fodrin by its molecular weight (240kd) and its calmodulin binding (e.g. Cohen et al, 1982;

Bartelt et al, 1982; Repasky et al, 1982). The interaction of the MS with the inner leaflet of the lipid bilayer is via membrane peripheral glycoproteins. Such proteins include band 4.1 and ankyrin which mediate MS interaction with glycophorin C and band 3, respectively (Lazarides, 1989; Steck, 1989). In addition, F-actin and an ezrin-like protein have been found at the cell periphery in the plane of flattening of chicken erythrocytes (Birgbauer et al., 1989).

THE MARGINAL BAND

The marginal band is a developmentally regulated bundle of microtubules (Ginsburg et al, 1989, Kim et al., 1987) found at the periphery of non-mammalian vertebrate erythrocytes and thrombocytes (Cohen, 1978; Fawcett and Witebsky, 1964), erythrocytes and clotting cells of invertebrates (Cohen and Nemhauser, 1985), mammalian blood platelets (Behnke, 1965), and primitive nucleated red cells of developing mammals (van Deurs and Behnke, 1973; Cohen et al, 1990). The MBs are thought to have a mechanical function in the morphological differentiation of immature nucleated erythrocytes (Barrett and Dawson, 1974) and in the maintenance of cell shape in mature red cells as they travel through vessels (Joseph-Silverstein and Cohen, 1984 and 1985). The difference in role of the MB according to the erythrocyte's stage of differentiation emphasizes the importance of the structure, composition and interaction

between elements of the cytoskeletal system throughout development. Existing studies on protein components of platelet MBs have revealed both similar structure and very similar SDS-PAGE patterns in the tubulin molecular weight region in comparison to erythrocyte MB protein (White et al, 1986). However, although the function of the MB has been explored, relatively little is known about its protein components and how these may determine its function in different cell types such as platelets and nucleated erythrocytes.

The cytoskeleton of nucleated erythrocytes provides a relatively simple and accessible system for the study of cytoskeletal interactions. It is also a good model system for the study of microtubules: their assembly dynamics, biochemistry, and structural features.

MB MICROTUBULES: TUBULIN ISOTYPES/ISOFORMS

Microtubules are composed primarily of tubulin, a protein heterodimer, and their formation is sensitive to changes in temperature and ionic conditions (with some exceptions: Schulze et al., 1987, Gibbons, 1981). Tubulins are highly conserved and are found in structures of great biological importance (Brinkley, 1985, Salmon et al., 1984, and Goslin et al., 1989). The function of MTs in vivo varies according to their arrangement, as well as the cell type in which they are found.

There are different isotypes and isoforms of α and ß

tubulin subunits. These are either products of different genes, differing immunologically and in primary sequence as determined by isoelectric focusing and peptide mapping, or can be a result of various post-translational modifications. Some of the α and ß isotypes have been described in neurons (Havercroft and Cleveland 1984), sea urchin flagellar microtubules (Lefebvre and Rosembaum, 1986), and the fungi Aspergillus (Moris et al., 1984) and Neurospora crassa (Hoang-Van et al., 1989). The presence of different isotypes and isoforms seems to correlate with the particular cell type and subcellular location of the microtubules, indicating that these isotype variations may somehow contribute to microtubule function.

The heterogeneity of tubulins can result from either posttranslational regulation, coding by different genes, or these two processes together. In many species there is a tubulin gene family spread among different chromosomes as shown by DNA hybridization analysis (Gwo-Shu Lee et al., 1983; Sanchez et al., 1980). Because there is a variety of genes encoding tubulin (Cleveland et al., 1980, and Cleveland and Sullivan, 1985), the question is raised as to whether there is differential tubulin isotype expression according to the particular MT structure formed. To date, there does not seem to be an exclusive isotype present within a particular MT structure, but rather a combination of several isotypes (from different α and ß subunits). If tubulin isotypes were direct functional determinants of

cytoplasmic MTs, the ratio of one isotype to the other could be as significant as the presence of one type exclusively.

Experiments done in Drosophila and in Aspergillus show that a mutation in one of the tubulin genes affects the function of many microtubule structures in these cells (May, 1989; Kemphues et al., 1979). Also a chicken/yeast chimeric form of ß tubulin was apparently incorporated indiscriminately into different microtubule structures within mammalian cells (Bond et al., 1986). However, by analyzing the expression of various α and ß tubulin isotypes throughout different tissues, a pattern seemed to emerge in which specific isotypes are overwhelmingly expressed in tissues with MT structures having specialized functions. An example of this phenomenon is the high level of expression of the Mß1 isotype of ß tubulin in mammalian hematopoietic tissue (Wang et al., 1988). The Mß1 tubulin is expressed in low levels in all the other tissues studied in mice and shows only 78% homology with other mammalian ß tubulins described. There are also differential levels of expression of ß tubulin genes throughout various tissues in the chicken (Goslin et al., 1989). A variant ß tubulin, cß6, found in chicken erythrocytes and thrombocytes, is immunologically different from ß tubulins of brain tissue (Murphy and Wallis, 1983), and makes up 75% of the total ß tubulin mRNA expressed in differentiating erythroblasts (Murphy et al; 1986). Although this isotype (cß6) may be specific for MB microtubules (Murphy et al., 1986; Murphy and Wallis,

1983b), it has been found to differ in amino acid sequence by 18% from its assumed counterpart in the mouse hemopoietic tissue, Mß1 (Murphy et al., 1987). A specific combination of tubulin isotypes may provide for favorable tubulin assembly kinetics according to the particular intracellular environment. An example of this could be the assembly of longer, more stable microtubules which would be required for MB biogenesis. Even though the arrangement of MTs in platelet and nucleated erythrocyte MBs is similar, the functional difference of this structure within the different cell types may account for the high divergence between the two ß tubulin isotypes. The ring of MTs in platelets is very dynamic, being able to respond to cell activation by rearranging its MTs. On the other hand, the MB in erythrocytes is very stable and remains as a circumferential bundle of MTs throughout the life of the cell.

Most recently another tubulin isotype, gamma tubulin, has been found in actively dividing tissue. Gamma tubulin has been found exclusively in MT organizing centers (MTOCs) in many different tissues and its properties suggest that it is involved in minus-end MT nucleation (Zheng et al, 1991; Burns, 1991).

The various isotypes\isoforms exhibit differences in biochemical properties, such as in their isoelectric point, which may determine the conditions under which tubulin polymerization kinetics are optimal or appropriate for MT function in the specific structure. Another source of

heterogeneity of tubulins is posttranslational regulation. The most studied posttranslational modifications of tubulins are phosphorylation and tyrosination of ß tubulin, and acetylation and tyrosination of α tubulin.

The α tubulin polypeptide is synthesized with a tyrosine at the C terminus. In vivo, this tyrosine (tyr) is cleaved by a carboxypeptidase, leaving the penultimate amino acid, glutamic acid (glu), at the carboxyl end. This peptide then serves as a substrate for the enzyme tyrosine ligase. Differences in the ratio of tyrosinated and non-tyrosinated (glu) α tubulin incorporated into MTs have been shown to correlate with changes in cell shape (Gundersen and Bulinski, 1986), and with stage of differentiation or of the cell cycle (Rodriguez and Borisy 1978). There are distinct populations of microtubules in vivo as described by the ratio of tyr and glu α tubulin, indicating that there are different subsets of cellular microtubules (Gundersen, et al., 1984; Kumar and Flavin, 1985; Webster et al., 1987b). This occurs, for instance, in TC7 mitotic cells as shown by immunofluorescence with antibodies against glu and tyr α tubulin respectively (Geyens et al., 1986). The polymerization kinetics of these two isoforms are basically the same in vitro. Alpha tubulin tyrosination and acetylation have also been found in the same cell types but within different microtubule subsets (Barra, et al., 1988). The acetylated and tyrosinated α tubulins are expressed reciprocally in rat L6 cultured cells as they undergo

myogenesis, indicating that their expression is developmentally regulated (Gundersen et al., 1989).

The MBs of immature chicken erythrocytes are sensitive to depolymerizing drugs such as nocadozole, while in mature cells the MB is not sensitive to these drugs. A post-translational modification such as tyrosination may have occurred (Kim et al., 1987; Joseph-Silverstein and Cohen, 1984). Baas and colleagues (1990) showed that a population of stable MTs exists in axons which contain detyrosinated tubulin. They proposed that these types of modifications occur in tubulins which are to form part of a stable structure and that these modifications occur after the MTs are assembled. MTs in clam, dogfish, and chicken erythrocyte cytoskeletons seem to be somewhat stable to MT depolymerizing drugs. This could be due to the small number of MT ends in MBs to which MT depolymerizing drugs such as colchicine bind, thereby inhibiting further tubulin dimer incorporation. In addition, the MTs of erythrocyte MBs may be stable and thus drugs affecting tubulin polymerization would have little or no effect on the assembled MBs.

Phosphorylation is known to play a major role in the regulation of protein function. The differentiation of N115 mouse neuroblastoma cells, for example, is accompanied by an increase in cellular tubulin polymerization, which in turn correlates with an increase in phosphorylation of a particular ß tubulin isotype (Gard and Kirschner, 1985). Drugs such as colcemid and colchicine which depolymerize

microtubules were shown to result in a decrease of ß tubulin phosphorylation while the microtubule stabilizing drug taxol had the opposite effect. The precise mechanism by which these drugs affect the phosphorylation state of ß tubulin is unknown. However, these MT depolymerizing drugs may increase the GTPase activity of tubulin incorporated into MTs, thereby increasing the proportion of GDP to GTP ß tubulin pool. In this respect phosphorylation may serve as a regulator specifically modifying the ability of ß tubulin to become incorporated into polymers in vivo.

Data indicate that both the differential expression and posttranslational modification of α and ß tubulin are developmentally regulated. They are under temporal control and, in some tissues, correlate with the presence of specific microtubule structures. The functional importance of the expression pattern, and the distribution of tubulin isotypes and isoforms in vivo, is still in question. It is interesting to note, however, that microtubules containing a high proportion of detyrosinated tubulin showed less binding to MAP2 (Kumar and Flavin, 1985), indicating that the differential binding of various microtubule associated proteins (MAPs) to a MT lattice may be regulated or specified by the proportional incorporation of a particular tubulin isoform. This could in turn serve in the functional regulation of microtubules.

MICROTUBULE ASSOCIATED PROTEINS

Microtubule associated proteins have been found in various microtubule systems such as mitotic spindles (Scholey et al., 1985b), flagella (Lefebvre and Rosenbaum, 1986), and brain microtubules (Sloboda and Rosenbaum, 1979; Shpetner et al., 1989). They have been classified on the basis of their molecular weight and their ability to co-localize with microtubules purified by cycles of assembly and disassembly in vitro (Weisenberg, 1972; Shelanski et al., 1973; Vallee, 1982; Dentler et al, 1975?). With the exception of dyneins and kinesin, which have motility-related functions, very little is known about the function of other MAPs in vivo (McIntosh and Porter, 1989; Porter, 1989). They are usually in very low concentration in the cell, and therefore are difficult to isolate.

The best characterized MAPs, isolated from brain tissue by temperature cycling, are the high molecular weight MAP-1 and MAP-2 and the low molecular weight tau protein (Vallee, 1984,; Herrman, et al., 1984; Cleveland et al., 1977b). The high molecular weight MAPs lower the critical concentration of tubulin, thereby promoting microtubule assembly, and also stabilize pre-assembled microtubules. Tau protein appears to be involved in nucleation without affecting the critical concentration. MAP-1 and MAP-2 are known to bind to MT surfaces and form projections of specific periodicity (Hirokawa et al., 1988 a and b). Tau has also been described as forming short projections from MTs in vitro

(Hirokawa et al., 1988b). The projection fragment as well as microtubule binding site of MAP-2 and tau have been described by analysis of proteolytic fragments which retain their function (Vallee and Borisy, 1977). Both thrombin cleavage of MAP-2 and chymotrypsin cleavage of tau protein produce 28KD proteolytic fragments with very similar amino acid sequence which contain the MT binding domain. Further analysis of this fragment has revealed the presence of three 18 amino acid imperfect repeats in the COOH terminal region of both MAP-2 and tau (Lewis et al., 1988). The second repeat was shown to be essential in both MAPs for MT binding and tubulin assembly promoting activity (Joly et al., 1989). However, the extent or efficiency of MT binding activity by the second repeat was affected by the presence or absence of the surrounding domains. MAP-2 and tau not only have the same MT binding motif but also may share the same binding site on the MT lattice (de la Torre et al., 1986; Serrano et al., 1985). On the other hand, MAP-1 contains a MT binding domain unrelated to that found in MAP-2 and tau (Noble et al., 1989), and presumably recognizes a different site on the MTs. The phosphorylation of MAPs has been shown to lead to more dynamic behavior of MT structures by causing the MAPs to bind less tightly to MTs (Lindwall and Cole, 1984a; Jameson and Caplow, 1981). MAP-2 contains phosphorylation sites in both the MT binding and projection regions (Akiyama et al., 1986). A calcium-calmodulin dependent kinase and a cAMP dependent kinase have been shown to phosphorylate MAP-2

at different sites (Shulman 1984b; Diaz-Nido et al., 1988). MAP-2 and tau are also able to interact with actin (Sattilaro et al., 1981), but this interaction is dependent on their respective phosphate content (Selden and Pollard, 1983). It is mainly the dephosphorylated form of these MAPs that retains the actin cross-linking activity. Calcium was shown to decrease the phosphorylation of tau using either ATP or GTP (Diaz-Nido et al., 1988). However in the presence of calmodulin, calcium inhibited MAP2-actin and tau-actin interactions. An assay done by phosphorylating tau and MAP-2, respectively, and then treating them with phosphatase showed that their actin cross-linking activity was restored although only half the labelled phosphate was removed. Therefore the actin cross-linking activity of these MAPs is not determined by the complete absence of phosphate groups but rather by their specific removal from particular sites within the protein. A similar conclusion was reached by Brugg and Matus (1991). They found that the affinity of MAP-2 for MTs appears to be regulated by the phosphorylation of specific sites on MAP-2.

The fact that these MAPs exist in different phosphorylation states and that they are phosphorylated at various sites by different kinases, resulting in a change in their properties, is extremely important when trying to understand the regulation of MT function through interaction with MAPs (Lindwall and Cole, 1984b; Hoshi et al., 1988).

Tau protein is a highly heterogenous, developmentally

regulated MAP with molecular weight in the range of 40KD to 70 KD (Binder et al., 1985; Grundke-Iqbal et al., 1986; Drubin et al., 1985). It is heat stable, indicating that it contains very little secondary structure in its native form. This has been confirmed by both circular dichroism (Cleveland et al., 1977b) and recently by low angle rotary shadowing (Hirokawa et al., 1988). Electron microscopy and image processing of tau paracrystals revealed that this protein is very elastic, being able to contract and stretch three-fold versus its original size (Lichtenberg et al., 1988). This elasticity depends on its phosphorylation state: when phosphorylated via a Ca/CaM dependent kinase, tau undergoes a conformational change which results in the protein becoming longer and less elastic (Hagestedt et al., 1989).

The sequencing data on tau protein indicate that its heterogeneity could also be derived from both posttranscriptional processing (RNA splicing) (Lee et al., 1988) and alternative translational initiation sites. Recently two tau cDNAs have been sequenced, revealing these to be different transcripts resulting in two tau isotypes which are homologous for the most part, differing only at the COOH terminus (Himmler, 1989). These cDNAs have also been shown to contain two methionine sequences at the amino terminus which could serve as alternative initiation sites for translation, as well as various poly A sequences at different distances from the stop codon. Further analysis

of these sequences shows that these tau isotypes may result from differential RNA splicing. Most recently at least six other isoforms have been sequenced (from bovine and human brain) which also differ at their amino termini (Goedert et al., 1989). Tau heterogeneity may possibly point to diverse functions in vivo.

MAP-2 and tau have been shown to produce MT bundles both in vitro and in vivo, but it is not clear whether this is actually a physical bundling involving cross-linking, or an artifact of overexpression of tau in vivo simply resulting in MT stabilization (Hirokawa et al., 1988; Kanai et al., 1989). Cowan and colleagues reported that MAP-2 and possibly tau contain a domain toward the COOH terminus which is involved in MT bundling activity, although the specific amino acid sequences involved have not been described (Lewis et al., 1989; Lewis and Cowan, 1990). The efficiency of MT bundling as described in vivo appears to be strongly affected by surrounding domains, especially by the second repeat which is involved in MT binding. The interaction between two tau proteins by means of their C-termini would allow the formation of cross-bridges between the MTs to which they are bound. Two models for the formation of cross-bridges have been proposed for MAP-2 and tau. One of the models involves direct interaction of the MAPs via a domain at their COOH termini and the other involves the presence of another (hypothetical) protein which would recognize the C-end domain of the MAPs and mediate cross-

bridge formation (Lewis and Cowan, 1990). Although the function of tau protein has been explored, its heterogeneity and possible role as a functional regulator of MTs in vivo requires further study.

MAPs can be differentially distributed within the same cell, as has been shown in neurons. MAP-2, for instance, is specifically present in dendrites but not axons. Although tau was originally thought to be found exclusively in the axon, it has since been shown that phosphorylated isoforms of the protein are found throughout the neuron (Binder et al., 1985; Matus, 1987). This indicates not only that MAPs may be compartmentalized according to type but also may be segregated according to their phosphorylation state. What determines the differential distribution of these MAPs within a cell is not known. In order to explore this question Hirokawa and Okabe (1989) microinjected labelled tau and MAP-2 proteins into cells in primary nerve cultures and found that they were differentially distributed within three to four days to the axons and dendrites, respectively. These researchers concluded that differential instability of the injected tau and MAP-2 within the dendritic and axonal compartments served as a mechanism for their specific distribution within the same cell. Their finding can also be applied to the presence of phosphorylated tau throughout the nerve cell, if tau phosphorylation results in its stability in both compartments. This is of great importance for studies on the developmental processes in cells, as well

as the understanding of morphological abnormalities apparently resulting from cytoskeletal disorganization in certain diseases (e.g. Alzheimer'disease) (Hardy, 1988; Kosik et al., 1986).

MAPs immunologically related to MAP-2 and tau have been reported in various nucleated erythrocytes. A high molecular weight protein found in the toad Bufo marinus was able to cross-link microtubules in vitro (Centonze and Sloboda, 1986), and a polyclonal anti-MAP2 antibody co-localized with anti-tubulin antibody at the MB in newt erythrocyte cytoskeletons by immunoflupresсence (Sloboda and Dickersin, 1980). However, Murphy and Wallis (1985) reported that they had not found the high molecular weight antigen in their chicken erythrocyte MT preparations, but rather only low molecular weight proteins which cross-reacted with hog brain anti-tau antibody. These tau proteins also elicited a polyclonal serum which recognized the erythrocyte tau but not brain tau protein, indicating that there may be some fundamental differences in these two protein isotypes. More recently, syncolin, a high molecular weight MAP that cross-links reassembled MTs in vitro, has been extracted from chicken erythrocytes (Feick et al., 1991). Syncolin was found to share some epitopes with MAP-2 but differed in its structure, being globular rather than filamentous. In addition to tau and syncolin, fluorescence co-localization of F-actin and ezrin-like protein with the MB has been observed in whole cytoskeletons of mature

chicken erythrocytes (Birgbauer et al., 1989). Although such data have been obtained with respect to the presence of various proteins in the cytoskeleton of nucleated erythrocytes, as yet there is no coherent picture of their specific distribution and function within this system.

A similar situation exists with respect to platelets. Removal of a MAP-2 like protein present in the platelet MB by salt extraction resulted in uncoiling of the band into straight bundles mostly composed of two MTs (Tablin et al., 1991). However, another laboratory found a 51kD protein in platelet MT preparations but no high molecular weight MAP2 immunoreactive protein (Kenney and Link, 1985). Although the MT bands in erythrocytes and platelets have different properties, the general protein components may be very similar.

The properties of MT structures may be determined by proteins that promote assembly, or induce disassembly, or regulate the dynamic state of assembled MTs. The biogenesis and maintenance of the MB in differentiated erythrocytes may actually involve a combination of all of these mechanisms.

MB biogenesis involves the assembly and bundling of particularly long and stable MTs (Kim et al., 1987), as well as their confinement to the periphery of the cell, thereby defining the plane of flattening. In order to establish and maintain the structural integrity of the MB during and after erythrocyte differentiation, factors involved in MT assembly, stabilization, and bundling are required. In

general some properties of the MT system in nucleated erythrocytes are similar to those of the axonal MTs. Both systems contain fairly stable bundled MTs which are essential during the morphological differentiation of the cells in which they are found. In addition, both neurons and erythrocytes contain bundles of MTs with essentially uniform polarity.

MAJOR APPROACHES AND GENERAL OBJECTIVES

The major approaches utilized in the research for this thesis were to develop a new MB isolation method and an in vitro system in which MT bundling could be analysed. By developing an isolation method that avoids the use of proteases, it was possible to isolate MBs from nucleated erythrocytes in large quantities while minimizing disruption to their structure. The isolation of MBs depends on the differential solubilization of the membrane skeleton (MS). Various proteases such as elastase and pepsin have been utilized for the digestion of the MS (Cohen and Ginsburg, 1986). Even previous methods which do not employ the addition of exogenous proteases may still involve endogenous activity (Bertolini and Monaco, 1974). These earlier methods have allowed initial studies on the isolated MB, but the detailed biochemistry and structural analysis may be complicated due to the nature of proteases. It was therefore desirable to develop a non-proteolytic method for the isolation of MBs, especially one that can be applied to

different species to allow comparative studies. By using isolated MBs as starting material, it is possible to obtain large amounts of tubulin from a biologically native microtubule structure. These preparations allow the analysis of the isotypes/isoforms of α and ß tubulin incorporated within the MBs as compared to the total tubulin pool in the cell. Isolation of MBs also permits the study of the microtubule arrangement within the band, and of MT-MT interactions possibly occurring via cross-bridge protein(s).

The work reported here differs from most previous studies in that it involves utilization of MT protein obtained by direct disassembly of marginal bands. In addition, it involves study of reassembled MT bundles that exhibit properties reminiscent of those observed in marginal bands. Such properties include the essentially parallel arrangement of MTs, mechanical stability, and flexibility.

In this thesis I have examined the protein composition of MBs, focusing on those proteins which are intrinsic to the MB structure. I have also studied F-actin and syncolin distribution in isolated MBs vs. whole cytoskeletons to determine whether these proteins are intrinsic MB components or are indirectly associated with the MB in the plane of flattening.

SPECIFIC OBJECTIVES

In summary, the major objectives of this work were the following:

a. To develop an improved marginal band isolation method that avoids the use of proteases.

b. To determine which of the proteins found in isolated MBs are intrinsic components of the MB.

c. To characterize the nucleated erythrocyte MB tau protein as compared with brain tau.

d. To study the assembly and bundling properties of the MB microtubule protein and the involvement of tau in these processes.

MATERIALS AND METHODS

LIVING MATERIAL

Most experiments were performed using erythrocytes of the smooth dogfish, Mustelus canis. Fish were obtained at the Marine Biological Laboratory, Woods Hole, MA, and maintained in a running sea water system until use. Blood was obtained via syringe from caudal vessels, mixed with heparinized Elasmobranch Ringer's (Cavanaugh, 1975), and the leukocytes removed quantitatively using sucrose density separation and subsequent Ringers washes as described previously (Cohen and Ginsburg, 1986).

Blood of the toad, Bufo marinus, was collected from a decapitated animal into heparinized amphibian Ringer's solution, and the leukocytes were removed by repeated centrifugation and resuspension in Ringer's. The white (upper) layer was discarded each time, and the leukocyte content of the cell suspension monitored using phase contrast microscopy. Chicken blood was freshly collected into heparinized (100U/ml) Hank's basic salt solution (HBSS) from young adult chickens after decapitation in a local market, and maintained at 38-39°C during collection and transport to the laboratory. The cells were centrifuged (Beckman, 1200g) and washed once more with heparinized HBSS (50U/ml) followed by two washes with HBSS at 38°C. Leucocytes were removed by aspiration of the buffy coat after each centrifugation step.

PREPARATION OF ERYTHROCYTE CYTOSKELETONS

Erythrocyte cytoskeletons were prepared by lysis of washed cell pellets in 25 volumes of Brij lysis medium consisting of 100mM PIPES (piperazine-n-n'-bis 2-ethane sulfonic acid), 5mM EGTA (ethyleneglycol-bis-(ß-aminoethyl ether) n,n'-tetraacetic acid), 1mM $MgCl_2$, pH 6.8 (=PEM) containing 10mM TAME (p-tosyl arginine methyl ester), 0.6% Brij-58, and 0.7µg/ml pepstatin A, 0.5µg/ml leupeptin, 10µg/ml aprotinin, and 2mM PMSF (phenylmethylsulfonyl fluoride), freshly added. The pH of PEM was adjusted with KOH or NaOH depending upon the particular experiment (=NaPEM or KPEM). These "Brij cytoskeletons" were sedimented in 2 min. at approx. 750g, then washed twice in 25 volumes of PEM. They were resuspended to 5 times their volume in PEM, an equal volume of glycerol was added, and the preparation was mixed thoroughly and stored at -20°C. Some "Brij cytoskeletons" were incubated for 20 min. at room temperature with 20µM taxol and then the glycerol was added before storage (Bartelt et al., 1983; Cohen et al., 1986).

Stored Brij cytoskeletons served as starting material for most of the experiments described here. A sample of cytoskeleton suspension (=one volume) was warmed to room temperature and, in most cases, washed free of glycerol by dilution with two volumes of NaPEM, centrifugation, and resuspension to two volumes in NaPEM. To survey various agents for their ability to release intact MBs from cytoskeletons, this suspension was then diluted 1:1 with PEM

containing test reagents at twice the desired final concentration. As described in greater detail in the Results section, such surveys eventually led to development of an MB isolation medium employing mixtures of detergents (such as 0.2% Triton X-100 and 0.05% SDS).

Chicken erythrocytes were freshly collected and the leucocytes removed as mentioned in the previous section. For preparation of chicken erythrocyte cytoskeletons, precautions were taken to avoid both temperature-induced disassembly of MBs in living cells and mechanical damage to MBs during preparation of cytoskeletons. The cytoskeletons were prepared without subjecting them to centrifugal packing, which causes breakage of their very thin MBs. Living cells were suspended in Brij lysis medium as described above, and layered over a 25/40/50% glycerol step gradient in PEM. The top two layers also contained 0.6% Brij, 10mM TAME, and other protease inhibitors as above. Cytoskeletons were centrifuged gently (Beckman Accuspin, 750g, for 10 min.) into the 50% glycerol layer which was then collected, brought to 10μM Taxol and incubated at room temperature for 30 min. before storage at -20°C.

PREPARATION OF ISOLATED MBs FROM DOGFISH AND CHICKEN ERYTHROCYTE CYTOSKELETONS.

MBs were isolated from cytoskeletons by detergent-based solubilization of the MS (Sanchez et al., 1990). Dogfish cytoskeletons stored in 50% glycerol in 50% PEM (taxol

treated) were washed by centrifugation (1500g for 3min) and resuspended with two volumes of NaPEM. The cytoskeleton suspension was then diluted 1:1 with 0.2% SDS, 0.8% Triton X-100 in NaPEM. After 20 min. at room temperature the nuclei were sedimented (18,000g, 30 sec.). For isolation of Bufo and chicken erythrocyte MBs, the procedure was modified slightly. Stored Brij cytoskeletons (in 50% glycerol) were diluted directly 1:1 in PEM and then 1:1 again with medium containing twice the desired final concentration of detergents (e.g., 0.4% Triton, 0.1% SDS in NaPEM). The final medium thus also contained 12.5% glycerol.

Cells, cytoskeletons, and isolated MBs were visualized using a Zeiss phase contrast microscope equipped with a DAGE?? video camara and a Hamamatsu Argus-10 image processor.

PROTEASE ASSAYS

Tests for the presence of proteases were conducted using the fibrin-based endoproteinase test kit obtained from Boehringer-Mannheim Inc. Radii of clear zones produced on the fibrin plates by test solutions and by control proteases (elastase, trypsin) were measured in photographs taken at various time intervals.

PREPARATION OF ERYTHROCYTES, CYTOSKELETONS AND ISOLATED MBs FOR ELECTRON MICROSCOPY

Cytoskeletons and isolated MB whole mounts for transmission electron microscopy were prepared on carbon-coated grids using polylysine for attachment. Cytoskeletons in 0.2% Triton in PEM, and MB suspensions in isolation medium, were placed on grids for 5 min., followed by a PEM wash and 15 min. fixation in PEM containing 1% glutaraldehyde. Grids were then washed in PEM and in water, stained with 1% aqueous uranyl acetate, and air-dried. In some preparations, isolated MBs on grids were treated with 0.1U/ml subtilisin (Carlsberg; type vIII, Sigma) or 1M NaCl in PEM with and without 10μM Taxol for 10 min., then fixed and stained.

For preparation of cytoskeletons for TEM thin sectioning, cells were simultaneously lysed and fixed initially in PEM containing 0.4% Triton X-100, 0.1% glutaraldehyde, then transferred into PEM containing 2% glutaraldehyde (2 hours minimum, room temperature). Dogfish isolated MBs were prepared for thin sectioning by fixation in PEM containing 2% glutaraldehyde, 5% tannic acid. Washed dogfish and chicken erythrocytes were fixed with 2% glutaraldehyde in Elasmobranch Ringers and HBBS, respectively for 2hr. at 22°C. The samples were then washed three times by centrifugation in PEM, post-fixed 1 hour in PEM containing 1% OsO4, and washed three times in PEM. The material was dehydrated in ethanol, and embedded in Polybed

812 (Polysciences Inc.). Thin sections were stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate. Whole mounts and thin sections were examined in the Hitachi HS-8, or Hitachi-600 transmission electron microscopes.

IMMUNOFLUORESCENCE

Specimens were placed on detergent-cleaned coverslips. After 10 min. incubation at room temperature (all incubations done in a moist chamber), coverslips were rinsed with PEM, and the adhering material fixed for 30 min. with 4% formaldehyde in PEM. Coverslips were then washed extensively in phosphate-buffered saline, pH 7.4 (PBS), incubated 20 min. in PBS containing 1% BSA and 0.05% sodium azide, washed in PBS, and incubated 40 min. with primary antibody in PBS. After washing in PBS, the material was incubated 40 min. with secondary antibody. It was then washed again in PBS, and mounted using fluoromount-G (Southern Biotech. Co.). MBs isolated from chicken erythrocytes were fixed with 1% glutaraldehyde in PEM for 30 min, incubated with 1mg/ml Na borohydride in PBS twice for 10min and then processed as the other samples. To check for non-specific binding of secondary antibody, some coverslips were processed with PBS substituted for the primary antibody.

Primary antibodies included sheep anti-bovine brain tubulin (Southern Biotech. Assoc., Inc.), mouse anti-chicken

brain α (DMA1) and ß (TUB.2.1) tubulin (Sigma Chem. Co.), rabbit anti-chicken brain tau (Sigma Chem. Co.), rabbit anti-chicken erythrocyte tau (gift of Dr. D. Murphy), 5E2 monoclonal anti-mammalian brain tau, 5F9 and 4F7 monoclonal anti-mammalian brain MAP-2 (gifts of Dr. K. Kosik), monoclonal tau-1 antibody to mammalian brain tau (gift of Dr. A. Frankfurter), monoclonal tau-2 anti-mammalian brain tau (Sigma, Chem. Co) and polyclonal anti-syncolin antibody (gift of Dr. G. Wiche). The secondary antibodies, fluorescein-conjugated rabbit (Fab')2 anti-sheep IgG and affinity purified goat anti-rabbit IgG were obtained from Southern Biotech. Rhodamine-labelled phalloidin was used to visualize F-actin (Molecular Probes).

SDS-PAGE AND IMMUNOBLOTTING

SDS-polyacrylamide gel electrophoresis was carried out using 7.5% gels, and 4-10% and 10-20% gradient Laemmli (1970) gels which were stained with coomassie blue or silver (Silver stain kit from BioRad; Merril et al., 1987). Molecular weight standards from BioRad included myosin (200KD), ß-galactosidase (116KD), phosphorylase-ß (92.5KD), BSA (66.2KD), and ovalbumin (45KD). Transfer to nitrocellulose paper was performed as indicated by Towbin et al., (1979) using Hoefer TE-transfer unit at constant current (100mA) overnight or the semi-dry Hoefer blotter (TE-70) for 40min. at 100mA. Blots were blocked with Blotto: 5% non-fat milk in PBS with 0.1% NP40 for the

polyclonal antibodies and with 10% goat serum in TBST or Blotto for the monoclonal antibodies. The antibodies used were monoclonal anti-mammalian brain tau 5E2, 46.1, 14 (Kosik et al., 1989), tau-1 (Binder et al, 1985); "DJ" polyclonal antibody against human fetal brain heat stable MAPs, 5F9 and 4F7 monoclonals to mammalian brain MAP2 (gifts of Dr. Kosik), and polyclonal anti-syncolin (Feick et al., 1991). The blots were treated with the antibodies overnight at 4°C, and an alkaline phosphatase labelled secondary antibody was used with BCIP-NCB developing system (Promega, Inc.).

TWO DIMENSIONAL GEL ELECTROPHORESIS

Proteins were separated according to their isoelectric points in a tube gel (Hoefer mini-tube IEF gel format). Servalyt pH 3-10 ampholines (Hoefer) were used to establish the pH gradient in the first dimension. The isoelectric points were determined by plotting the pH of IEF gels run simultaneously without samples. Each gel was cut into 1cm strips and the ampholines of each gel strip were diluted into 0.1M KCl in water, and the pH of the solutions was determined. In addition, carbamylated proteins comercially available used as pI standards were used to corraborate the pI determined for the proteins in the cytoskeleton and MB protein samples (Bio-Rad). Two dimensional separation was achieved by using 7.5% SDS-PAGE in the Hoefer "Mighty Small" slab gel. IEF and two-dimensional gel

electrophoresis were performed according to the method of O'Farrell (1975).

HEAT-STABLE ERYTHROCYTE MICROTUBULE PROTEINS

Low temperature extracts from cytoskeletons were brought to 0.35M NaCl, 2% mercaptoethanol, and 1mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin A, and 1mg/ml TAME. Also taxol treated dogfish cytoskeltons were extracted with .35M NaCl in PEM for 20min and the salt extract was obtained after cetrifugation for 10min at 18,000g. Preparations were then heated at 95°C for 5 min. The heat stable proteins were obtained from the supernate after centrifugation at 18,000g for 30 min. at 4°C. This supernate was concentrated by using Centricon-30 filters (Amicon, Co) and dialysed against PEM containing protease inhibitors (as above) at 4°C.

BRAIN HEAT STABLE PROTEINS

Dogfish brains were removed, rinsed in Elasmobranch Ringers and immediately immersed in liquid nitrogen and stored at -70°C until use. The frozen brains were weighed, cut into pieces, and homogenized for 10 min. in ten volumes of PEM buffer containing 0.5μg/ml leupeptin, 0.7μg/ml pepstatin A, 0.2μg/ml aprotinin, and 2mM PMSF. The homogenate was centrifuged twice at 18,000g for 25 min. and the supernate was brought to 1mM GTP, 0.5M NaCl and 2% mercaptoethanol and heated for 5 min. at 95°C. The

suspension was centrifuged at 18,000g for 30 min. and the supernate containing heat stable proteins was concentrated using concentrator resin (Bio-Rad) and dialysed against PEM with protease inhibitors as above.

Bovine brain heat stable proteins were obtained from commercially available bovine brain acetone powder (Sigma, Co). The acetone powder was resuspended in NaPEM containing 0.5M NaCl, protease inhibitors (as above), incubated for 1hr at 4°C, and centrifuged (30 min., 18,000g). The supernate was then heated at 95°C for 5 min., centrifuged, and concentrated using Centricon-30 filters (Amicon, Co).

HEAT-STABLE PROTEINS FROM ISOLATED MBS

Isolated MBs were centrifuged for 3 min. at 18,000g (Fisher microfuge) and the pellet resuspended in an equal volume of 0.5M NaCl, 2% mercaptoethanol, 2mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin A, 0.2μg/ml aprotinin. The salt extract obtained after centrifugation as above was then heated at 95°C for 5 min. The heat-stable proteins were obtained from the supernate after centrifugation (18,000g, 30 min.).

MT PROTEIN AND BUNDLING ASSAY

The stored cytoskeletons were washed with 10 volumes of NaPEM, resuspended in 1/20 original volume NaPEM containing 5% glycerol and incubated for three to 10 hours at 0°C. The low temperature extracts of cytoskeletons were obtained

after centrifugating twice for 5 min. each at 18,000g, 4°C. The extract was then passed through an Acrodisc .2μm filter (Amicon, Co.) to remove any vesicles present. Extracts obtained in this manner did not contain MTs as determined by negative staining (TEM).

Low temperature MT protein preparations were also made from detergent-isolated MBs (Sanchez, et al., 1990). The protein concentration was estimated by the Bradford method (Bradford, 1976) using BSA as the standard.

Microtubule protein preparations were brought to 0.3mg/ml, 5% glycerol, and 0.1mM GTP. This suspension formed MT bundles when rewarmed at room temperature (22°C). The MT bundles were visualized by using a Zeiss microscope with phase and DIC optics, a DAGE video camera and Argus 10 video image processor (Hamamatsu Argus). Negatively stained samples were observed utilizing a Hitachi-600 transmission electron microscope.

PHOSPHATASE TREATMENT OF CHICKEN ERYTHROCYTE CYTOSKELETONS

Chicken cytoskeletons were sedimented by centrifugation at 1,500g for 3 min. and resuspended in 1/10 volume with TBS (tris basic salt, pH 7.4) with 25U/ml alkaline phosphatase from calf intestine (Boehringer, Co.), 1mM PMSF, 7μg/ml pepstatin A, and 5μg leupeptin. A control was done by resuspending cytoskeletons in the same buffer mixture lacking the alkaline phosphatase. These samples were incubated for 4 hrs. at 37°C. After this incubation

the samples were washed three times with 10 volumes of TBS, and then washed twice with PEM. The cytoskeletons were resuspended in 1/20 volume PEM containing 0.1mM GTP, 5% glycerol and placed at 0°C for 1 hr. The low temperature extract was obtained by centrifuging the cytoskeleton suspension at 18,000g at 4°C twice for 5 min. each. The supernate was placed at 37°C for 1 hr. and the MTs recovered by centrifugation at 18,000g for 15 min.

EFFECT OF ANTIBODIES ON MICROTUBULE ASSEMBLY AND BUNDLING

Low temperature extracts from dogfish cytoskeletons were incubated with antibodies against tau (tau-1 and 5E2), anti-actin antibody (as an indifferent control), and anti-syncolin, separately at 0°C overnight. Other controls included the use of antibodies pre-absorbed with whole cytoskeletons. The MT protein was rewarmed to 22°C and MT bundling was monitored by phase contrast and DIC, using video-enhanced light microscopy.

IMMUNOPRECIPITATION

The antibodies 5E2, tau-1 (Kosik *et al.*, 1988), anti-syncolin (Feick *et al.*, 1991), and anti-actin (Sigma Immunochemicals) diluted in PBS were incubated overnight at 4°C with protein-A beads with gentle shaking. The beads were then washed several times with PBS and incubated with aliquots of the erythrocyte cytoskeleton MT protein overnight at 0°C with gentle shaking. The protein-A beads

containing bound antibodies and the respective immunoprecipitated protein were centrifuged (18,000g) and the supernate was rewarmed to room temperature and incubated for up to 3 hrs. at 22°C.

ION EXCHANGE AND GEL FILTRATION COLUMNS

The low temperature MT protein obtained from cytoskeletons was passed through a Whatman P11 phosphocellulose 10x2cm column (Bio-Rad). The void volume was collected and the bound fraction was recovered by washing the column with 1M NaCl (Sloboda et al., 1976).

The protein components of cytoskeleton low temperature extracts were further separated by the use of a Sephadex G-200 column run at an average rate of 0.1ml/min. The column was calibrated with amylase, a 200kD protein which, in SDS gels, runs as several bands in the 50-60kd region. Fractions of 0.8mls were collected at 0°C and aliquots were warmed for 3 hrs. at 22°C to test for MT assembly.

Protein concentrations were determined by the Bradford method (Bio-Rad reagent) using BSA as standard.

MEASUREMENTS OF MICROTUBULE ASSEMBLY

The polymerization of low temperature extracts from dogfish and chicken erythrocyte cytoskeletons was monitored by turbidity changes (350nm absorbance) at temperature close to physiological for the animal. A Beckman model DU-6 spectrophotometer and a water jacketed cuvette was used to

maintain the temperature at 22°C for the dogfish and at 37°C for the chicken samples, respectively.

DETERMINATION OF MT LENGTH AND MT NUMBER PER BUNDLE

Equal aliquots (10μl) of low temperature extracts from dogfish cytoskeletons were incubated at 22°C and negatively stained at different time intervals. Electron microscope negatives of random fields were projected, and the number of MTs per bundle and their respective lengths were determined in the projected images.

UNBUNDLING ASSAY

Dogfish erythrocyte MT protein was rewarmed at 22°C for 60 min. in the presence of 5% glycerol and 0.1mM GTP, then diluted 1:1 in the same PEM buffer containing one of the following:

a. 50 U/ml and 500 U/ml of the catalytic subunit of protein kinase II (Sigma Co.), 1mM ATP.
b. Alkaline phosphatase from calf intestine, 44U/ml (type Boehringer Mann. Co.).
c. 1-4mM ATP
d. various dilutions of mAbs tau-1, 46.1, 14, and 5E2.
e. anti-chicken syncolin polyclonal Ab.
f. DTT, 2mg/ml
g. 1M-2M NaCl with and without 10μM taxol

The samples above were incubated at 22°C and monitored by phase contrast, video enhanced microscopy for up to 5hrs.

USE OF PROTEASES IN UNBUNDLING

Bundles were allowed to form as previously described, then were centrifuged at 18,000g for 10 min. and resuspended to approximately 4mg/ml. The bundle suspension was brought to a final concentration of 2mg/ml MT protein with 5% glycerol in NaPEM containing one of the following enzymes: 10-50U/ml trypsin (type III, Sigma Co.), or 1% w/w subtilisin (type VIII, Sigma Co.) to MT protein (Serrano et al., 1985).

EFFECT OF TAXOL AND SALT ON MT STABILITY

Erythrocyte cytoskeleton low temperature extracts were rewarmed at 22C for 1 hr., during which time MT bundles formed. A sample of the bundle suspension was brought to 10μM taxol, incubated for 30 min. at 22°C, and divided into two equal aliquots. One aliquot was diluted 1:1 with 2M NaCl, 10μM taxol, 0.1mM GTP, 5% glycerol in NaPEM and then 1:1 again with 1M NaCl, 10μM taxol 0.1mM GTP, 5% glycerol in NaPEM. The second aliquot was diluted in the same manner with 10μM taxol, 0.1mM GTP, 5% glycerol in NaPEM. Another sample of bundle suspension was also divided into two equal aliquots, diluted in the same manner with the exception that taxol was omitted and DMSO (taxol solvent) was added instead, as control. The samples were then incubated at 22°C for 60 min. The absorbance at 350nm was obtained using a Beckman DU-6 spectrophotometer.

Each sample was then centrifuged for 15 min. at 18,000g

and the protein concentration in the supernate was determined by Bradford assay (BioRad, Co).

MT BUNDLING RECONSTITUTION ASSAYS

Low temperature extracts from dogfish cytoskeletons were rewarmed to 22°C for 1hr., and the MT bundle suspension was centrifuged for 10 min. at 18,000g. The pellet of MT bundles was resuspended in an equal volume of PEM containing 2M NaCl, 5% glycerol and incubated for 5 min., by which time unbundling had occurred. The single MTs were then sedimented by centrifugation for 10 min. at 18,000g. The resulting supernate was brought to 1mM PMSF, 0.5μg/ml leupeptin, 0.7μg/ml pepstatin A and then heated at 95°C for 5 min. The sample was then centrifuged for 30 min. at 18,000g and the "heat-stable protein supernate" was recovered. The single MTs in PEM containing 1M NaCl, 5% glycerol (obtained above) were aliquoted into four tubes. An equal volume of 1M NaCl, 5% glycerol in PEM with protease inhibitors (as above) was added to tubes b and d, while heat-stable protein supernate was added to tubes a and c. All preparations were then diluted 1:10 in PEM containing 5% glycerol and 0.1mM GTP. Tubes a and b were incubated at 22°C. Tubes c and d were incubated at 0°C for 60 min. and then rewarmed to 22°C for 40min. MT assembly and bundling was monitored by phase contrast, video enhanced light microscopy (VEM). Taxol (10μM) was added to an aliquot of tube d, as no apparent MT assembly was observed. Several

grids were prepared from all samples for TEM (negative staining), and counts were made of single and bundled MTs in projected TEM negative images as described above.

RESULTS

STRUCTURE AND PROTEIN COMPONENTS OF THE NUCLEATED ERYTHROCYTE CYTOSKELETON

Structural Organization of MBs

The organization of chicken and dogfish MBs was examined by thin sectioning (TEM) in whole cells and cytoskeletons (Fig.1 and 2). MBs in mature chicken erythrocytes contained 10-14 MTs while dogfish erythrocytes had 30-35 MTs, as expected. Dogfish MBs contained a lower proportion of outer MTs to total number of MTs than did chicken MBs, ~25% compared to ~40% for chicken. In addition, the clear zone of about 10nm was observed surrounding each MT of the MBs in both chicken and dogfish erythrocytes. In cross sections through MBs in whole erythrocytes, 86% of the MTs were separated from one or more other MTs by a distance of 17-33nm. Measurements of distance between MTs in the MB and the counts of number of outer MTs in the MB were obtained from five different erythrocytes of chicken and of dogfish. To optimize conditions for preservation and visualization of possible MT-MT cross-bridges, hemoglobin was removed during cytoskeleton preparation by means of simultaneous lysis and fixation. Nevertheless, cross-bridges were observed only occasionally, and these were thin and filamentous, < 20nm in length (Fig.2).

Figure 1. Thin sections of whole nucleated erythrocytes (TEM). (a and c) Sections through erythrocytes of dogfish and chicken, respectively, perpendicular to plane of flattening. n = nucleus; arrowheads denote location of MB. (b and d) Higher magnification view of areas in (a) and (c) respectively, showing MTs comprising the MB at the cell periphery. (a) X 4,000; (c) X 8,000;(b and d) X 60,000.

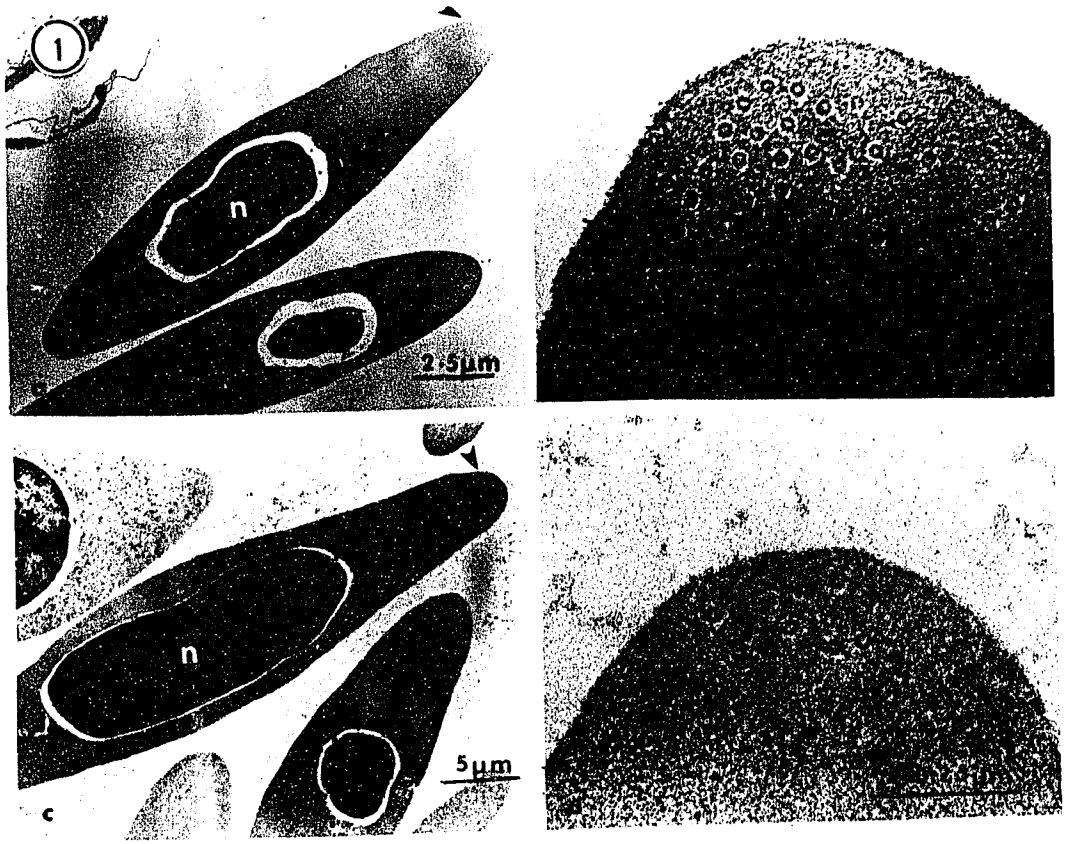
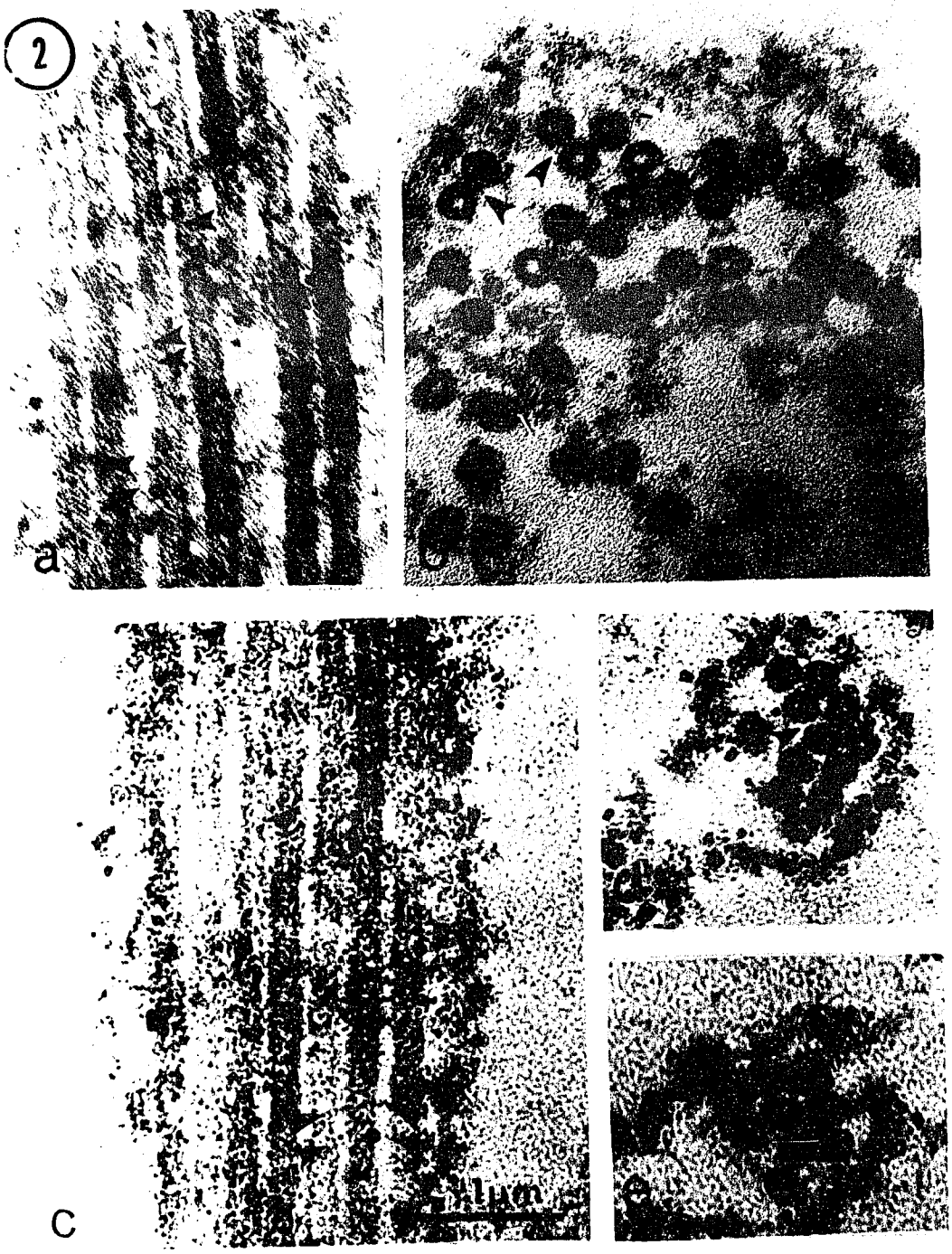

1
n
2·5µm
n
5µm
c

Figure 2. Cytoskeletons of dogfish and chicken erythrocytes (thin sections, TEM). (a and c) Longitudinal and (b and d) cross-sectional view of microtubules of the MB in dogfish and chicken cytoskeletons, respectively. The cytoskeletons were prepared by simultaneous Triton lysis and glutaraldehyde fixation of cells, showing a few filamentous cross-links between MTs (arrowheads). (a-d) X 120,000.


2
a
1µm
C

Storage of Dogfish cytoskeletons

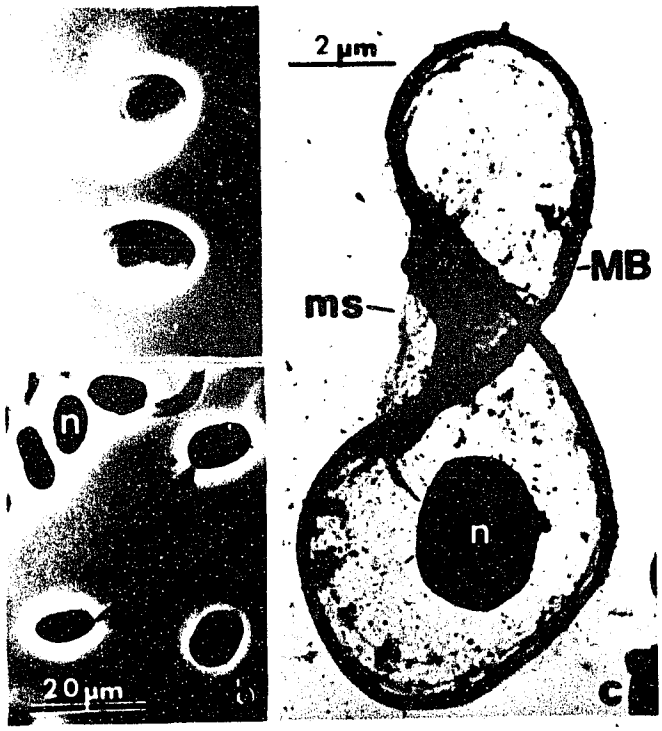
Dogfish (Mustelus canis) cytoskeletons prepared in the presence of protease inhibitors and stored in 50% glycerol in PEM with 10μM taxol at -20°C were used as starting material (Fig.3). Taxol, known to stabilize microtubules (Schiff and Horowitz, 1980), was found to preserve the otherwise cold-labile MB microtubules at low temperature (Fig.4). It was noted that this stored material showed no change in major protein components over long periods of storage under the conditions stated above (Fig.5). As observed in TEM (negative staining), the MBs of cytoskeletons stored in the absence of taxol contained less MTs as compared to the MBs of taxol-treated stored cytoskeletons.

Erythrocyte cytoskeleton proteins

The major proteins of cytoskeletons of nucleated erythrocytes include spectrin, actin, and tubulin (Cohen et al., 1982). The erythrocyte α spectrin is actually a calmodulin-binding protein more similar to α fodrin (Levine and Willard, 1981; Bartelt, et al., 1982). Cytoskeletons of chicken and dogfish erythrocytes contained similar immunoreactive proteins as shown by immunoblotting with polyclonal antibodies against chicken erythrocyte α spectrin (Fig.6b). In contrast, they contained somewhat different ß spectrins, as indicated by SDS-PAGE molecular weight pattern (Fig.6).

Figure 3. Dogfish erythrocytes and erythrocyte cytoskeletons. (a) Living cells in Ringer's solution, phase contrast. (b) "Brij cytoskeletons", starting material for MB isolation experiments; n= one of many nuclei visible. (c) Brij cytoskeleton after further extraction with Triton X - 100, uranyl acetate staining, TEM; n= nucleus. Figure eight twisting of MB increases visibility of membrane skeleton (ms) in overlap region. (a and b) X 850; (c) X 8,500.

Figure 4. Effect of taxol on dogfish cytoskeleton storage. Cytoskeletons stored in 25% glycerol in PEM with (a) or without (b) 10μM Taxol, at -20°C. MBs of the cytoskeletons stored without taxol were notably thinner, indicating that the MTs may have dissasembled. (a and b) X 1,500.



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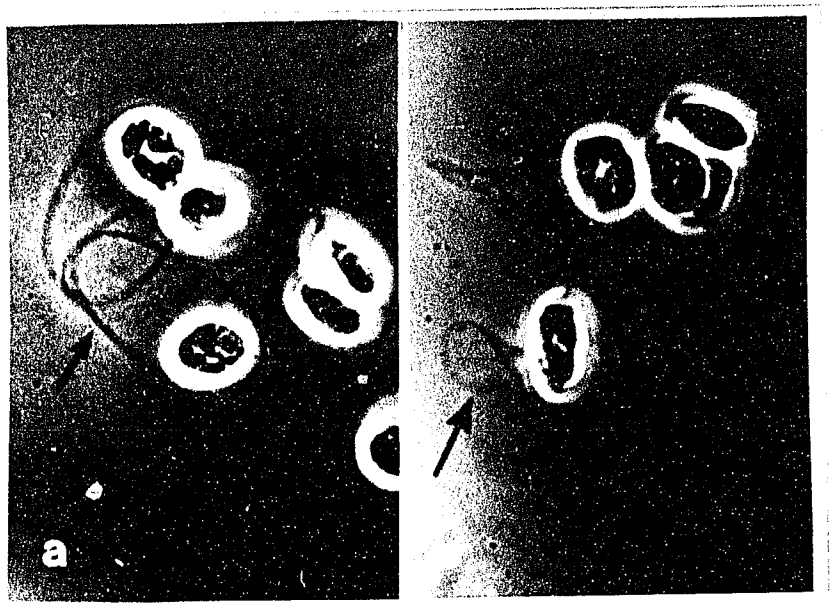
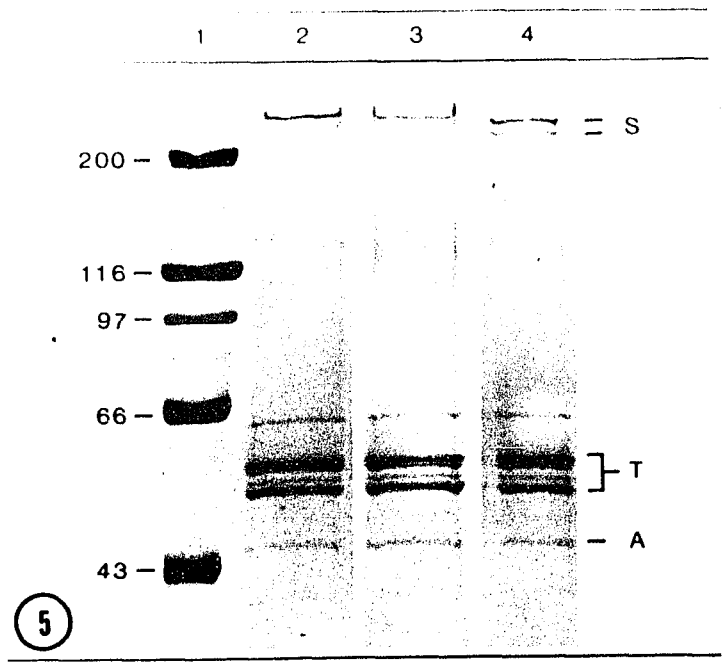


4

Figure 5. SDS-PAGE profiles of stored dogfish erythrocyte cytoskeletons. Lane 1: molecular weight standards. Lane 2: No storage. Lanes 3 and 4: after six and twelve weeks of storage, respectively, pattern remains essentially unchanged. S= Spectrin. T= Tubulin. A= Actin. Several other bands are also present.

Figure 6. Dogfish and chicken erythrocyte cytoskeleton α spectrin. a) SDS-PAGE; Lane 1: dogfish and lane 2: chicken whole cytoskeletons. b) Immunoblotting of samples in a with antibody against chicken erythrocyte cytoskeleton α spectrin; lane 1: chicken cytoskeletons Lane 2: dogfish ctoskeletons. Cytoskeletons of these two species contain immunologically similar α spectrin. (Arrowhead denotes position of α spectrin).

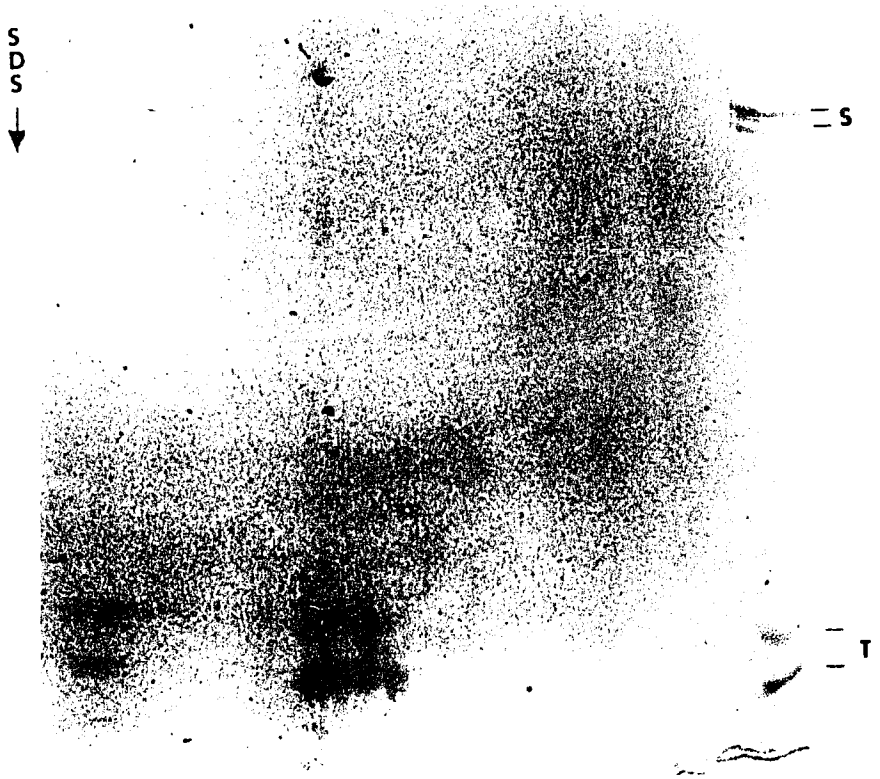


5

6

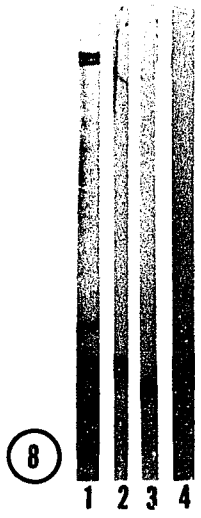
Figure 7. Two dimensional SDS-PAGE of dogfish cytoskeleton protein. Single lane on the right shows protein pattern separated by SDS-PAGE only. Two major groups of proteins with different pIs in the tubulin molecular weight region (T) are observed.

Figure 8. Tubulin in dogfish cytoskeletons. Lane 1: blot of dogfish cytoskeleton protein stained with india ink. Lane 2 and 3: immunoblot of cytoskeleton protein treated with monoclonal antibodies against α and β tubulin, respectively. Lane 4: immunoblot of bovine brain (used as standard) proteins with mixture of antibodies against both α and β tubulin.



7

6.8



8

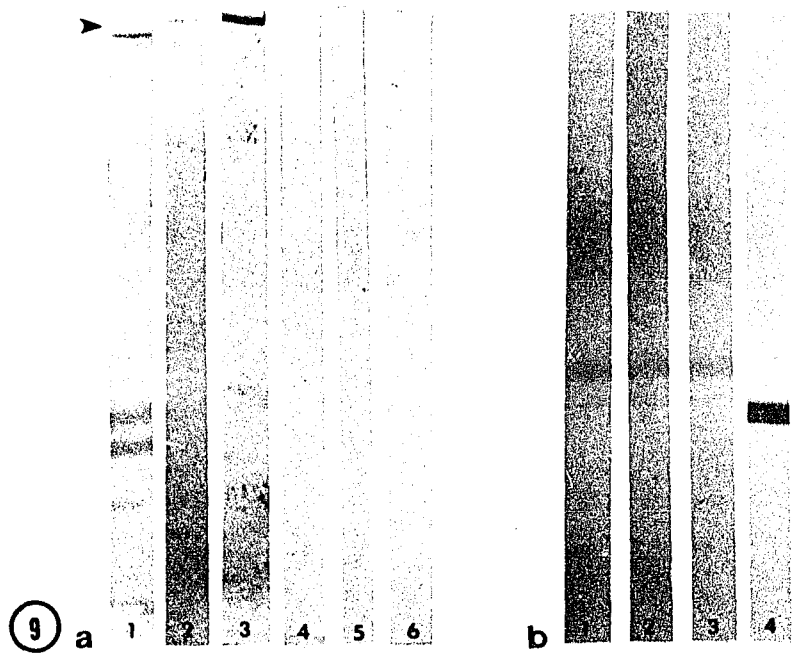
Two dimensional separation of dogfish erythrocyte cytoskeleton proteins revealed several proteins with pIs corresponding to those reported for spectrin (pI 5.6), actin (pI 5.5), tubulin (pI 5.7-5.2), and tau (pI~6.8) (Fig.7).

As previously demonstrated, dogfish cytoskeletons contained four protein bands in the tubulin molecular weight region (Fig.5) which may account for the multiple spots in the two dimensional gel. Immunoblotting with monoclonal antibodies against α and ß tubulin showed the upper and lower groups of protein bands in the tubulin molecular weight region to be α and ß tubulin, respectively (Fig.8, lane 2). Dogfish erythrocyte cytoskeletons contained several isoforms of both α and ß tubulin as indicated by two dimensional electrophoresis (Fig.7).

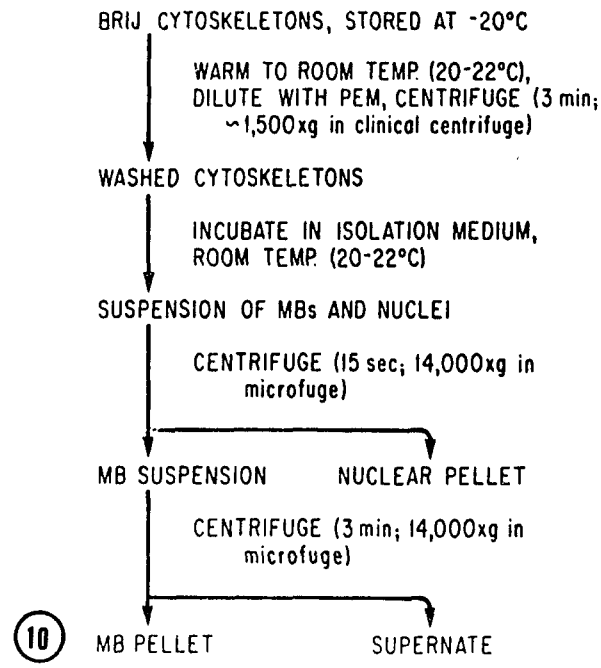
Immunoblotting of dogfish cytoskeleton proteins with antibodies 5F9, 4F7, or RPN.1194 to MAP-2 showed no cross-reactivity with high molecular weight protein (Fig.9a, lanes 4-6). However, a 290kD protein reacted with "DJ" anti-mammalian heat stable proteins and anti-chicken erythrocyte syncolin (Fig.9a, lane 2 and 3). Interestingly, antibodies 5F9 and 4F7 bound in the tubulin molecular weight region, as was observed for antibody against tau (Fig.9b).

Figure 9. Immunoblotting of dogfish cytoskeleton proteins. a) Lane 1: amido black stain. Lane 2: anti-syncolin antibody. Antibodies against mammalian brain MAP-2: lane 3, "DJ"; lane 4, "5F9"; lane 5, "4F7"; lane 6, RPN.1124. b) Higher loading of cytoskeleton protein treated with anti-MAP2 and anti-tau monoclonal antibodies. Lane 1, "4F7" anti-MAP2; lane 2, "5F9" anti-MAP2; lane 3, 5E2 antibody; lane 4, anti-ß tubulin antibody. A 290kD protein in dogfish cytoskeletons cross-reacts with antibody to heat stable brain proteins and anti-syncolin but no reactivity was observed with anti-MAP2 monoclonal antibodies (4F7 and 5F9). The latter antibodies, however, cross-reacted with proteins in the tubulin molecular weight region in the same pattern as anti-tau.

Figure 10. Summary of detergent based method for isolation of MBs from dogfish erythrocytes.


a
1
2
3
4
5
6
b
1
2
3
4

(9)


BRIJ CYTOSKELETONS, STORED AT -20°C
WARM TO ROOM TEMP. (20-22°C), DILUTE WITH PEM, CENTRIFUGE (3 min; ~1,500xg in clinical centrifuge)
WASHED CYTOSKELETONS
INCUBATE IN ISOLATION MEDIUM, ROOM TEMP. (20-22°C)
SUSPENSION OF MBs AND NUCLEI
CENTRIFUGE (15 sec; 14,000xg in microfuge)
MB SUSPENSION
NUCLEAR PELLET
CENTRIFUGE (3 min; 14,000xg in microfuge)
MB PELLET
SUPERNATE

(10)

DEVELOPMENT OF AN IMPROVED MB ISOLATION METHOD THAT AVOIDS THE USE OF PROTEASES.

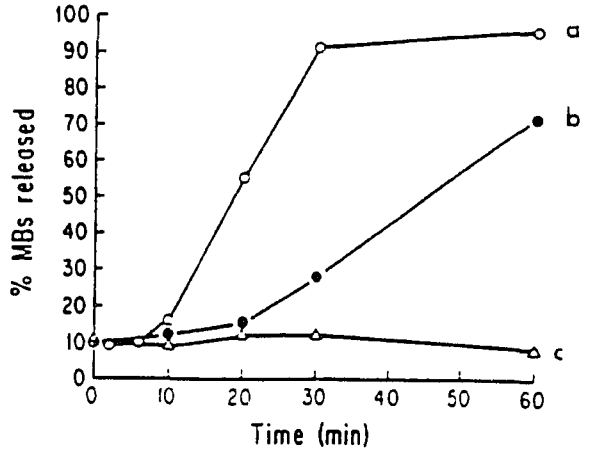
MB isolation method

In order to find a non-proteolytic method for the isolation of marginal bands, many agents which had been reported to solubilize the MS at least partially (mostly in studies of mammalian erythrocytes) were tested systematically. These reagents included several phosphate compounds, reducing agents, and organic mercurials (Sanchez et al., 1990). However, these compounds were unable to solubilize the MS of the dogfish cytoskeletons. Since detergents had been used for membrane extraction in a variety of cells, and for MS dissolution in platelets (Kenney and Link, 1985) several detergents were tested individually. These also proved ineffective. Based on the "hook forming" medium described for the determination of MT polarity (Heidemann and McIntosh, 1980) which includes a mixture of detergents, several combinations of detergents were tested for their possible ability to solubilize the erythrocyte MS while providing MT stabilizing conditions. Only a specific combination of ionic and non-ionic detergents was found effective. A mixture of SDS and Triton in a 1:2 molar ratio, and within a particular concentration range (0.025% SDS, 0.1% Triton - 0.1% SDS,.0.4% Triton) was able to liberate the MB from the whole cytoskeleton. The MB isolation procedure developed using this and similar

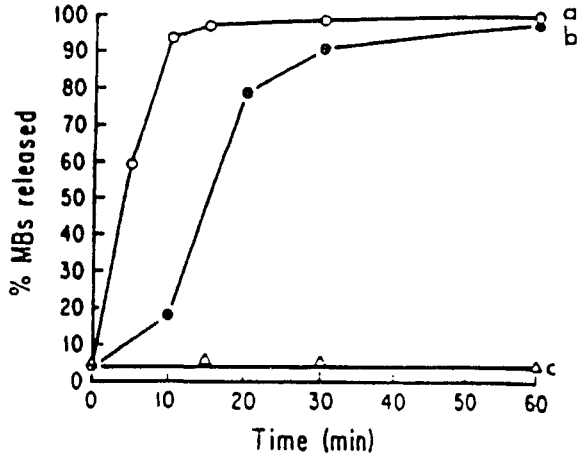
Figure 11. Effect of excess Triton and of glycerol on MB release from cytoskeletons. Conditions: Na +PEM containing (curve a) 0.025% SDS, 0.1% Triton X-100; (curve b) 0.025% SDS, 0.1% Triton X-100, 25% (v/v) glycerol; (curve c) 0.025% SDS, 0.25% Triton X-100. Presence of glycerol slows MB release but does not block it (b vs a); excess Triton X-100 blocks MB release (c).

Figure 12. Effect of detergent concentration on MB release from cytoskeletons. Conditions: Na^+PEM containing (curve a) 0.05% SDS, 0.2% Triton X-10; (curve b) 0.025% SDS, 0.1% Triton X-100; (curve c) 0.01% SDS, 0.04% Triton X-100. Within a certain range (curves a, b) MB release is slower at the lower detergent concentration, but final percentage MB release is the same. If detergent concentration is too low (c), no MB release occurs.

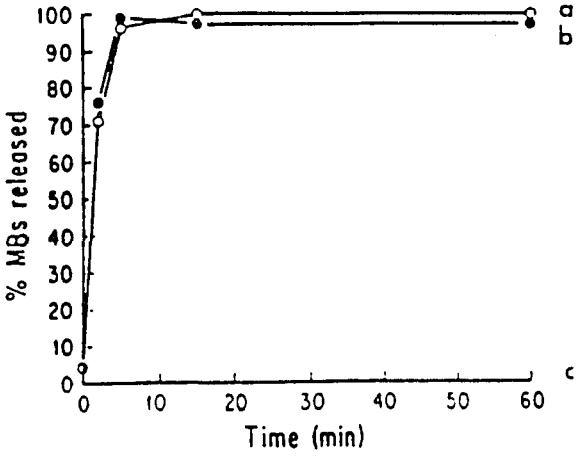
Figure 13. Effect of protease inhibitors on MB release from cytoskeletons. Conditions: NaPEM containing 0.1% SDS, 0.4% Triton X-100 (curve a) and 0.1% SDS, 0.4% Triton X-100, 0.5μg/ml leupeptin, 0.1mM PMSF, 0.7μg/ml pepstatin A (curve b). Presence of protease inhibitors has no effect on time course of MB release.


% MBs released
Time (min)
a
b
c

(11)


% MBs released
Time (min)
a
b
c

(12)


% MBs released
Time (min)
a
b
c

(13)

detergent mixtures as the isolation medium is summarized in Fig. 10.

MB release during different isolation conditions

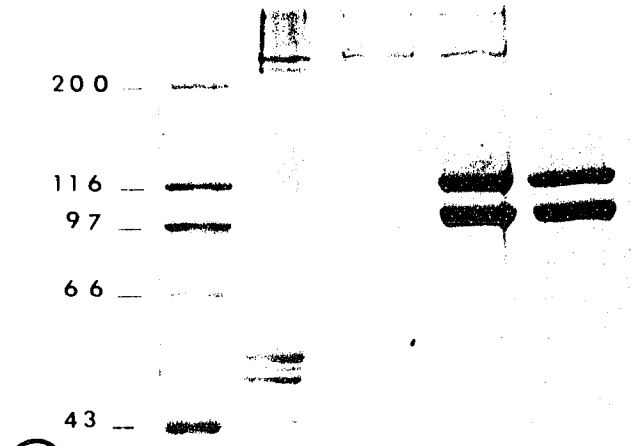
Glycerol, which is known to stabilize microtubules, slowed down the MB release, and the addition of excess Triton stopped the reaction (Fig.11). In order to quantitate MB release, the reaction was stopped by 1:10 dilution into a "stop mixture" containing glycerol and excess Triton, and the number of nuclei without a surrounding MB was determined at different time intervals. Counting of the nuclei was more accurate than directly counting isolated MBs because there were many isolated MBs at different tilt angles and at different planes of focus. This permitted quantitative assays of the time course of MB release under different conditions. By this method, it was found that 90% of the MBs can be liberated from cytoskeletons in 5 to 30 min. depending upon detergent concentration (Fig.12).

Tests for proteolytic activity during MB isolation

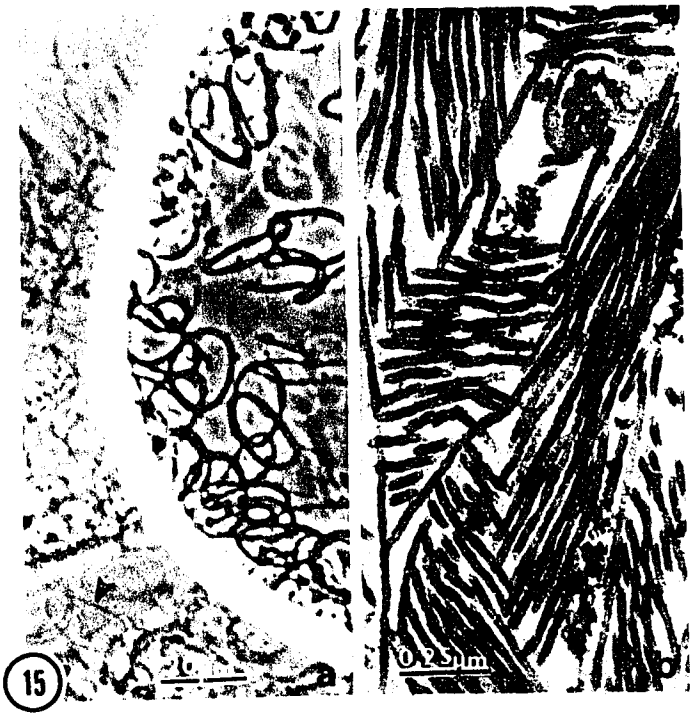
Several kinds of experiments showed that proteolytic activity from endogenous proteases was not involved in MB release from cytoskeletons. The MB release reaction occurred in the presence of protease inhibitors (Fig.13), and standard proteins added to the isolation medium were not proteolyzed (Fig.14). In addition, the Boehringer test kit

Figure 14. Test for proteolytic activity during MB isolation. Lane 1: molecular weight standards (kD). Lane 2: whole cytoskeleton (starting material). Lane 3: supernatant containing material solubilized during MB isolation. Lane 4: supernatant from experiment in which MBs were isolated in the presence of ß-galactosidase (116kD) and phosphorylase B (97kD); the added proteins were not proteolyzed. Lane 5: the added proteins alone.

Figure 15. Pellets of isolated dogfish erythrocyte MBs. (a) In phase contrast MBs are barely visible in liquid phase (arrowhead, left), but are clearly seen in air bubble (right). (b) In TEM thin section, isolated MB pellet consists mainly of microtubules.
(a) X 500 (b) X 40,000.



14



15

for endoproteinases was used and revealed no proteolytic activity during the isolation reaction.

General application of the MB isolation method

The detergent-based MB isolation procedure allowed mass isolation of dogfish MBs which could be monitored by phase contrast (Fig.15). In addition, the method was found to be applicable to other species such as toad (Bufo marinus), and chicken (Gallus domestica) (Fig.16).

MBs ISOLATED FROM ERYTHROCYTE CYTOSKELETONS

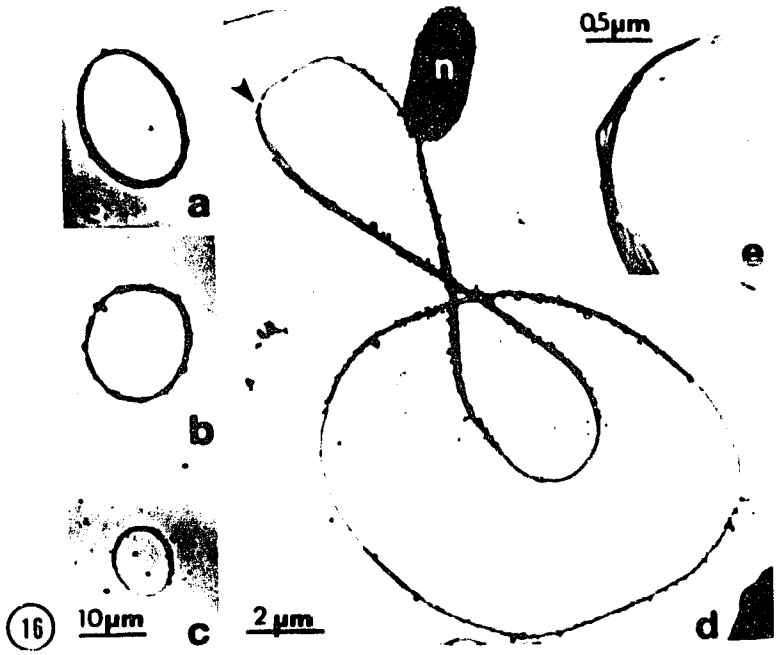
Structure of isolated MBs

As seen in negatively stained whole mounts, the MTs of isolated MBs remained bundled (Fig.16, 17b), but no cross-links between the MTs were visible, even at high magnifications. Most MTs in isolated MBs showed parallel arrangement similar to a ribbon (Fig.16e and 17b). The MT arrangement was easier to observe in isolated chicken MBs, due to their small number of MTs.

In TEM, negatively stained whole mounts as well as thin sections revealed MTs to be the major component. Structures resembling cross-links were seldom observed in these preparations, even with the use of tannic acid during fixation (Fig.18). However, when cross-links were seen they appeared filamentous (Fig. 18, arrowheads).

Figure 16. MBs isolated from erythrocytes of three different vertebrate classes by the same method. (a) Smooth dogfish. (b) Toad. (c) Domestic chicken. Phase contast (bubble method; see Fig. 15). (d) Uranyl acetate-stained whole mount of isolated chicken erythrocyte MBs, TEM. Released nucleus is present (n). (e) Higher magnification view of region near arrowhead in (d), showing a ribbon-like structure of the MB. (a-c) X 1,000; (d) X 5,500; (e) X 20,000.

Figure 17. Isolated MB (dogfish) in uranyl acetate-stained whole mount, TEM. (a) Complete MB; arrowhead denotes region seen at higher magnification in (b), in which individual microtubules are visible. (a) X 3,200; (b) X 80,000.


0.5µm
n
a
b
e
d
16
10µm
c
2 µm

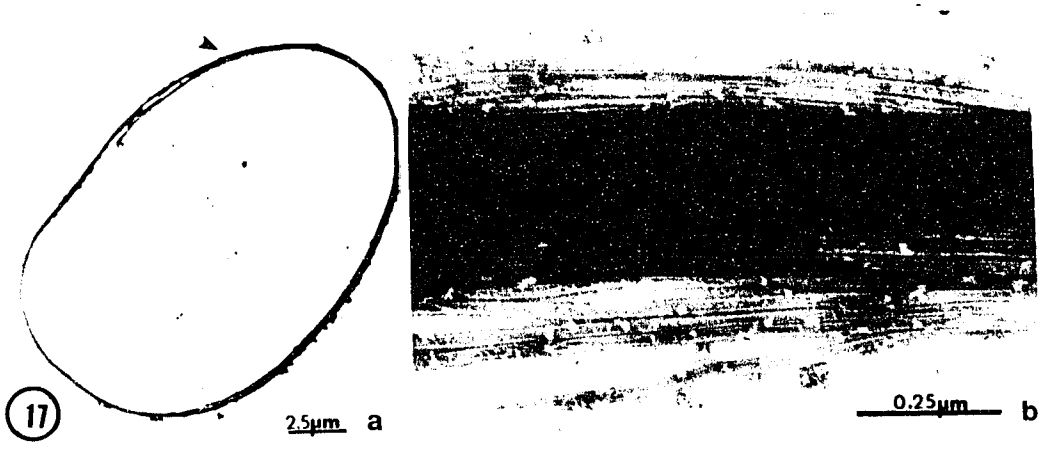
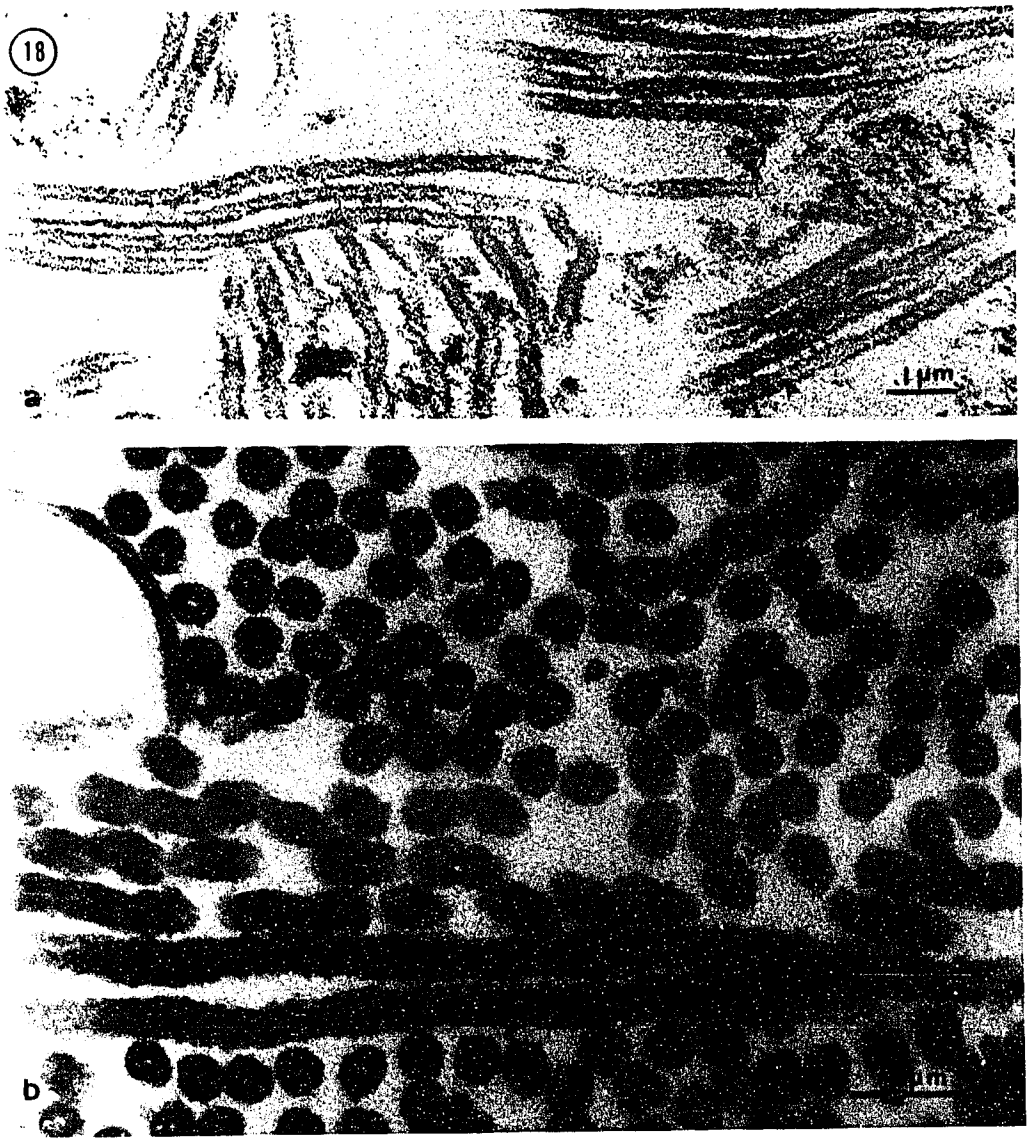

17
2.5µm
a
0.25µm
b

Figure 18. Thin section of dogfish erythrocyte isolated MB, tannic acid fixation. Longitudinal and cross-sectional view of MTs in isolated MB pellet. Filamentous cross-links between MTs are occasionally seen (arrowheads). a) X 100,000, b) X 200,000.


18
a
.1 µm
b
.1µm

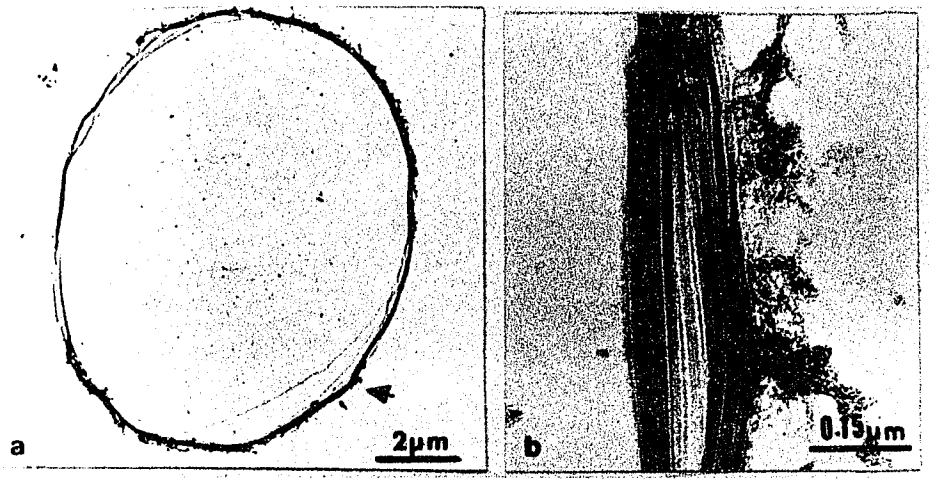
Stability of isolated MBs

Because chicken MBs are very thin and therefore more sensitive to mechanical manipulation, chicken erythrocyte cytoskeletons were prepared in such a way as to minimize the effects of centrifugation which cause MT breakage (refer to Materials and Methods). On the other hand, MBs in dogfish cytoskeletons seem to be less sensitive to centrifugation and remained intact when cytoskeletons were prepared by the usual method previously published (Cohen and Ginsburg, 1986) as visualized by video enhanced microscopy phase contrast (VEM). Under the same isolation conditions, the MBs of dogfish erythrocytes were found to be more stable than those of chicken. In 0.1% SDS, 0.4% Triton in PEM, for example, as followed by video enhanced phase contrast microscopy, dogfish MBs remained intact for up to 1 hr. In the same medium, chicken MBs disappeared within 10 minutes, but when 12.5% glycerol was included during isolation the MBs remained intact for up to 1 hr. However, when these preparations were diluted 1:10 with PEM, the chicken MBs disappeared. Taxol did not prevent the effect of dilution on isolated chicken MBs. After such dilution however, isolated dogfish MBs remained stable with or without taxol.

MS remnants associated with isolated MBs

Remnants of the MS were observed associated with MBs isolated from both chicken and dogfish erythrocytes (Figs.17a and 19). These remnants were not randomly

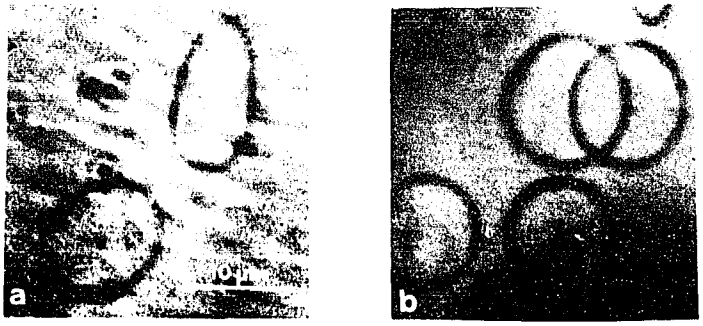
Figure 19. Isolated MBs from chicken erythrocyte cytoskeletons. a) Negative staining, TEM of whole isolated MB. b) Higher magnification of area denoted by arrow. The ribbon-like arrangement of individual MTs is visible (b), as well as MS remnants still attached to the MB. a) X 5,500; b) X 80,000.



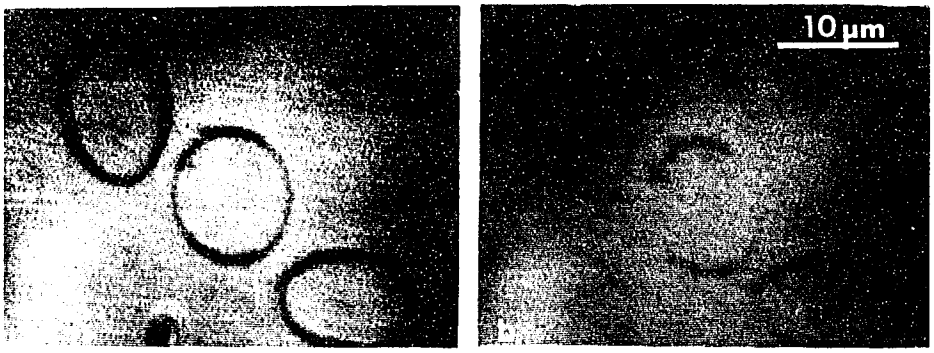
(19)

Figure 20. Effect of the MS on the isolated chicken MBs. Phase contrast, video enhanced microscopy (VEM) of chicken MBs isolated with different concentrations of detergent mixture: (a) 0.1% SDS, 0.4% Triton X-100, 12.5% glycerol in PEM; (b) 0.5% SDS, 2% Triton X-100, 12.5% glycerol in PEM. At the same time point (60min), MBs isolated with lower detergent concentrations are more oval.

Figure 21. Dilution effect on isolated erythrocyte MBs. (a) Isolated chicken MBs adhering to detergent-cleaned coverslips. PEM containing taxol was then perfused underneath the coverslips and the same MB observed (b). Chicken MBs disassembled due to dilution leaving behind patches of MS still attached to the coverslip. This was not observed with dogfish MBs.



(20)



(21)

adhering contaminants, but rather were usually attached to the outer edge the isolated MB (Fig.19).

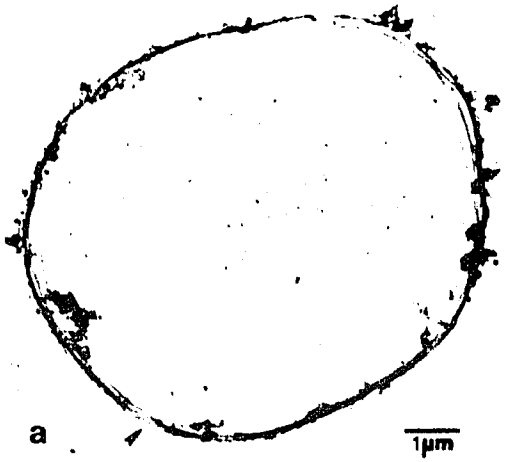
When MBs were prepared using isolation media with two different concentrations of the detergent mixture, more of the MS remnant was present at a given time point at the lower detergent concentration. These MBs retained their elliptical shape, whereas, at the higher detergent concentration, the MBs became circularized more rapidly (Fig.20).

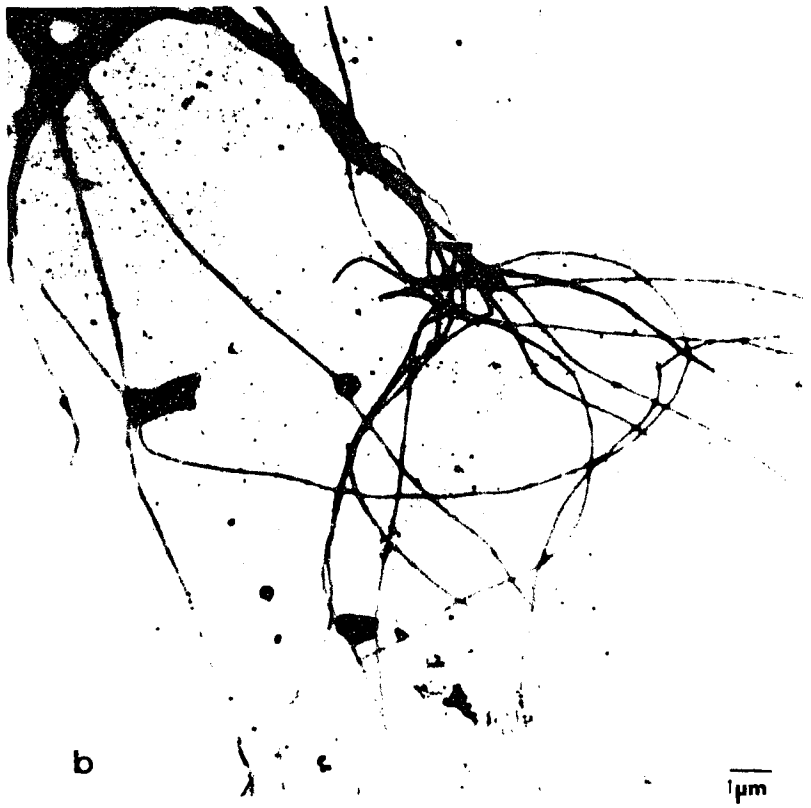
The instability of isolated chicken MBs to dilution was useful in demonstrating that the attachment of the MS remnant was not random. PEM was perfused under coverslips to which isolated chicken MBs had been attached, causing the MTs to dissassemble. Only the MS remnant remained on the coverslip, in patches corresponding to the shape of the original isolated MB (Fig. 21).

Unbundling of MTs in isolated MBs

The MTs of isolated MBs from both dogfish and chicken erythrocytes were unbundled by limited treatment with subtilisin (Fig.22). Similarly, isolated MBs exposed to 0.5M-1M NaCl were observed to start unravelling and eventually unbundle. A 54kD heat-stable protein was found to be present in such salt extracts (Fig. 23). In the case of both subtilisin and 0.5M NaCl, unbundling occurred with or without 10 μM taxol present. However, taxol was normally included in these experiments because, as observed for salt

Figure 22. Unbundling of isolated MBs by subtilisin. (a) Dogfish and (c) chicken controls treated with subtilisin in the presence of protease inhibitors. MBs remained intact. (b and d) Isolated dogfish and chicken MBs treated with subtilisin. MBs are unravelled. a) X 7,000; b) X 6,000; c) X 14,000; d) X 6,000.


a
1µm


22
b
c
1µm

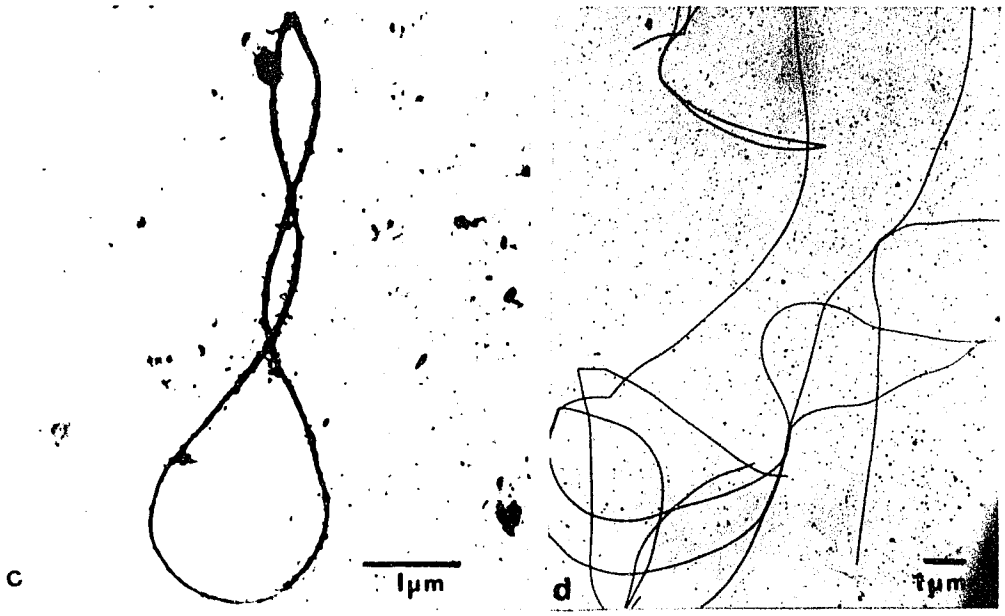
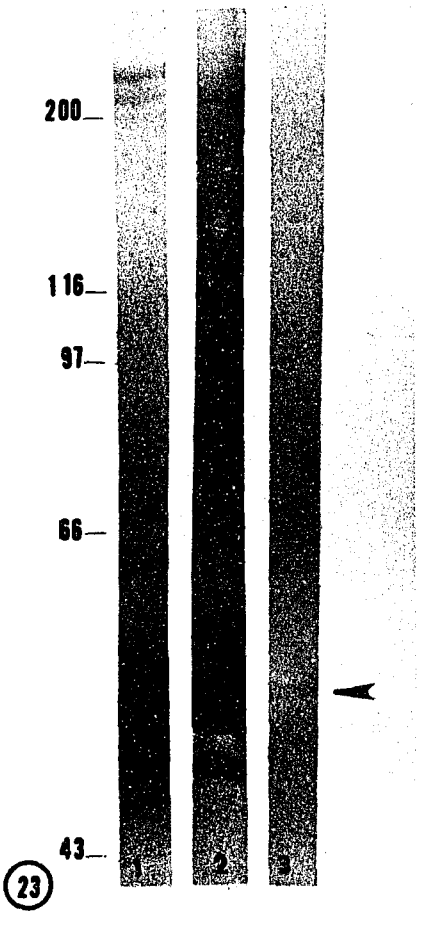

c
1µm
d
1µm

Figure 23. Heat stable protein in 1M NaCl extract of isolated dogfish MBs. Lane 1: whole cytoskeletons; lane 2: salt-extracted isolated MB preparation; lane 3: heat stable protein from isolated MBs. The major heat-stable protein extracted from isolated MBs is ~54kD (arrow= faint band).


200
116
97
66
43
1
2
3
23

treatment, it prevented MTs from shortening after unbundling. From a given MB, a relatively small number of very long, single MTs were produced, some of which were long enough to traverse the MB circumference one or more times (Fig.22 b and d).

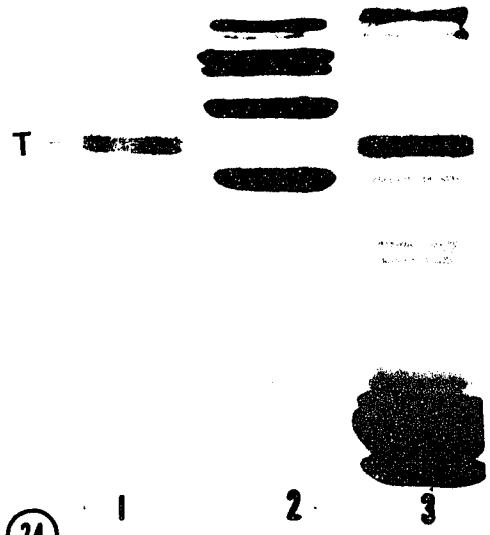
Protein components of isolated MBs

Studies on the proteins present in the isolated dogfish MBs revealed tubulin as their major component as shown by SDS-PAGE (Fig.24 and 25). Four tubulin-region bands (55,58,60,61KD) were present in the same "apparent" stoichiometric ratio as in the whole cytoskeleton (Fig.25, lanes 1 and 2). Small amounts of actin and spectrin remained in the isolated MB preparations, with ß spectrin being predominant (Fig.25,lane 2,4, and 6). A high molecular weight protein of approximately 280-290kD was present in the whole cytoskeleton but was solubilized together with actin and spectrin by the MB isolation medium (Fig.25, lane 1 and 5), and thus was not enriched in MB pellets (lane 6). The same pattern was observed for MB isolation from chicken erythrocytes (Fig.26) with the exception of a ~80kD protein doublet in chicken MB preparations. When the sample was centrifuged over a 30% sucrose cushion, the same proteins were observed in the MB pellets.

It was possible to identify the proteins in the MS remnants of chicken MBs by diluting isolated MBs 1:10 with

Figure 24. SDS-PAGE gradient gel (10-20%) of dogfish cytoskeleton and isolated MBs. Lane 1: isolated MB pellet. Lane 2: molecular weight standards. Lane 3: whole cytoskeleton. T= tubulin.

Figure 25. Protein components of MB pellets and supernatant obtained during MB isolation. Cytoskeletons freshly prepared. Lane 1: whole cytoskeleton (starting material). Lane 2: isolated MB pellet with tubulin loading equivalent to lane 1. Lane 3: components solubilized by the isolation medium. Lane 4: isolated MB pellet, overloaded. Fractions from stored cytoskeletons. Lane 5: components solubilized by the isolation medium. Lane 6: isolated MB pellet, overloaded. In both fresh and stored preparations, 220kD is the predominant minor band in MB pellet (lanes 4, 6). Arrowheads denote the position of 240 and 220kD polypeptides for lanes 5 and 6. A 280kD protein is solubilized by MB isolation medium (arrow).



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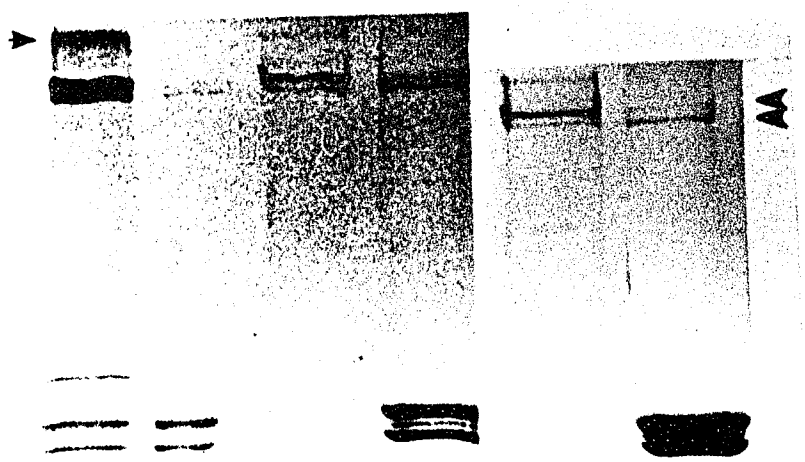
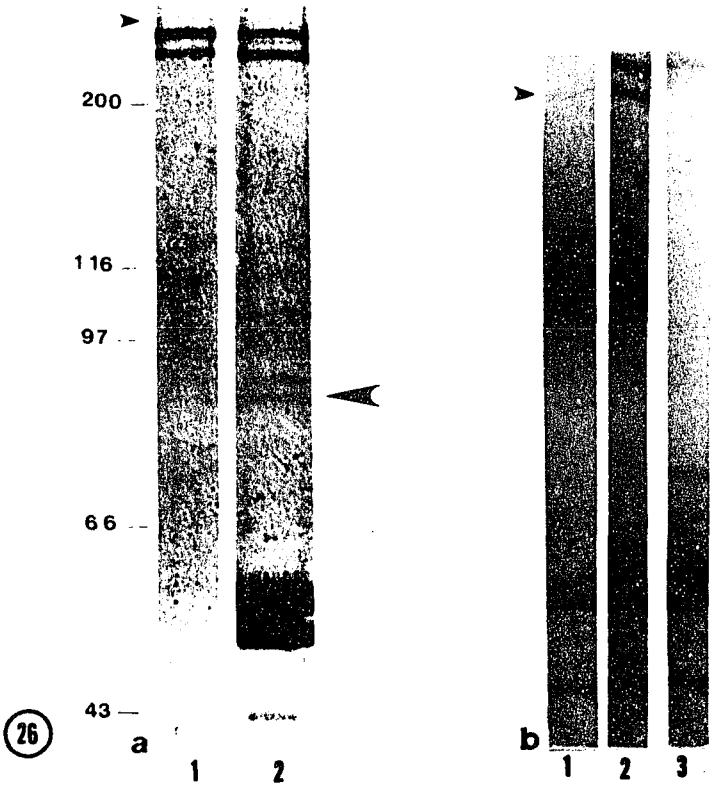


(25) 1 2 3 4 5 6

Figure 26. SDS-PAGE of the components of the isolated MBs and cytoskeletons from chicken erythrocytes. a) Coomassie blue stained gel. Lane 1: Supernate after MB isolation. Lane 2: chicken erythrocyte MB pellet. b) Silver stained gel. Lane 1: isolated MBs. Lane 2: chicken whole cytoskeletons. Lane 3: dogfish whole cytoskeleton (comparison standard). The pellet obtained after isolated MB disassembly contains actin, spectrin, and 80kD doublet. Arrowheads denote the position of the 290kD protein band, and the 80kD doublet.

Figure 27. SDS-PAGE gel of MS remnant proteins from chicken isolated MBs. Lane 1: pellet after MT disassembly of isolated MBs by 1:10 dilution. Lane 2: supernate after same dilution. The major proteins in MS remnant are spectrin and high molecular weight protein (~290kD) denoted by arrowhead. As expected, tubulin (T) is present in the supernate after dilution.



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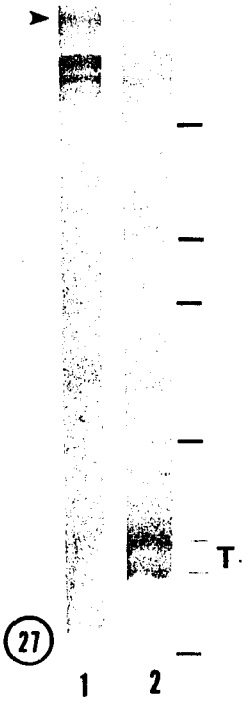


(27)

PEM, thereby disassembling the MTs. The pellet obtained after centrifugation of diluted isolated MB suspension contained 290kD protein and actin, but no tubulin, as expected (Fig.27, lane 1).

Tau protein in MBs

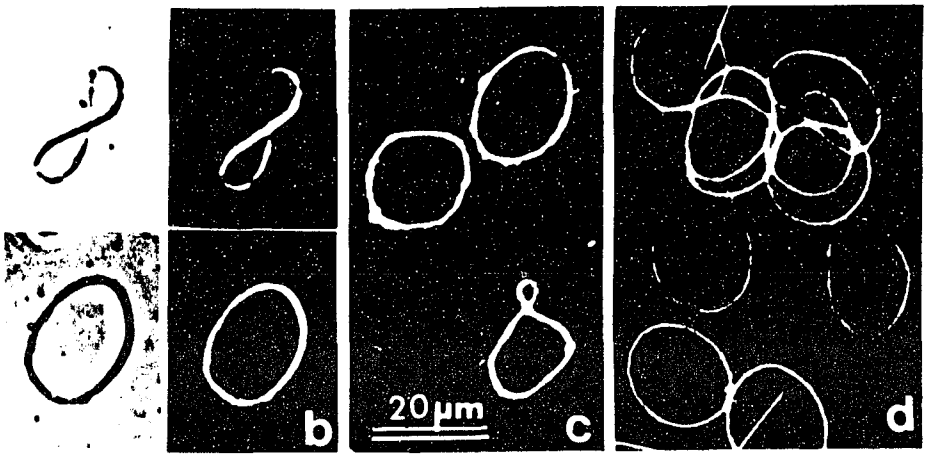
Because the major proteins in isolated MB preparations were in the tubulin molecular weight range, and proteins cross-reacting with anti-tau antibodies were found in whole dogfish cytoskeletons, I tested for the presence of tau protein in dogfish isolated MBs. Isolated MBs showed reactivity with chicken brain and erythrocyte tau, and with mammalian brain tau as demonstrated by continuous binding along the MB observed by immunofluorescence (Fig.28).

Better separation of the isolated MB proteins was obtained on the basis of their isoelectric points. Denaturing isoelectric focusing (IEF) and two dimensional gels of dogfish erythrocyte MBs revealed protein bands of pI~6.8 in addition to α and ß tubulin isoforms (pI 5.7-5.2) (Fig.29). Slab IEFs of pH range 3.5-10 and reverse polarity IEF showed protein bands with the same pIs as above.

Immunoblotting of dogfish erythrocyte whole cytoskeleton and isolated MB preparations using polyclonal antisera to chicken erythrocyte tau resulted in major binding to protein bands in the 40-62kD region. In MB overloads the anti-chicken erythrocyte tau antibody also bound to the 68kD region (Fig.30). The presence of the 68KD

Figure 28. Immunofluorescence localization of tubulin and tau in isolated MBs (dogfish). (a, b) Phase contrast (VEM)/fluoresence pairs showing binding of anti-tubulin. (c) Anti-chicken brain tau. (d) Anti-chicken erythrocyte tau. (a-d) X 900.

Figure 29. Two dimensional gel electrophoresis of isolated dogfish MB proteins. There are two major groups of proteins in the tubulin molecular weight region (~50-60kD). The more acidic group appears in the pI range expected for tubulin (5.2-5.7); the other has a more basic pI (~6.8) compatible with values previously reported for brain tau.



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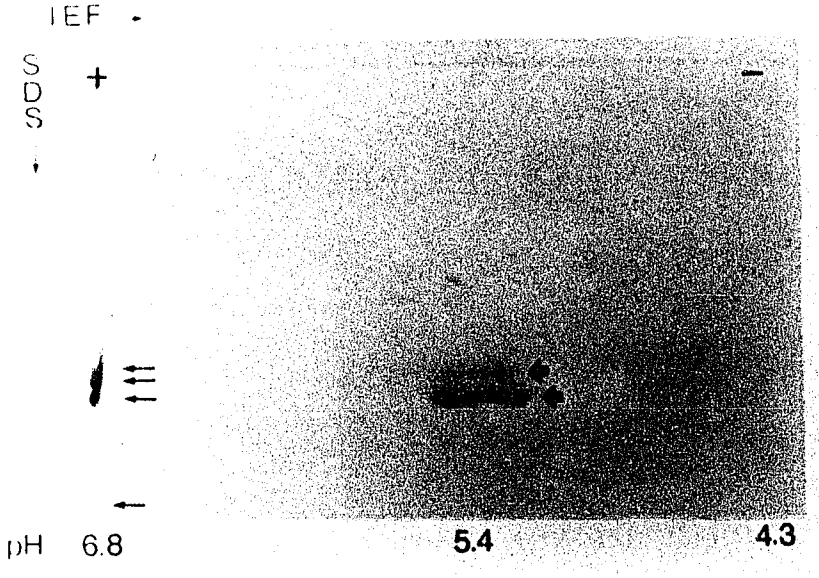

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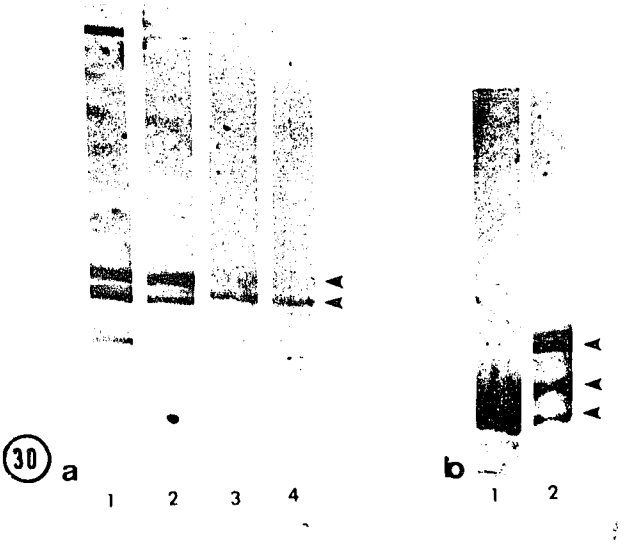
Figure 30. Immunoblots of cytoskeleton and isolated MB proteins from dogfish erythrocytes. MT proteins treated with antibody against chicken erythrocyte tau. a: lanes 1 and 2, Coomassie blue stained blot; lanes 3 and 4, Immunoblot. (1 and 4) Whole cytoskeleton, (2 and 3) isolated MB pellet. (b) Overloaded MB preparation. Lane 1: Amido black stained blot, lane 2: Immunoblot. The antibody (gift of Dr. Doug Murphy) recognizes bands of 57KD to 62DK, but in overloads also reacts with bands of 68kD.

Figure 31. Isolated MB proteins, antibodies against mammalian brain tau. Lane 1: India ink, blot. Lane 2, 3 and 10: monoclonal antibodies to bovine brain tau; 46.1, 14, and Tau-1, respectively. Lane 4 and 5: antibodies against human fetal brain heat stable MAPs: 5E2 and "DJ", respectively. Lane 6: polyclonal anti-chicken erythrocyte tau antibody. Lane 7: monoclonal against chicken brain tau. Lane 8: anti-ß tubulin; lane 9: anti-α and anti-ß tubulin reference mixture. Major binding of anti-tau antibodies was observed at ~54kD region. Lines at left indicate location of 200, 116, 97, 66, and 43kD molecular weight markers.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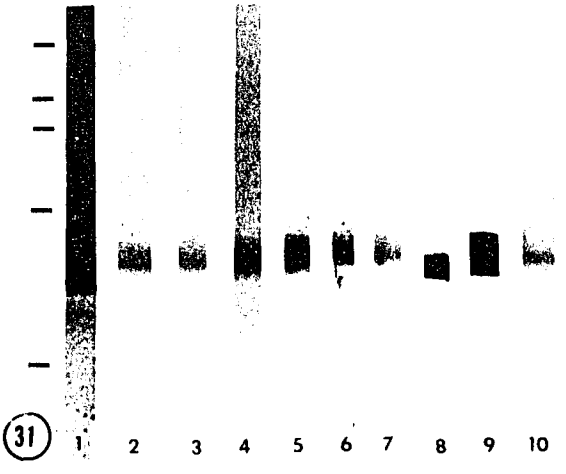
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tau isoform in dogfish erythrocyte MBs was observed only in SDS-PAGE overloads of preparations made from non-stored cytoskeletons.

To describe further erythrocyte tau protein in dogfish MBs, its immunoreactivity was tested with several monoclonal antibodies against mammalian brain tau. The epitopes recognized by the antibodies used have been sequenced by various laboratories and were located throughout the length of mammalian brain tau (Murphy and Wallis, 1985; Kosik et al., 1988). All the anti-tau antibodies showed major immunoreactivity at approximately the 54kD region (Fig.31). Because these antibodies bind in the tubulin region, the immunoreactivity of some control samples was also tested. These controls included salt-extracted heat denatured tubulin, and bovine brain acetone powder. No binding was observed to the tubulin samples and only four bands in the tau molecular weight region were observed to bind the antibodies in the bovine brain acetone (data not shown). When very large overloads of isolated MB samples were immunoblotted, either lower reactivity or no reactivity was observed. This suggested that these antibodies were not binding unspecifically to tubulin, but rather were binding to minor protein bands in the tubulin molecular weight region which become unaccessible for antibody binding in such large overloads. In addition, different blocking solutions were tested, including 10% goat serum in TBS, 2% BSA in PBS, and 5% dry milk in PBS with .1% NP40, but the

binding pattern of these antibodies to the isolated MB proteins remained the same to that shown in Fig. 31.

Only antibodies against chicken erythrocyte tau and DJ made against human fetal brain MAPs cross-reacted with the higher molecular weight (68KD) taus of isolated MBs (Fig.30b); however, these did not bind mAb tau-1 or 5E2 (data not shown).

Although isolated MBs contained protein bands in the 40-62KD region which bound antibodies against mammalian brain tau, these also reacted with anti-MAP2 mAbs "4F7 and 5F9" in the same pattern (data not shown).

Localization of MB-associated proteins

In addition to rhodamine labelled phalloidin, antibodies against tubulin, syncolin, and tau bound to whole chicken cytoskeletons in a pattern coinciding with the MB (Fig.33, b-d). The isolated MBs, however, contained patches which bound both phalloidin and anti-syncolin (Fig.33, d) while antibodies against tubulin and tau bound in a pattern along the length of the MB (Fig.33, c'and d').

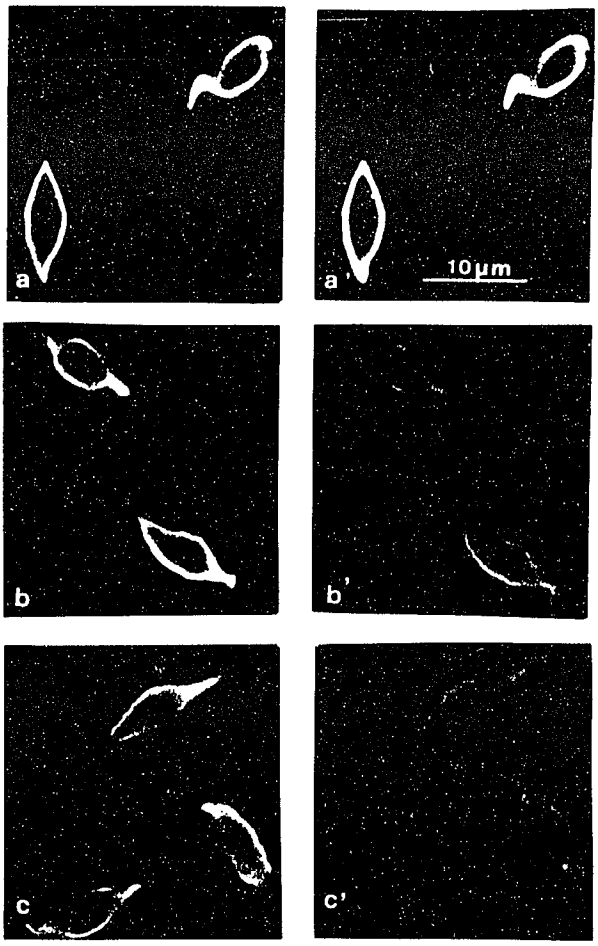
Advantage was taken of the instability of MTs in the chicken MB to examine the immunoreactivity of MS remnants adhering to the coverslip after MT disassembly due to dilution (Fig.34). The MS remnants bound phalloidin, as well as anti-syncolin, but did not bind anti-tau antibody (Fig.34 a-c). There was no immunoreactivity with antibody against tubulin, as expected (Fig.34, a).

ERYTHROCYTE TAU PROTEIN

Tau aggregates

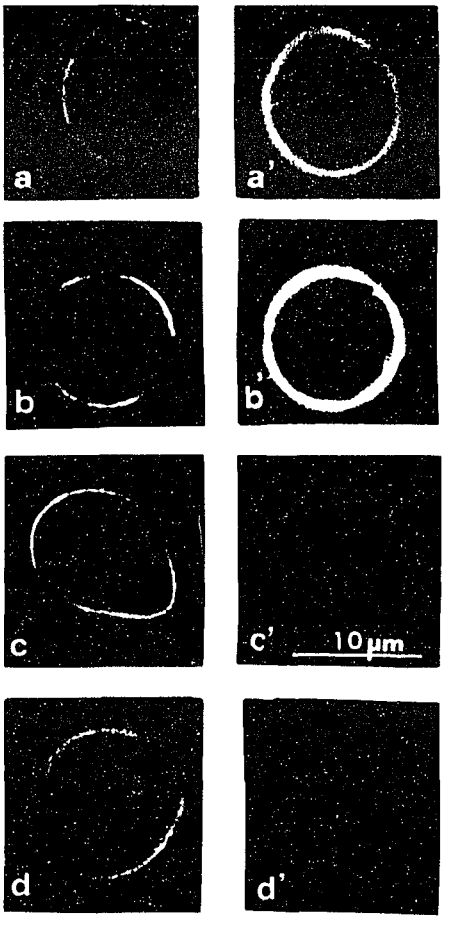
Salt (0.5M NaCl) extracts of taxol-treated dogfish erythrocyte cytoskeletons contained major heat stable protein bands of 40-65kD and a minor band of 290kD. When the extracts were concentrated and desalted, aggregates formed, appearing as groups of 2 to 6 amorphous filaments with a diameter of ~7nm (Fig.35, a). The aggregates shown in Fig.35a were still present after heating in sample buffer, but aggregates which were soluble in sample buffer had similar appearance in TEM. Aggregates that were not soluble in sample buffer were only obtained when salt was completely removed by dialysis. It was necessary to run the samples right after heating, and at higher voltage than usual (400V), in order to get the protein into SDS-polyacrylamide gels. Figure 35b, lane 1 shows the aggregate pellet in sample buffer (after centrifugation) containing protein bands in the 65-42kD range. These aggregates did not react with antibody against tubulin but showed weak immunoreactivity with 5E2 anti-tau antibody in dot blots (not shown). What caused these aggregates to be partly insoluble in SDS buffer remains to be studied. It was noted that the formation of these aggregates was inhibited by the presence of salt (0.5-1M NaCl). However, aggregates would form in the presence of ~.1M NaCl which allowed for their sedimentation prior to preparation for SDS-PAGE.

Figure 32. Fluorescence localization of proteins in chicken erythrocyte cytoskeletons. (a and a') double labelling with rhodamine-labelled phalloidin and anti-tubulin, respectively. (b and b') Labelling with phalloidin and monoclonal antibody "tau-2". (c and c') Phalloidin and polyclonal anti-syncolin antibody, respectively. Whole cytoskeletons react with anti-syncolin, anti-tau antibodies and phalloidin in a pattern corresponding to the the MB.



(32)

Figure 33. Isolated chicken MBs.
(a, a'and b, b') Double labelling with rhodamine-phalloidin and anti-tubulin, respectively.
(c, c') Phalloidin and tau-2 antibody.
(d, d') Phalloidin and anti-syncolin. Chicken isolated MBs interact with the MS and this interaction seems to involve a ring of F-actin. In the most isolated MBs shown only patches of F-actin remain (b-d).



(33)

Figure 34. Fluorescence localization of F-actin and syncolin in MS remnants from chicken isolated MBs by fluorescence. (a, a') Rhodamine-phalloidin and anti-tubulin, respectively. (b, b') Phalloidin and anti-tau antibody "tau-2". (c, c') Phalloidin, and anti-syncolin. Patches of MS remaining after MB disassembly contained syncolin and F-actin.

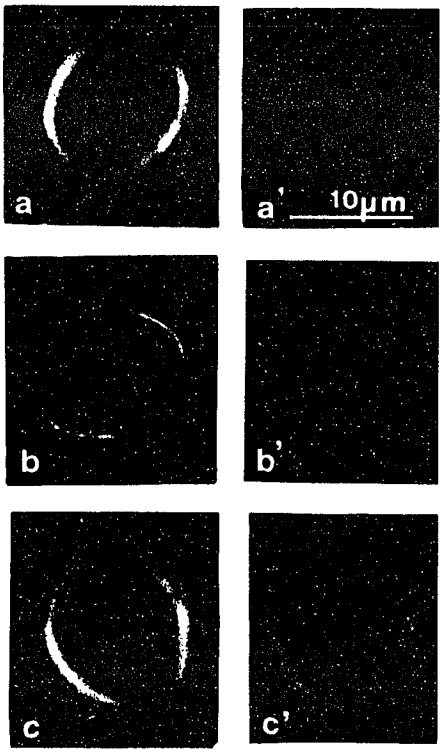


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Figure 35. Heat stable protein aggregates from salt-extract of dogfish cytoskeletons. Aggregates after concentrating and desalting were collected by sedimentation and prepared for gel electrophoresis. After heating in sample buffer aggregates remained which were then collected by centrifugation and visualized by negative staining, TEM (a). Aggregates contain filaments of ~7nm width. (b) SDS-PAGE of protein in aggregates. Lane 1: aggregate pellet in SDS-PAGE sample buffer. Lane 2: sample buffer after centrifuging down aggregates. (c) Aggregate-forming heat stable proteins from dogfish cytoskeleton salt-extracts immunoblotted with "DJ" polyclonal antibody against mammalian heat stable MAPs.

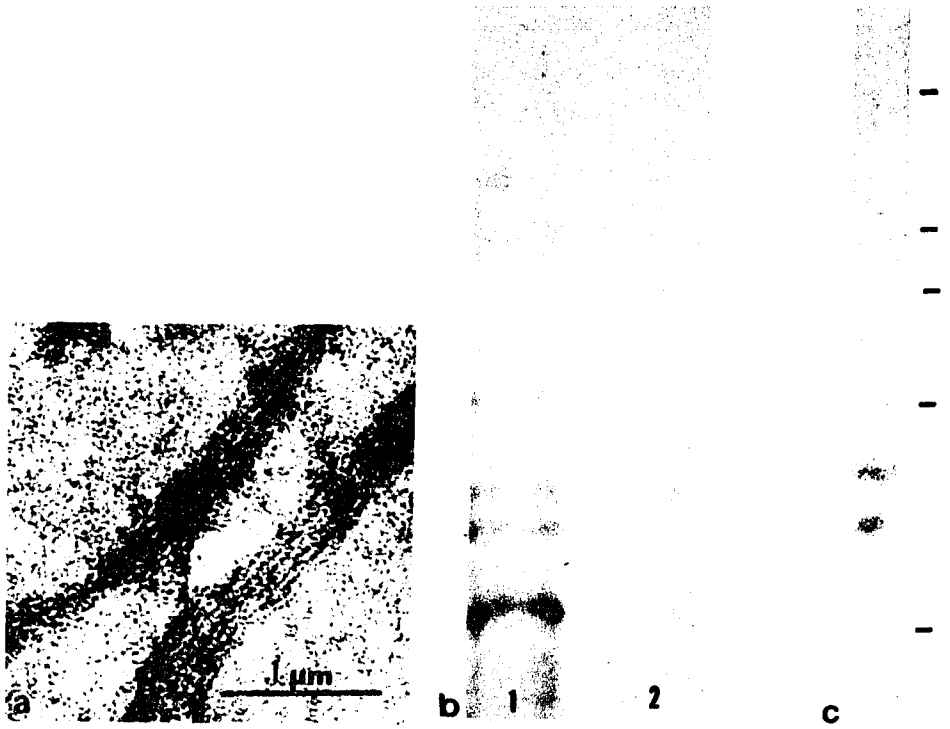

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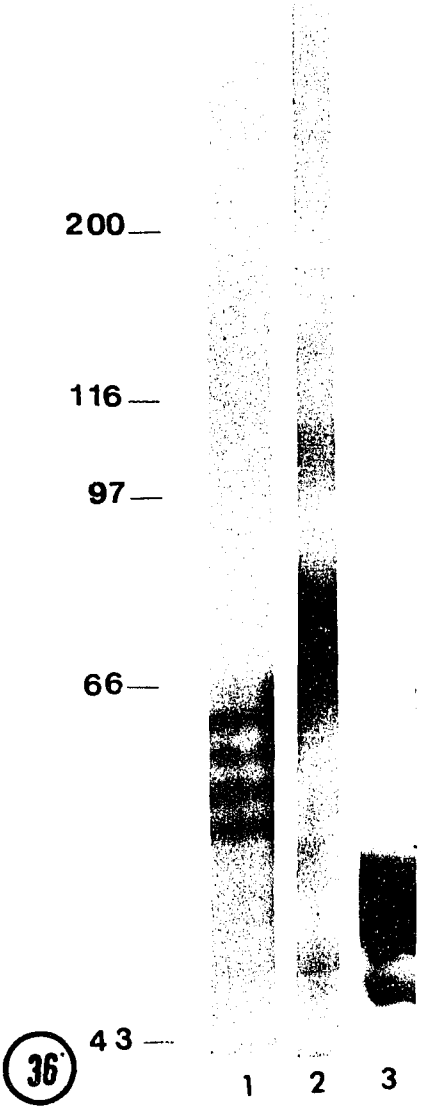
Figure 36. Brain and erythrocyte heat stable MAPs. Immunoblot with polyclonal antibody against mamalian heat stable MAPs, "DJ". Lane 1: Bovine brain tau. Lane 2: dogfish brain heat stable proteins. Lane 3: heat stable protein from taxol-cytoskeleton salt extract. Heat stable protein from dogfish erythrocyte cytoskeletons has a lower molecular weight than bovine brain tau but shows strong cross-reactivity to anti-mammalian brain heat stable MAPs.



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In addition, inclusion of nucleotide triphosphates (ATP and GTP) during erythrocyte tau preparation had inhibitory effects on the formation of aggregates. This was especially noticed with inclusion of ATP (2.4mM), but it has not been determined whether this was just a salt effect or whether phosphorylation was actually involved.

Sedimentation of aggregates prior to SDS-PAGE electrophoresis revealed 40-60kD proteins, while the 290kD protein remained in the supernate. Aggregates cross-reacted with "DJ" antibody to mammalian heat stable brain MAPs, as shown by immunoblotting (Fig.35c and 36, lane 3).

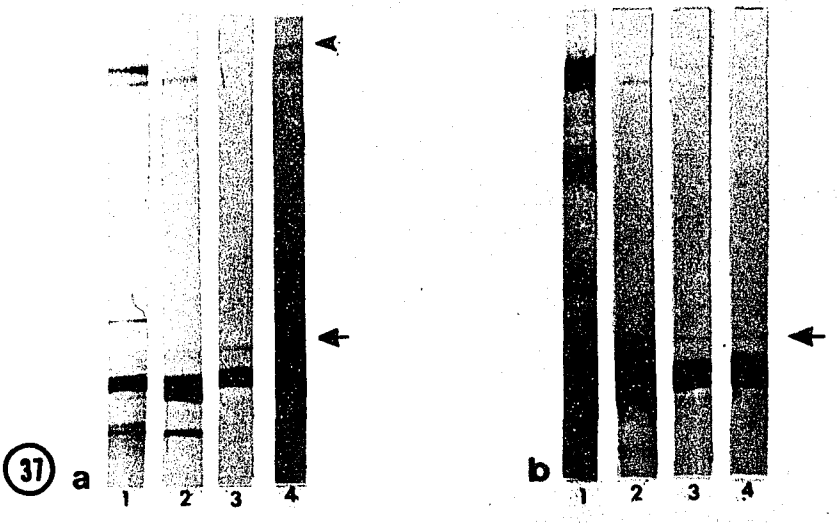
MT PROTEIN FROM ERYTHROCYTE CYTOSKELETONS

Temperature cycling experiments

Low temperature extracts and cycled MT protein from dogfish erythrocyte cytoskeletons contained only proteins in the tubulin molecular weight region. The 290kD protein remained in the cytoskeleton after temperature-induced MT disassembly. However, when either 0.1M NaCl or KCl was included during low temperature incubation some of the 290kD protein was also extracted (Fig.37a vs 37b, lane 3 and 4). In the latter preparations the 290kD protein recycled with MTs during temperature-induced reassembly (Fig.37a, lane 4).

As demonstrated by immunoblotting, low temperature extracts and reassembled MTs contained tau in the 40-60kD region (Fig. 38 and 39). The 290kD protein reacted with

Figure 37. Proteins of the cytoskeletal system of the dogfish erythrocyte separated by 4-15% gradient SDS-PAGE. Low temperature extract of cyto- skeletons in the presence (a), and absence (b) of 0.1M KC1. Lane 1, whole cytoskeleton. Lane 2, isolated MBs. Lane 3, low temperature extract of cytoskeletons. Lane 4, MT pellet obtained from sample shown in lane 3 after rewarming to room temperature. There are several bands of molecular weight 61KD to 70KD which are enriched together with tubulin (arrow). The high molecular weight band seems to be preferentially removed from cytoskeletons by the presence of KC1 (arrowhead).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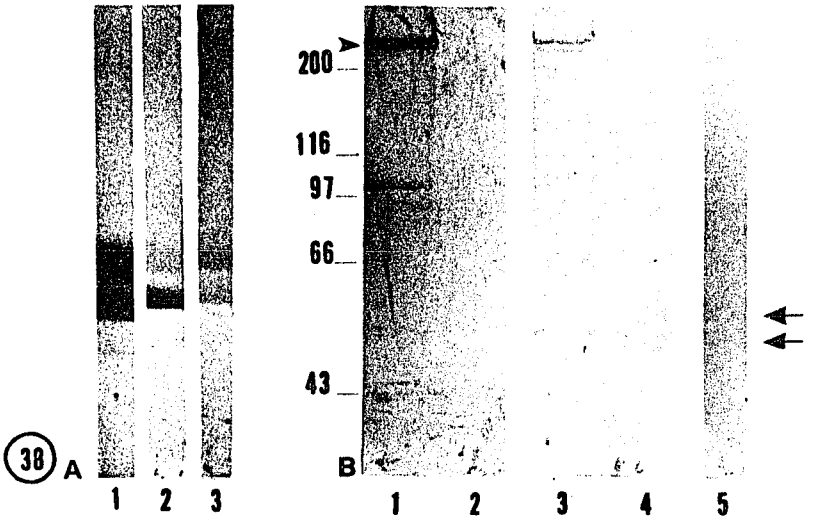
"DJ" anti-heat stable brain MAPs and anti-syncolin antibodies but remained in the cytoskeleton after MT disassembly. The 290kD protein was not detected in reassembled MT bundles in this system as shown by immunoblots with anti-heat stable MAPs or anti-syncolin antibodies (Fig.38a and 38b, lanes 2,4,5). The pattern of anti-tau antibody binding to temperature-cycled MT preparations was similar to that of the original low temperature extract, indicating the immunoreactive protein bands to be MAPs (Fig.39).

In contrast, the chicken cytoskeleton MT protein contained a major tau protein of 68kD (Fig.40a). To test whether the phosphorylation state of this tau was involved in its high apparent molecular weight, the SDS-PAGE pattern of MT protein obtained from alkaline phosphatase (AP) treated cytoskeletons was examined. A shift in mobility of chicken cytoskeleton tau from higher to lower apparent molecular weight on SDS-PAGE was observed (Fig.40b).

In both chicken and dogfish MT preparations the higher molecular weight tau proteins seem to be lost during temperature induced assembly-disassembly cycles (Fig.41). That is, more of the higher molecular weight tau in both chicken (68kD) and dogfish (62kD) preparations was left in the supernate after MT protein assembly (Fig.41a and b, lane 1).

Figure 38. Immunoblotting of MT bundle protein and cytoskeleton residue after low temperature extraction. a) Blot of dogfish MT bundle protein. Lane 1: india ink stained; Lane 2: chicken erythrocyte anti-tau antibody (polyclonal; Dr. D. Murphy). Lane 3: "5E2" mammalian brain tau monoclonal. (b) Blot of dogfish MT bundle protein and cytoskeleton residue. Lane 1: Cytoskeleton residue, chicken erythrocyte syncolin antibody. Lane 2: MT bundles, syncolin antibody. Lane 3: Cytoskeleton residue, mammalian brain heat-stable MAPs antibody (polyclonal, DJ; Dr. K. Kosik). Lane 4: MT bundles, tau antibody (DJ). Lane 5: MT bundles, mammalian brain tau antibody (5E2). DJ and 5E2 antibodies cross-react with proteins in the 50-60kD region (faint in blot photo, arrows). Both DJ and anti-syncolin bind to high MW protein in the cytoskeleton residue. Anti-syncolin does not recognize any protein in the MT bundles.

Figure 39. Dogfish MT protein MAP. Low temperature extract from cytoskeletons (lane 1 and 4) and cycled MT protein pellet (lane 2 and 3). Lane 1-2: Coomassie blue stained blot. Lane 3-4: immunoblot with anti-chicken erythrocyte tau antibody. Major cross-reactivity was observed to ~54kD protein. The binding pattern and immunoreactivity in proportion to tubulin was similar suggesting that this protein is a MAP.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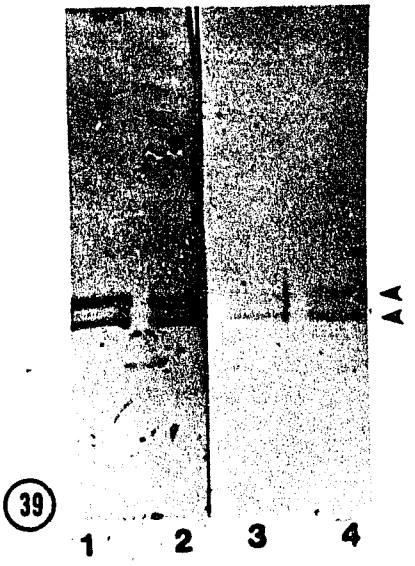
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Properties of MT bundles assembled in vitro

In living dogfish and chicken erythrocytes the MB disassembles at 0°C and reassembles upon rewarming the cells to physiological temperature (Cohen et al., 1982; Behnke, 1970). Similarly, MTs of the MB in cytoskeletons and in isolated MBs disassembled at low temperature in vitro (Cohen et al., 1982 a and b). As observed by video-enhanced light microscopy, MT protein obtained by low temperature disassembly of either the dogfish cytoskeleton MBs or of isolated MBs formed MT bundles upon temperature-induced reassembly (Fig.42). MT bundles formed from cytoskeleton low-temperature extracts that had been obtained either in the presence or absence of salt (0.1M KCl or 0.1M NaCl). The bundles were initially relatively short and thin, but increased in thickness and length with time as observed by video enhanced light microscopy and negative staining (Fig.43). The Student-Newman-Keuls and Kruskal-Wallis multiple comparison tests indicated that the increase in MT length as a function of time was statistically significant ($P<.0001$). In vitro assembled MT bundles exhibited flexibility (Fig.44) and mechanical stability in flow, maintaining their structural integrity even after vigorous pipetting and coverslip tapping, and they were also observed to withstand bending without breaking (Fig.45).

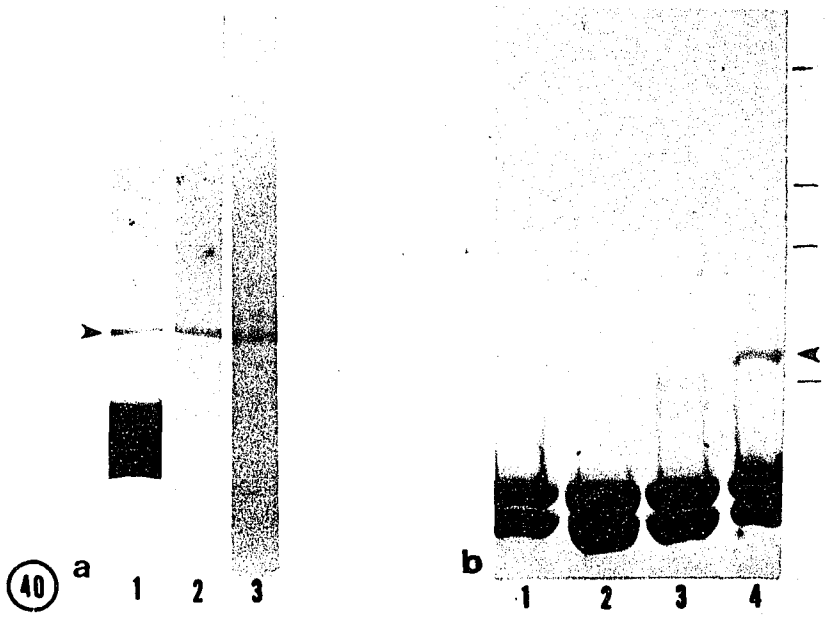
A difference in the pattern of bundle formation was observed depending on whether MT protein was prepared by low temperature MT disassembly in the presence or absence of

Figure 40. Chicken erythrocyte MT protein.

(a) Lane 1: low temperature extract, india ink. Lane 2: Immunoblot of sample in lane 1, DJ polyclonal antibody against mammalian brain heat stable MAPs was used. Lane 3: Immunoblot of heat stable protein from cytoskeleton low temperature extract. Arrowhead denotes the position of the 68kD protein.

(b) SDS-PAGE of alkaline phosphatase (AP) treated MT protein. Lane 1: Supernate after sedimentation of assembled MT. Lane 2: pellet after sedimentation of assembled MT protein. Lane 3 and 4: Pellet and supernate of assembled MTs from alkaline phosphatase treated cytoskeletons. Arrow shows the 68KD protein which is decreased in those preparations which had been treated with AP. Arrowhead denotes the position of the 68kD protein.

(c) SDS-PAGE of chicken erythrocyte MT protein. Lane 1: Low temperature extract from cytoskeletons. Lane 2: heat stable proteins from PC-salt fraction. Lane 3: heat-denatured pellet of assembled MTs. Lane 4: heat stable protein from salt extract of assembled MTs. Chicken erythrocyte MT protein contains a major tau protein of 68kD (arrowhead) and minor tau of lower molecular weight which are enriched during MT assembly. The higher molecular weight tau seems to have lower affinity for MTs especially during assembly in the presence of taxol.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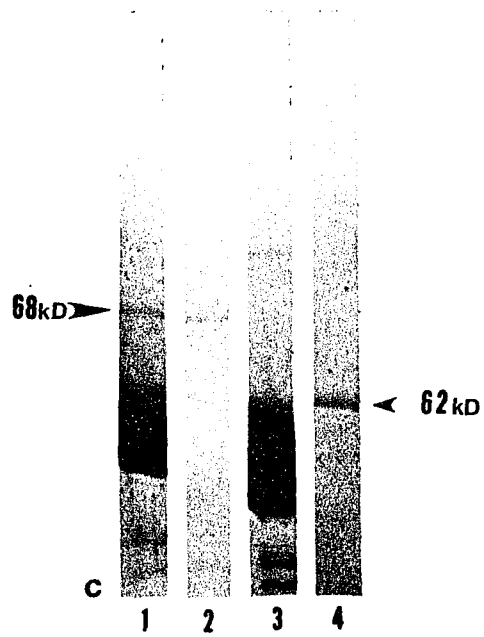
68kD
62kD
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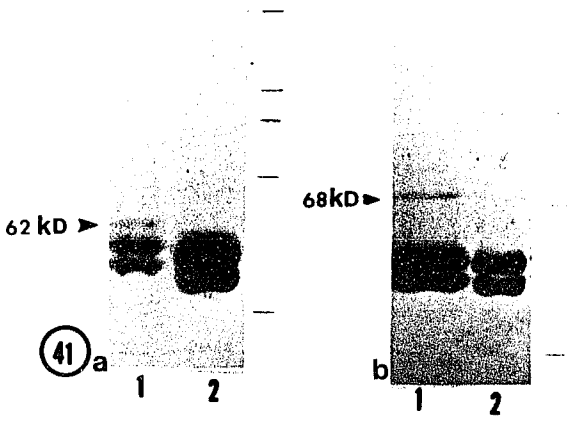
Figure 41. Temperature cycling of chicken and dogfish MT protein from cytoskeletons. (a) Dogfish and (b) chicken MT preparations. Lane 1: supernate after sedimentation of MTs; lane 2: temperature-induced assembled MTs, pellet. The high molecular weight taus in dogfish (62kD) and chicken (68kD) proteins are left in supernate. Lines at thr right of the gels represent molecular weight standards; 200, 116, 97, 66, and 42 kD.

Figure 42. Microtubule bundles assembled in vitro by warming of MT protein obtained by low temperature disassembly. (a) MT bundles as visualized by phase contrast, and (b) same bundles in DIC. (c) High magnification of a bundle of MTs after several hours of rewarming to room temperature (TEM, negative staining). Phase-dense portions of bundles in (a) correspond to thickened regions in (b), and are interpreted as areas of MT overlap.


62 kD
68kD
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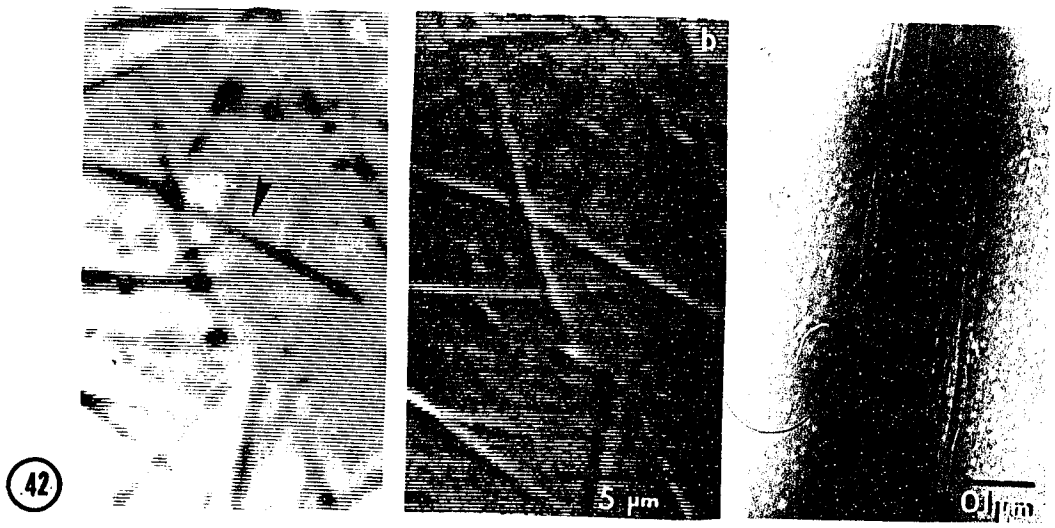
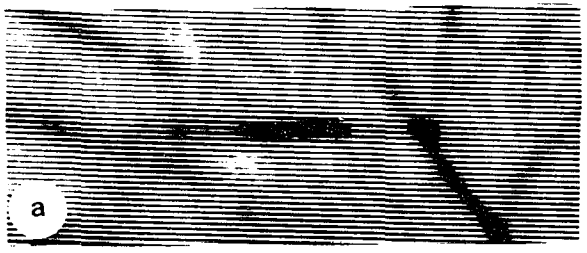
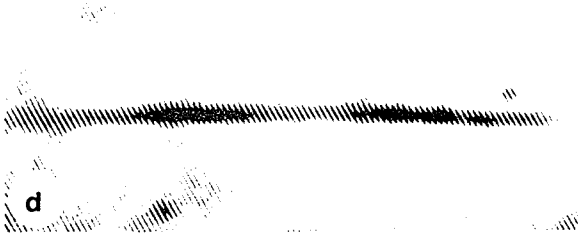

42
b
5 µm
0.1 µm

Figure 43. Video images of bundles and possible interpretation in terms of MT overlap. MT bundles after ~10 min. assembly (phase contrast, VEM). a and d: phase density patterns of MT bundles, as seen by phase contrast, VEM. b and e: Interpretation of possible MT overlap patterns (1-2-1 pattern) and (2-1-2 pattern), respectively. MT bundles with dense areas may be the result of MT overlap (b and e), as has been observed in negatively stained TEM preparations (c and f) X 30,000.

(43)





b

e

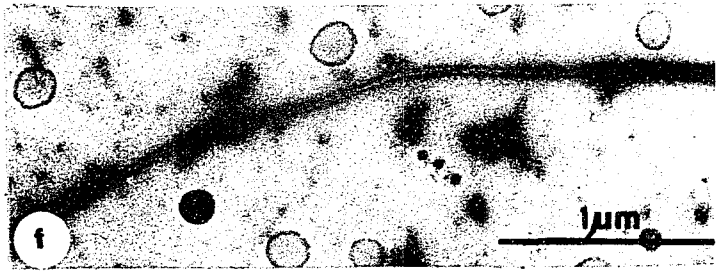
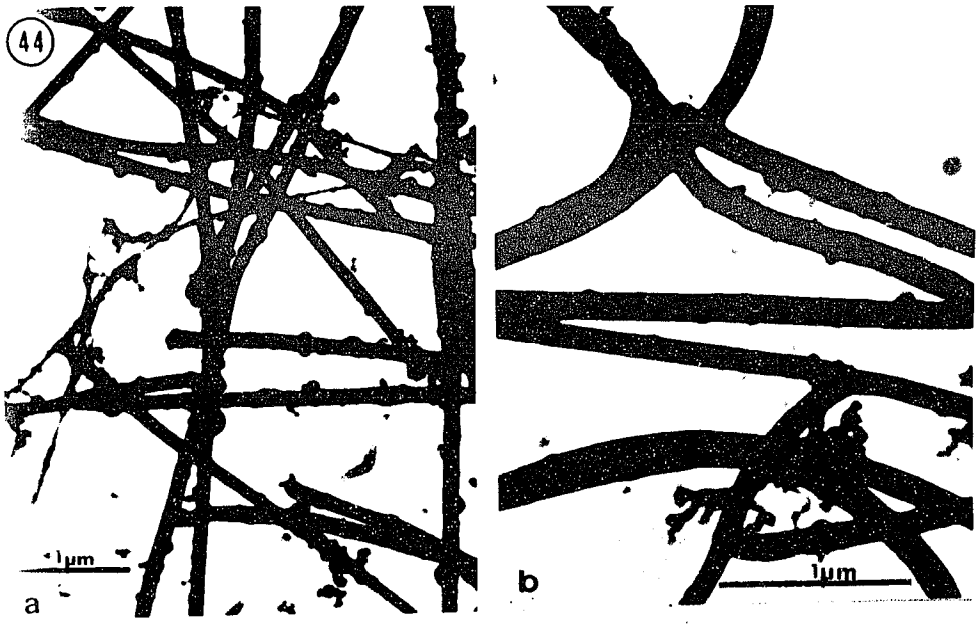
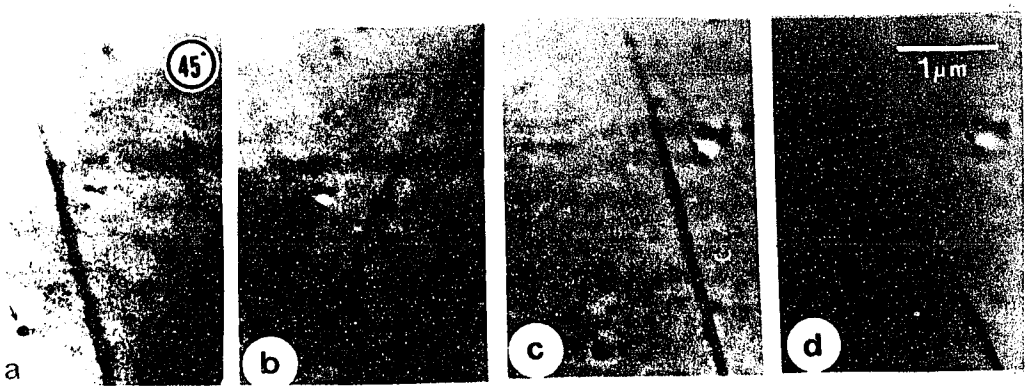


Figure 44. MT bundles, critical-point dried and examined in TEM (Prestained with uranyl acetate in ethanol). (a) Survey view, showing bundles of various thicknesses; most bundles are straight (linear). (b) Higher magnification of several bundles exhibiting curvature. a) X 18,000; b) X 26,000.

Figure 45. MT bundles assembled in vitro show flexibility and mechanical stability. (a) MT bundle with typical linearity at "rest" state. This bundle was stuck to the slide (tethered) at one end; (b) Bending of same bundle to the right, when coverslip is tapped; (c) Spontaneous return of same bundle to linearity afterward; (d) The same bundle bending to the left when coverslip is tapped in another location. Bundle returned to linearity afterward. Arrows denote stable background markers. All phase contrast (VEM). (a-d) X 1,500.


44
1µm
a
b
1µm


45
a
b
c
d
1µm

0.1M KCl. The low temperature extracts of cytoskeletons obtained in the presence of 0.1M NaCl or KCl contained ~40% more protein than extracts obtained without inclusion of salt. At the same concentration of MT protein and under the same conditions, the bundles formed from low temperature extracts obtained in the absence of KCl formed longer bundles. On the other hand, MT bundles assembled from extracts obtained in the presence of 0.1M KCl were shorter in length and assembled faster. The difference in the MT assembly pattern was not due to the presence of salt during temperature-induced MT assembly, as preparations brought to the same salt concentration (0.1M KCl) prior to assembly showed the different pattern upon rewarming.

To examine whether the 290kD protein was required for MT bundle formation in this system, MT protein extracted at low temperature in the presence or absence of 0.1M KCl was fractionated by gel filtration, and the fractions tested for their ability to form bundles. In both preparations, the late fractions devoid of high molecular weight protein were able to form many bundles (Fig.46).

In contrast to dogfish preparations, the reassembly of MT protein extracted at low temperature from chicken cytoskeletons only occasionally resulted in MT bundle formation. Chicken MT protein bundle formation was not found to be directly correlated with any particular assembly condition. It was observed, however, that when bundles did form, inclusion of 20% glycerol during temperature-induced

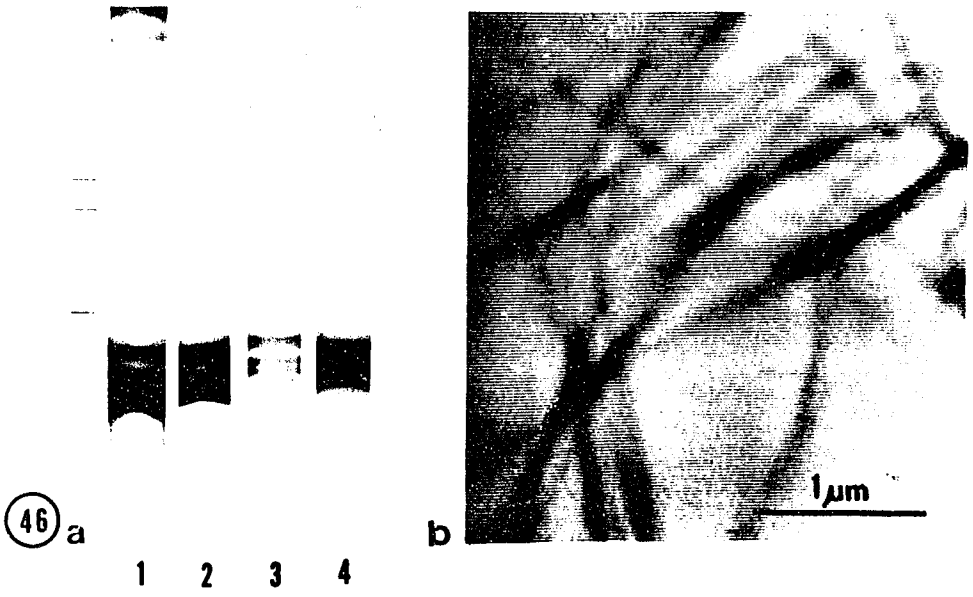
reassembly resulted in thicker, longer bundles as compared to 5% glycerol. The occasional formation of bundles did not correlate with concentration of GTP used, presence of 290kD protein, or inclusion of taxol during reassembly. However, when the bundles did form, it was mostly in preparations with high protein concentrations (~1mg/ml). Because chicken MT protein had a lower critical concentration than that of dogfish, tests were made to determine whether the low bundling activity was due to low nucleation. When MT seeds prepared in the presence or absence of taxol were added to chicken cytoskeleton low temperature extracts, no increase in MT bundling activity was observed.

Unbundling of MT bundles assembled in vitro

Different reagents were tested to perturb MT-MT interaction in bundles produced by in vitro assembly to learn about factors involved in the bundling of these MTs. Assembled MT bundles were incubated with anti-tau and anti-syncolin antibodies, alkaline phosphatase, the catalytic subunit of protein kinase II, DTT, ATP, subtilisin, and NaCl (refer to Materials and Methods). Only 0.5M NaCl and 1% w/w subtilisin resulted in MT unbundling.

Single MTs varying in length were observed after exposing MT bundles to either subtilisin or salt (Fig.47 and 48). Unbundling occurred in the presence or absence of taxol, but without taxol MTs started to shorten after incubation in this high salt concentration. Taxol was

Figure 46. MT bundling of fraction obtained from gel filtration column. (a) Low temperature extract prepared in the presence of 0.1M KCl was loaded onto a Sephadex G-200 column and 0.5ml fractions were collected at 0C. A standard 200kD protein (ß-amylase) was added. This protein has four subunits which run at ~50kD in SDS-PAGE. Lane 1: Early fraction contains ß-amylase as well as some high molecular weight protein. Lane 2: Fraction 5 contains some ß-amylase and tubulin. Lane 3: Fraction 9 underloaded to show several major tubulin bands; no standard protein is present. Lane 4: Fraction 9 more heavily loaded; no high MW protein is present. (b) MT bundles formed by rewarming fraction 9; phase contrast, VEM. b) X 2,170.


46 a
1 2 3 4
b
1 µm

included in the preparations shown (Fig. 47, 48).

Unbundling resulting after MT bundles were exposed to salt (0.5-1M NaCl) was reversible. Many thick MT bundles were observed after decreasing the salt concentration to at least 0.2M NaCl either by dialysis or by dilution. It was noted, however, that this re-bundling occurred only when the salt-extracted single MTs were allowed to disassemble at room temperature by incubation in the salt medium (0.5M-1M NaCl) for at least 20 min. This was discovered accidentally, and accounted for variable preliminary results. Because many single MTs were observed immediately after salt extraction of the MT bundles, I was not taking into account the time the MTs were left in the high ionic strength medium. When MTs were dialyzed immediately after unbundling, mostly single MTs were observed.

The controls for subtilisin-induced unbundling were MT bundle samples treated with subtilisin in the presence of protease inhibitors (Fig.48b). In addition, the effect of trypsin was tested on MT bundles. No unbundling by trypsin was observed at several concentrations tested (10-50U/ml) (Fig. 48c, lane 5-8).

Effect of GTP on assembly and bundling of MT protein

Assembly of dogfish low-temperature extracts obtained from stored cytoskeletons exhibited much faster nucleation as compared with assembly of MT protein from freshly prepared cytoskeletons (Fig.49a vs 51). Non-stored

cytoskeleton MT protein showed a 5 min. lag time before assembly, while no lag phase was observed during MT protein assembly from stored cytoskeletons (Fig.49a vs 51). Increasing concentrations of GTP during assembly had no apparent effect on the nucleation of MT protein from stored fresh cytoskeletons (Fig.51). Steady state was achieved at the same time by MT protein from stored or fresh cytoskeleton MT protein, but the extent of polymerization varied.

Reassembly of MT protein from stored and freshly prepared cytoskeletons with lower concentrations of GTP or no GTP present resulted in thicker MT bundles (mean 4+/-2 vs 2+/-1 MTs per bundle).

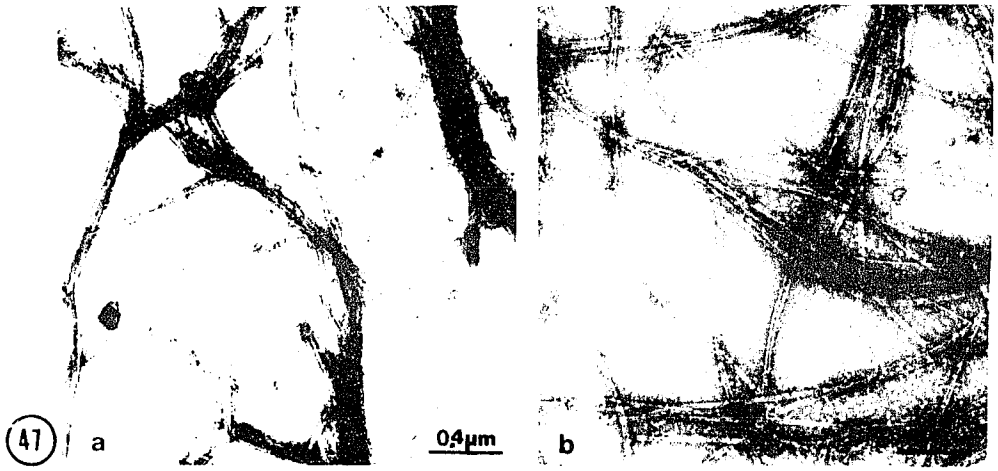
Assembly and bundling of MT protein from cytoskeletons

MT protein from stored cytoskeletons showed faster nucleation, although the extent of polymerization was lower (80% vs 38%). For practical reasons (dogfish are not available during most of the academic year), MT protein from stored cytoskeletons was used for all remaining experiments. Nucleation of either chicken or dogfish erythrocyte MT protein did not require the presence of MT seeds, as previously reported for dogfish preparations (Cohen et al., 1982b). Dogfish erythrocyte MT protein (0.3mg/ml, 1mM GTP, 5% glycerol in NaPEM) achieved steady state after approximately 30 min. at 22°C (Fig.49 and 51).

Turbidity assays of the polymerization of MT protein

Figure 47. "Unbundling" induced by presence of .5M KCl. After assembly of MT bundles, KCl was added to a final concentration of 0.5M, and the preparation examined by VEM and by negative staining for TEM. The population shifted toward thinner bundles and many single MTs. (a and b) X 25,000.

Figure 48. MT bundles assembled in vitro from dogfish MT protein. (a) Unbundling of MTs by treatment of bundles with subtilisin, TEM (negative staining). (b) Control sample, bundles treated simultaneously with subtilisin in the presence of protease inhibitors. (c) SDS-PAGE of protease treated MT bundles. Subtilisin-treated sample, supernate (lane 1) and pellet (lane 2); Subtilisin control (protease inhibitors), supernate (lane 3) and pellet (lane 4); Trypsin-treated sample (25U/ml), supernate (lane 5) and pellet (lane 6); Trypsin control (protease inhibitors), supernate (lane 7) and pellet (lane 8). MT unbundling occurs after treatment with subtilisin but not trypsin. (a and b) X 70,000.


47
a
0.4μm
b

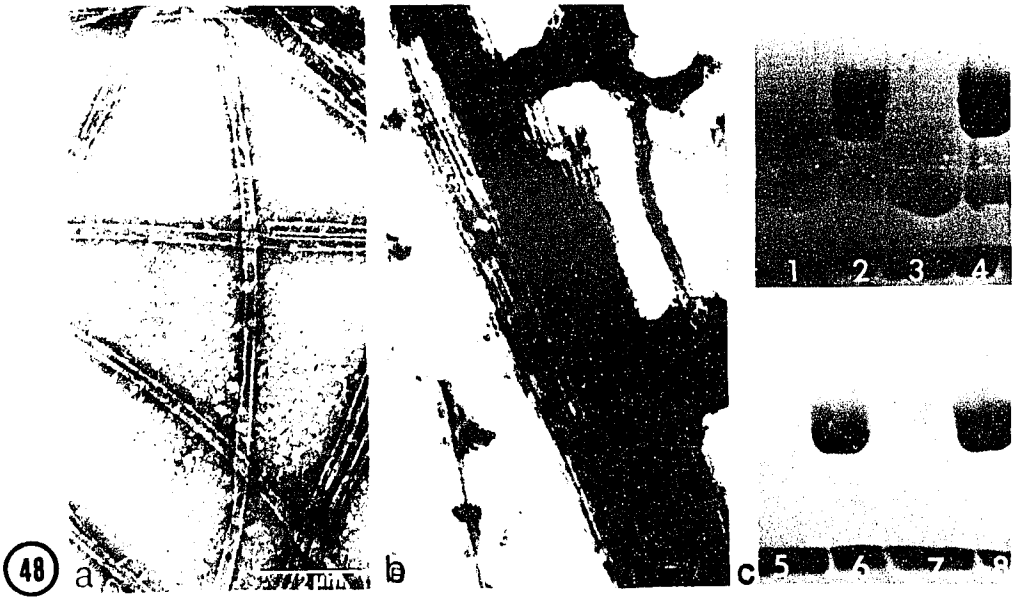
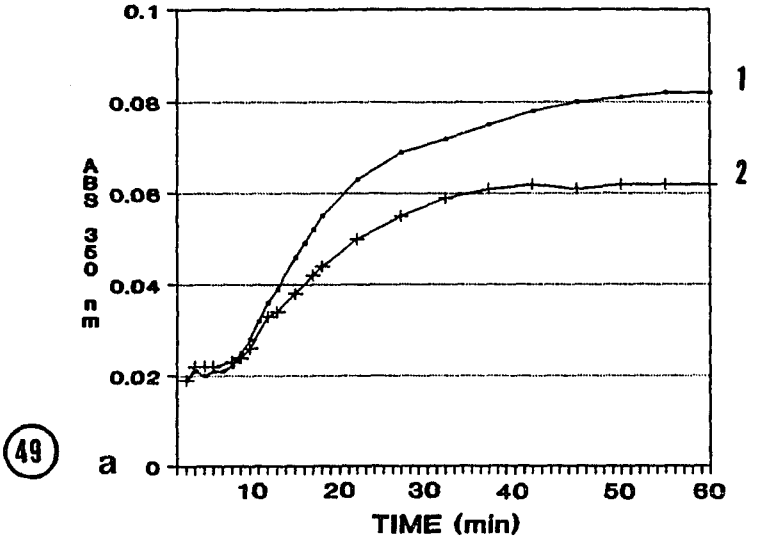
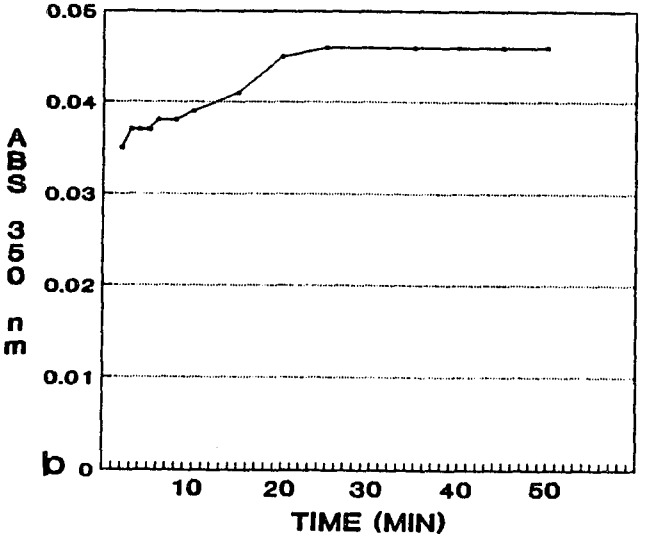

48
a
b
c
1
2
3
4
5
6
7
8

Figure 49. MT assembly in low temperature extracts prepared from non-stored cytoskeletons from (a) dogfish and (b) chicken erythrocytes. Absorbance (350 nm) as a function of time during reassembly MT protein. a) Curve 1; assembly conditions: 0.3mg protein/ml, 5% glycerol, 0.1mM GTP, 22°C. Curve 2: same, except 1mM GTP. b) assembly conditions: 0.3mg protein/ml, 5% glycerol, 0.1mM GTP, 38°C.

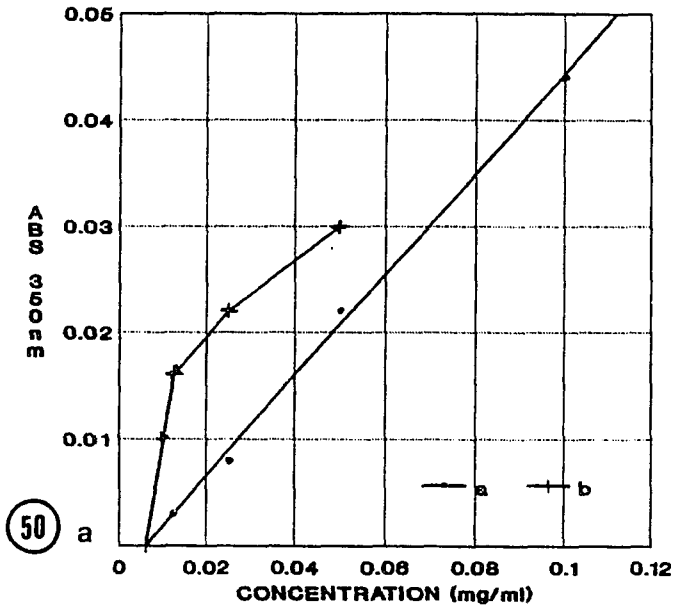

0.1
0.08
0.06
0.04
0.02
0
ABS 350 nm
1
2
10
20
30
40
50
60
TIME (min)

(49) a

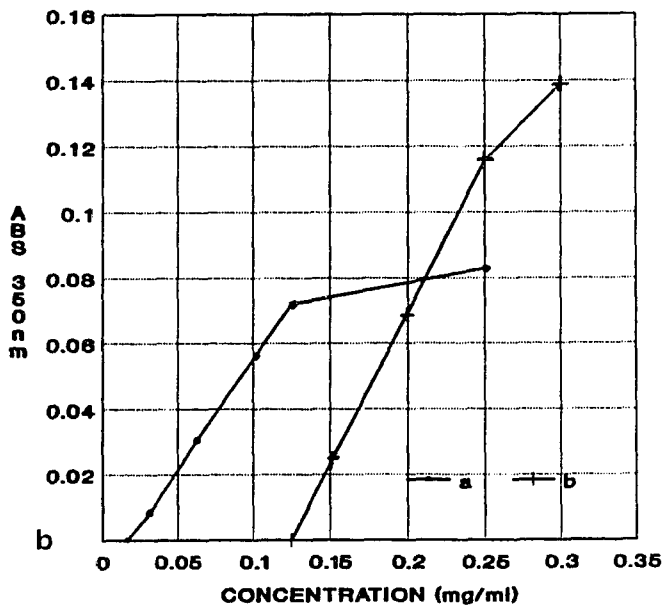

0.05
0.04
0.03
0.02
0.01
0
ABS 350 nm
10
20
30
40
50
TIME (MIN)

b

Figure 50. Critical concentration of dogfish (a) and chicken (b) erythrocyte MT protein, respectively. (a and b) Curves a: Determination by dilution of assembled MTs (0.3mg/ml, 5% glycerol, 0.1mM GTP, 22°C.). Curves b: Determination by assembly at concentrations shown. Note: Abs (350nm) = change in O.D. X 10^5/sec.


0.05
0.04
0.03
0.02
0.01
ABS 350nm
a
b
0
0.02
0.04
0.06
0.08
0.1
0.12
CONCENTRATION (mg/ml)

(50) a


0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
ABS 350nm
a
b
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
CONCENTRATION (mg/ml)

b

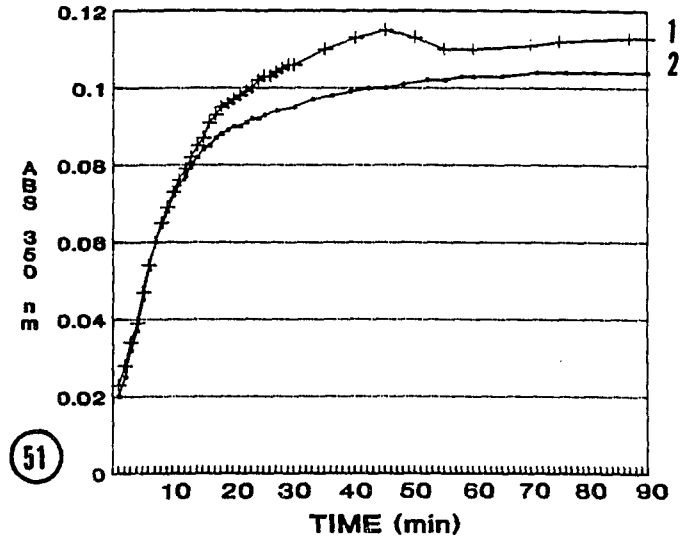
from dogfish samples showed fast nucleation, though no MT seeds were present. As described above, temperature-induced assembly of dogfish MT protein produced both MT assembly and MT bundling. As observed by negative staining (TEM), the mean MT length and the thickness of MT bundles increased with time during assembly (Fig.52 and 53). Inclusion of 0.1M NaCl or 20% glycerol during MT reassembly had an inhibitory effect on the rate and extent of polymerization (Fig.54, table I). Both the relative number of polymers and amount of protein incorporated into polymers was lower in those samples as compared to standard conditions which contained 5% glycerol in NaPEM.

Critical concentration of cytoskeleton MT protein

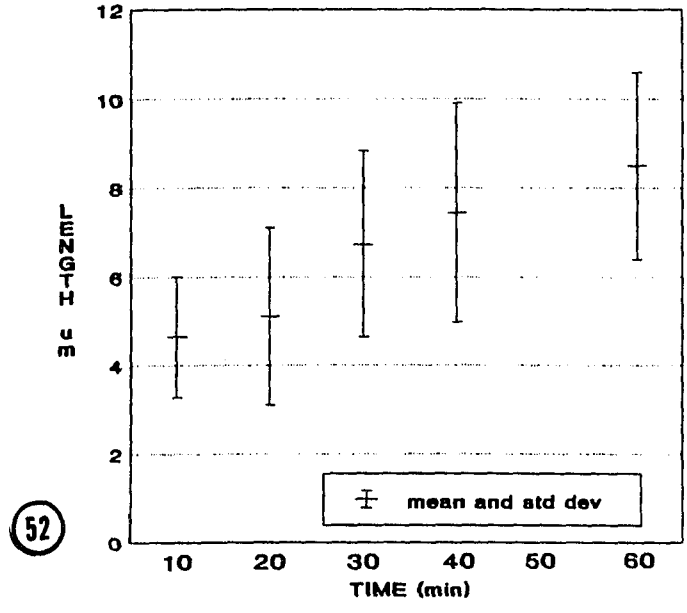
The turbidity (350nm) of temperature-induced MT protein reassembly at different concentrations was determined. The minimum MT protein concentration required for assembly was also determined by dilution of pre-assembled polymers. Both assembly and dilution experiments of dogfish cytoskeleton MT protein revealed the same critical concentration,~0.01mg/ml (Fig.50a). In contrast, the critical concentration determined by assembly and dilution of chicken MT protein differed. The value obtained for chicken MT protein by assembly was ~0.125mg/ml while dilution experiments revealed ~0.025mg/ml (Fig.50b).

Figure 51. Dogfish erythrocyte MT protein assembly (stored cytoskeletons). Effect of GTP Concentration on MT assembly. Curve 1: Conditions: 0.3mg/ml, 5% glycerol, 0.1mM GTP, 22°C. For protein incorporated into polymers, see table 1. Curve 2: Conditions: 0.3mg/ml, 5% glycerol, 1mM GTP, 22°C.

Figure 52. Mean length of single MTs and MT bundles during assembly (negative staining, TEM). The mean length of MTs increases as a function of time.


0.12
0.1
0.08
0.06
0.04
0.02
0
ABS 360 nm
1
2
10 20 30 40 50 60 70 80 90
TIME (min)

51

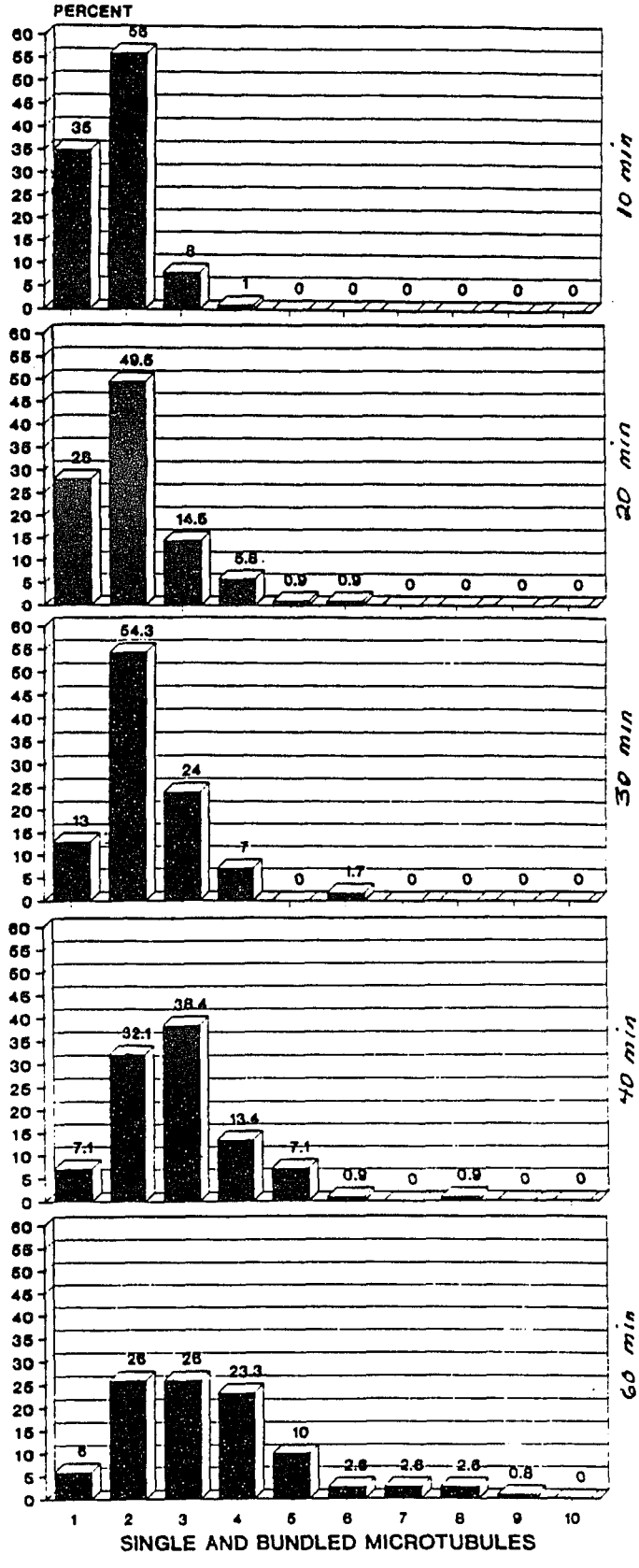

12
10
8
6
4
2
0
LENGTH um
mean and std dev
10 20 30 40 50 60
TIME (min)

52

Figure 53. Distribution of single and bundled MTs as a function of time. MT assembly conditions: MT protein 0.3 mg/ml, PEM containing 5% glycerol, 0.1mM GTP, 22°C (same preparation as in Fig. 51, curve 1). Time course samples were negatively stained with uranyl acetate. Measurements were made on enlarged projections of negatives representing non-selected fields.

The % bundles with 2 MTs remained relatively high (~50%) during the first 30 min., then declined. The % single MTs dropped steadily, indicating that single MTs are contributing to the formation of bundles. The % bundles with 3 or more MTs steadily increased.

The mean number of MTs per bundle and the mean bundle length (figure 52) both increase with time.


PERCENT
10 min
20 min
30 min
40 min
60 min
SINGLE AND BUNDLED MICROTUBULES

(53)

Figure 54. Effect of salt and glycerol on MT assembly. Turbidity increase at 350nm as a function of time.

Curve 1: 0.3mg/ml, 5% glycerol, 0.1mM GTP, 22°C.

Curve 2: same as curve 1, except 20% glycerol

Curve 3: same as curve 1, except + .1M NaCl

Table 1: Proteins incorporated into polymers under different assembly conditions as in Fig.53.

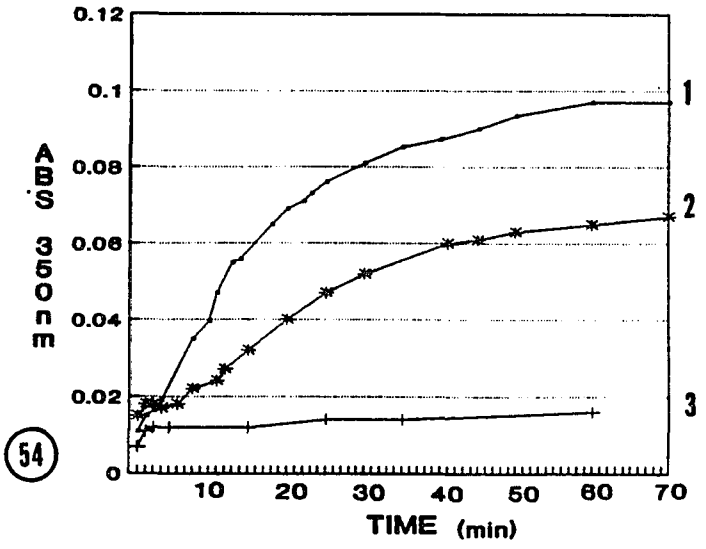


TABLE I

EFFECT OF ASSEMBLY CONDITIONS ON THE EXTENT OF POLYMERIZATION

Assembly media*	Appx. Protein incorporation into polymers	Extent of polymerization ABS 350nm
5% GLYCEROL	37.4%	.085
20% GLYCEROL	31.4%	.055
.1M NaCl, 5% GLYCEROL	22.7%	.009

* ASSEMBLY CONDITIONS OF ALL SAMPLES INCLUDED; 0.3mg/ml MT PROTEIN, 0.1mM GTP NaPEM, 22°C.

TAXOL SEEMS TO INCREASE THE EXTENT OF POLYMERIZATION BY INCREASING THE INCORPORATION OF MT PROTEIN INTO POLYMERS. HOWEVER, WHEN THE SAMPLES WERE BROUGHT TO 1M NaCl, THE AMOUNT OF PROTEIN PER BUNDLE DECREASED REGARDLESS OF THE PRESENCE OF TAXOL IN THE MEDIUM. ASSEMBLED MTS BUNDLES SHOW STABILITY AGAINST THE EFFECTS OF DILUTION SINCE THE SAMPLE RETAINED THE SAME AMOUNT OF PROTEIN INCORPORATED INTO POLYMERS AND PROPORTIONAL TURBIDITY (350NM) ACCORDING TO THE DILUTION FACTOR.

Effect of salt and taxol on stability of MT bundles

The absorbance at 350nm of different preparations of MT bundles diluted in PEM was examined with or without taxol, and with or without high salt (Table II). In addition to the turbidity assays, the amount of protein incorporated into polymers was estimated by determining the amount of protein remaining in the supernate after sediemntation of MTs. MT bundles diluted with PEM with or without taxol showed the same absorbance at 350nm, but the amount of protein incorporated into polymers was much higher in samples containing taxol. Dilution of MT bundle preparations without salt or taxol maintained the same amount of protein incorporated into polymers as was estimated for the sample before dilution. MT depolymerization resulted after dilution of MT bundle preparations in media containing high salt regardless of inclusion of taxol. However, taxol had an effect on the extent of MT depolymerization. It was noted that taxol retained its MT assembly promoting activity in the presence of 1M NaCl since taxol added to disassembled MTs in 1M NaCl was able to promote MT assembly.

Role of erythrocyte tau during MT assembly and bundling

MT assembly (and thus also bundling) was blocked by incubation of the MT protein with antibody to mammalian brain tau prior to and during assembly. However, both MT assembly and bundling were observed when antibodies against

TABLE II

EFFECT OF TAXOL AND SALT ON POLYMER STABILITY AFTER DILUTION

SAMPLES	DILUTION MEDIA		PROTEIN INCORPORATION INTO POLYMERS	EXTENT OF POLYMERIZATION
SALT	TAXOL	% FROM INITIAL PROTEIN CONC.		ABS 350NM
A	+	+	8%	.027
B	-	+	50%	.027
C	+	-	11%	.013
D	-	-	38%	.020

STARTING CONCENTRATION: 0.3MG/ML MT PROTEIN, 0.1MM GTP, 5% GLYCEROL, PROTEIN INCORPORATION INTO BUNDLES BEFORE DILUTION 37%.

FINAL CONC. IN ALL SAMPLES AFTER DILUTION IS 0.075 MG/ML MT PROTEIN, 5% GLYCEROL, 0.1MM GTP.

Table III

EFFECT OF ANTIBODIES ON MT PROTEIN ASSEMBLY AND BUNDLING.

Exp't #	Antibody	Relative Amt. Bundling (VEM Obs.)
1	dilution control	+ + +
	anti-tau, 5E2	-
	preabsorbed 5E2	+ + +
2	dilution control	+ + +
	anti-tau, tau-1	-
	preabsorbed tau-1	+ + +
3	dilution control	+ + +
	anti-syncolin	+ + +
	preabsorbed anti-syncolin	+ + +
4	dilution control	+ + +
	anti-actin	+ + +
	preabsorbed anti-actin	+ + +

Conditions: Dogfish MT protein 0.3 mg/ml, PEM, 5% glycerol. Within each experiment, the dilution control was the standard, with + + + representing maximal bundling. In each experiment, a control was performed using the same antibody preparations pre-absorbed with protein-A beads. The anti-actin (expected to be indifferent) was anti-sarcomeric monoclonal, from Sigma.

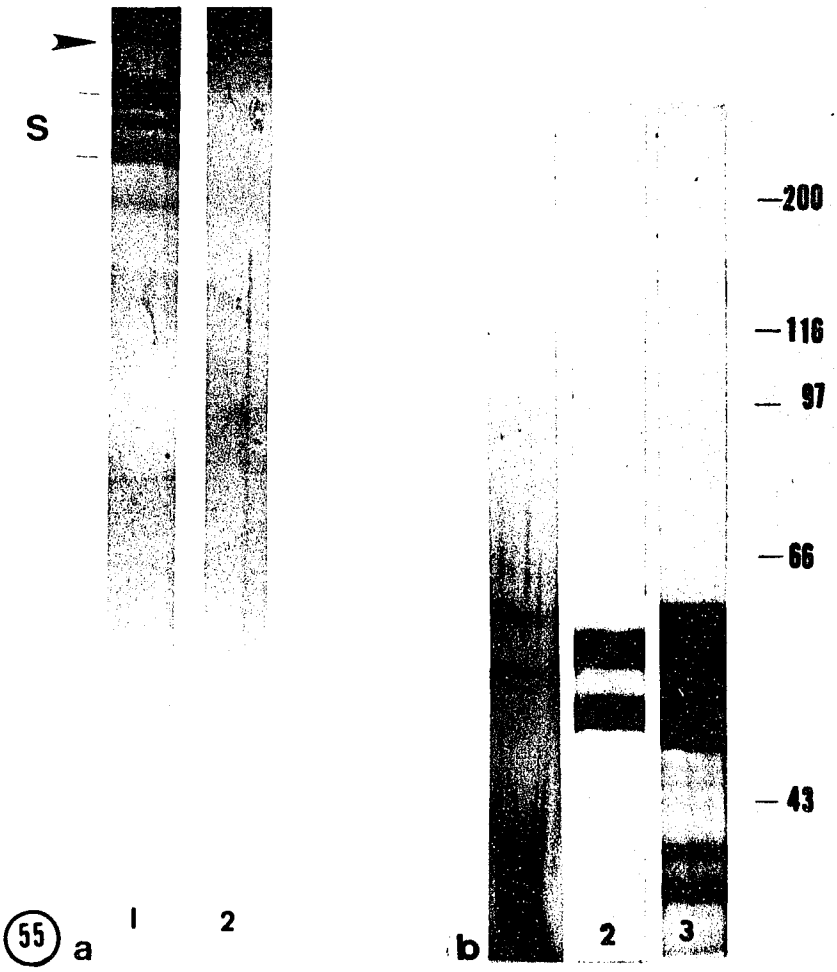
Immunoprecipitation of low temperature extracts with protein A-bound antibodies mentioned above (see materials and methods) prior to temperature-induced MT assembly gave the same results.

syncolin and actin were used (Table III). In addition, immunoprecipitation of MT protein with protein A bound antibodies to syncolin, actin, and tau prior to assembly produced the same results.

Advantage was taken of salt-induced MT unbundling to test for the presence of factors involved in bundling. MT depolymerization resulted after sedimented MT bundles were exposed to 0.5M NaCl for 15 minutes. However, when the MTs unbundled by salt were immediately sedimented, single MTs were recovered. This provided one source of tubulin for reconstitution assays. No high molecular weight protein was detected in the MT bundle pellets (Fig.55b, lane 2,3). Heat stable proteins were obtained from the salt extract of MT bundles with apparent molecular weight of 54-61kD (Fig.55b, lane 1). Aggregates formed if the salt was removed from these heat stable protein preparations. To avoid such aggregate formation, which might interfere with their interaction with MTs, the preparations were left in the buffer containing 5% glycerol, 1M NaCl in PEM. The bundling activity of these heat stable proteins was then tested by a reconstitution assay. Single MTs (2mg/ml) and the heat stable proteins (0.1mg/ml) were incubated together in the presence of 0.1M NaCl. As controls, two samples were prepared containing only 1M NaCl in PEM instead of the heat stable proteins. These preparations were diluted 1:10 with PEM containing 5% glycerol and 0.1mM GTP to decrease the salt concentration to 0.1M NaCl. The MTs of such

preparations, with or without the heat-stable proteins, remained single even when left at 22°C for over 1 hr (Fig.56a and b). In contrast, when preparations containing single MTs (0.2mg/ml) and the heat stable proteins were instead incubated together at 0°C for 1 hr., and then rewarmed, bundles of MTs appeared during temperature-induced reassembly (Fig.56c). No MT assembly was observed in any preparations lacking the heat-stable proteins (not shown). In order to test whether MT assembly alone in the latter preparation could result in MT bundling, taxol was added to 10μM. Many single MTs were observed, but no bundles (Fig.56d).

Figure 55. Profile of proteins from bundled MTs. (a and b) Silver stained 5% and 7.5% SDS-PAGE, respectively. a) Lane 1: cytoskeleton residue after 0°C extraction. Lane 2: Overload of MT bundle protein (corresponding to twice the loading of that in b, lane 3). Tubulin was lost due to use of 5% gel and extended run to improve separation of high molecular weight proteins. b) Lane 1: heat stable proteins from the salt extract of in vitro assembled bundles. Lanes (2,3) Normal loading and overloaded MT bundles. Nearly all of the heat stable protein from the MT bundle salt extracts (b, lane 1) is in the tubulin region of the gel. A high MW protein (280kD; arrowhead) remains in the residue after low temperature extraction (a, lane 1), and is not present in the MT bundles (a, lane 2; b, lanes 2 and 3).



55

Figure 56. Reconstitution experiment. a) Single MTs obtained after salt extraction of MT bundles were incubated without (a and d) and with (b and c) heat stable proteins from the salt extract.

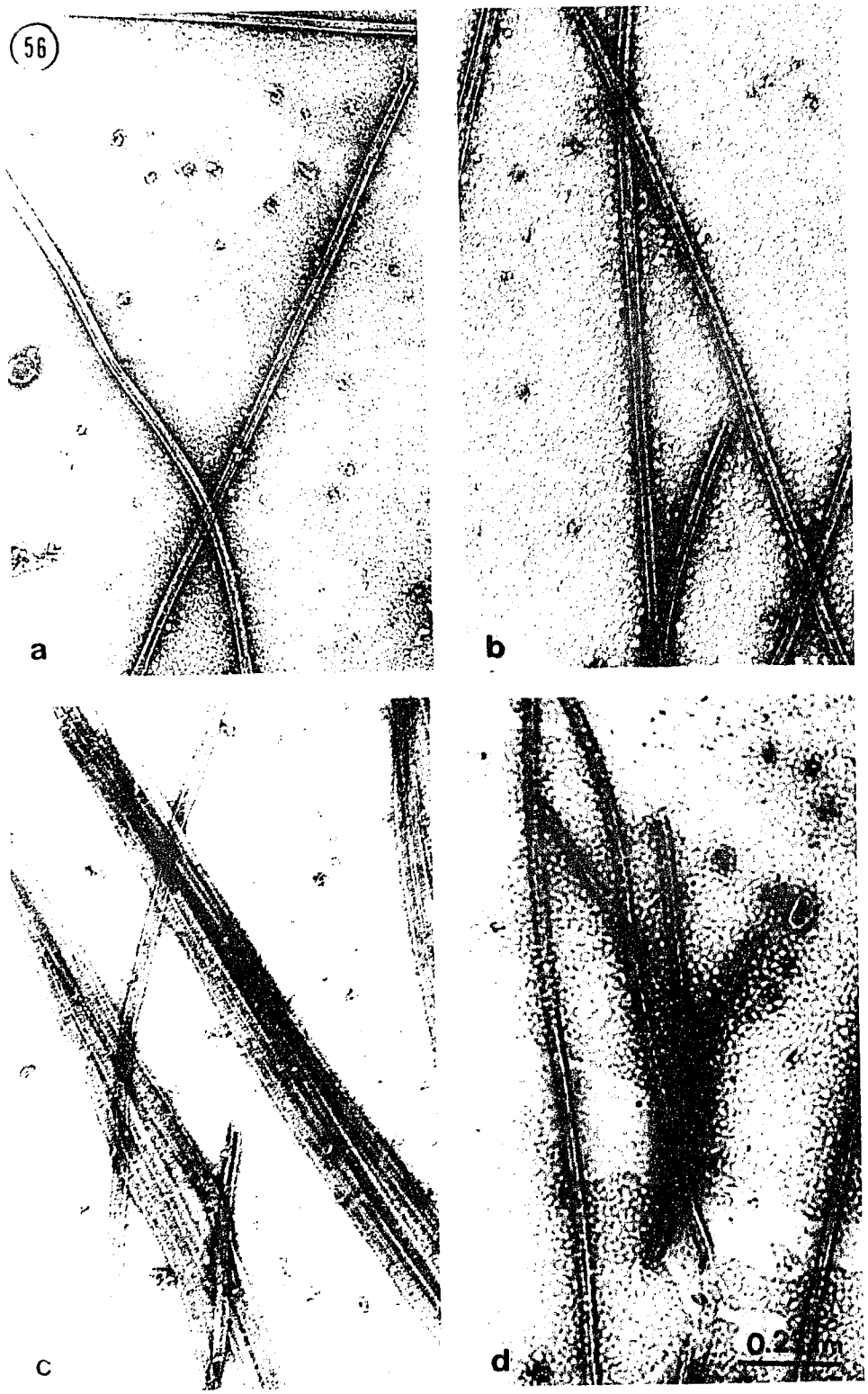
(a) Single MTs, incubated at 22°C

(b) Single MTs, heat stable proteins,incubated at 22°C.

(c) " ", heat stable proteins, temperature cycled.

(d) " ", temperature cycled, 10μm taxol.

Many MT bundles were observed in sample (c) after temperature induced assembly, but no MTs were observed in similar preparations in the absence of heat stable proteins (not shown). Addition of taxol (10μM) to the temperature cycled preparation lacking heat stable proteins, resulted in assembly of many single MTs but no MT bundles (shown in d). All preparations contained 5% glycerol, 0.1M NaCl, 0.1mM GTP in PEM. Heat stable proteins obtained from MT bundle salt-extract retained bundling activity.


56
a
b
c
d
0.2 µm

DISCUSSION

Intrinsic vs extrinsic components of the MB

The proteins present in isolated MBs and in the cytoskeleton remnant were examined to determine which proteins might be intrinsic to the MB and which might be localized at the MB/MS interface region. Fluorescence microscopy and analysis of proteins present in isolated MB preparations indicated that tubulin and tau protein are intrinsic MB components. The minor components in isolated MBs included some spectrin, actin, and a 290kD protein. These proteins were found in the MS remnants associated with the isolated MB, and their localization was consistent in isolated MBs of dogfish and of chicken.

Although the 290kD protein in dogfish erythrocyte cytoskeletons was immunologically similar to syncolin, it exhibited heat stability unlike syncolin (Feick et al., 1991). Although monoclonal antibodies against MAP-2 (5F9, 4F7 and RNP.1194) did not bind to the 290kD, crossreactivity was observed with "DJ" polyclonal antibody against mammalian brain heat stable proteins. Kosik and colleagues (1988) reported that they observed no cross-reactivity of the MAP2-like protein in mouse optic and rabbit sciatic nerves with 5F9. The epitopes of both 5F9 and 4F7 were described to be highly hydrophobic and conformation dependent (Kosik et al., 1984).

During low temperature disassembly of MBs most of the 290kD protein was found in the remaining cytoskeleton, but when 0.1M KCl or NaCl was included during low temperature incubation, the 290kD protein was also extracted. According to temperature cycling experiments the 290kD, when present in the low temperature extracts, bound to reassembled MTs and therefore acted as a MAP. Thus, the MBs of nucleated erythrocytes contain two MAPs which interact with their MTs in different ways. The factors determining the distribution of MAPs within different cellular compartments are unknown. The localization of these two MAPs may result from differential expression during MB biogenesis, level of affinity to MTs, or different MT binding sites. A more detailed description of the 290kD protein in dogfish cytoskeletons remains to be done to understand its similarity to syncolin and/or MAP-2.

Although nucleated erythrocytes contain a circumferential ring of F-actin, it did not appear to be an intrinsic component of the MB. By using varying concentrations of detergent mixture, isolated MBs were obtained which contained F-actin throughout the MB, or which had only patches of F-actin or no F-actin at all. The patches attached to the isolated MB were shown to be remnants of a specialized MS region containing not only F-actin but also a syncolin immunoreactive protein as shown by fluorescence microscopy. These proteins were also present in the MS remnants which remained attached to coverslips

even after the MTs of chicken erythrocyte MBs had disassembled.

The MB/MS Interface

Cellular morphogenesis in nucleated erythrocytes is believed to involve a sequence in which spheroidal cells become flattened discoids and then attain elliptical shape (Cohen, 1991). MB biogenesis is essential for the morphological differentiation of erythrocytes and is responsible for the transition of developing erythrocytes from spheroids to flattened discs. As the MB forms, amphibian erythrocytes have been found to exhibit "single" and "double" pointed morphology correlating with the shape of MT bundles in the cells MB biogenesis (Ginsburg et al., 1989). During such shape changes, the stability of assembling MTs and MT-MT interaction presumably play an important role in establishing the flattening of the cell.

The discoid shape of immature erythrocytes has been shown to be dependent on the presence of the MB (Barrett and Dawson, 1974; Sanchez, unp obs.), but after differentiation the MB is involved primarily in maintaining cell shape under conditions of mechanical stress. In discoid cells the shape is cold-labile, while cells that are already elliptical retain their flat shape even after MB disassembly (Barrett and Dawson, 1974; Sanchez, unpub. obs). Proteins associated with the MB at the plane of flattening may comprise a specialized region of the MS which is involved, together

with the MB, in establishing and maintaining the shape and some of the mechanical properties of erythrocytes. Erythrocyte differentiation includes changes in cytoskeleton protein isoforms (Lazarides 1987) and the MB/MS interface may be one of the areas in which most changes occur. For instance, the ezrin-like protein has been shown to be associated with the plane of flattening in whole cytoskeletons after MB formation (Birgbauer et al., 1989).

The effect of the MS on the elliptical shape of MBs had been examined previously by isolation of MBs with methods involving proteases. By such methods isolated MBs were circular and contained no remaining spectrin, but a small amount of actin was observed (Bartelt et al., 1982). Using the detergent-based method, it was also possible to isolate circular MBs. However, at lower detergent concentrations, isolated MBs still retained their oval shape, even though most of the MS had been removed. These MBs contained MS remnants which appear to represent a specialized region containing F-actin and syncolin. These results point to a significant role of the MB/MS interface region in maintaining the elliptical shape of MBs. Waugh and colleagues (1976) pointed out that the force required to cause the deflection in the MB which brings about the change from circular to elliptical conformation might depend on the rigidity of the cell membrane complex. The lipid bilayer apparently contributes little to elliptical cell shape, as detergent extracted erythrocytes retain their elliptical

shape. This suggests that the MS might have a much larger contribution in providing an applied force which could result in the localized MB deflection. In order to account for such force, the rigidity of the membrane complex of the nucleated erythrocyte would have to be greater than that measured for mammalian erythrocytes. Also there may be other cytoskeletal elements at the plane of flattening responsible for the maintenance of the elliptical shape. In fact, it has previously been observed that unlike immature erythrocytes, low-temperature induced disassembly of the MB does not affect the shape of mature erythrocytes (Barrett and Dawson, 1974). The changes in MS components during erythrocyte differentiation may result in the specialization of the MS at the plane of flattening responsible for MB-independent elliptical erythrocyte shape.

The specialized region at the MB/MS interface contains F-actin, syncolin, and possibly ezrin-like protein according to data from other laboratories and that presented here. The fact that oval MBs could be obtained after isolation by the detergent based method brings into question the contribution of the complete MS as a force determinant responsible for the elliptical shape of the MB, since the isolated MBs contained only MS remnants. Thus, the specific interaction of proteins in the MB/MS interface "ring" may be involved in the maintenance of their elliptical shape. The possible source of force resulting in the deflection of the MB in elliptical cells remains to be determined. It is not

clear according to the data obtained which elements of the MB/MS region are responsible for the oval shape of these isolated MBs. However, circular isolated MBs which contained a continuous ring of F-actin according to rhodamine-phalloidin fluorescence were observed. It would be interesting to study in detail the protein arrangement in the MB/MS interface region and to measure its flexural and extensional rigidity independent of the MB.

Mechanical properties of the MB

The MB is an important cytoskeletal component involved in restoring cell shape after deformation which may result from mechanical stress during flow. This function requires a flexible system capable of sustaining applied force without breaking and able to return to its original conformation. The mechanical properties of isolated MBs from the newt (Notopthalmus viridescens) have been studied by Waugh and colleagues (1989). Both the flexural and extensional rigidity was determined for these MBs isolated by four different procedures involving proteases and high salt. They had also previously found that MBs were much more resistant to bending per unit area than the same area of cell membrane (Waugh and Evans, 1976), which would account for the MB having a major role in maintenance of cell shape during circulation. In this respect, MT-MT interaction may also contribute to the flexibility properties of the MBs. Specific measurements of

the contribution of MT-MT interaction have not yet been possible. For such measurements one would have to be able to make preparations of intact MBs and of MBs from which associated proteins have been removed. In addition, most of the MB isolation procedures used involved proteases which may damage the protein content of the isolated MBs. The studies reported here may help to understand the mechanical properties of the MB, and how its structural integrity is established and maintained for the proper functioning of erythrocytes.

MT stabilization in MBs

The MB contains relatively stable, non-dynamic MTs with a structural role in differentiated erythrocytes. One of the most important contributing factors to the mechanical properties of the MB is maintenance of the structural integrity of its MTs. The MB tau may be involved in the stabilization of these MTs.

MAP interaction with MTs has been proposed to be responsible for MT cold stability in brain (Sloboda et al., 1976). It was previously reported that dogfish erythrocyte MBs are highly cold labile in vivo (Bartelt et al, 1982), but isolated MBs are slightly more cold resistant. When 0.1M NaCl or KCl is included in the medium with these isolated MBs during low temperature incubation, they become much more cold labile (Cohen, 1978). In fact, about 40% more MT protein is obtained from dogfish cytoskeletons when

0.1M KCl is included during low temperature incubation (Sanchez, unpublished obs). This phenomenon may be partly due to more dynamic interaction of MAPs with MTs in the presence of higher ionic strength medium, as may be the case in vivo.

The effect of MAPs on MT stability has been proposed to result from a decrease in the disassembly rate (Sloboda and Rosenbaum, 1979; Murphy, 1977). Chicken erythrocyte cytoskeletons contain primarily a higher molecular weight tau protein (68kD), but most of this tau seems to be lost during detergent-based MB isolation. The interaction of MAPs with MTs has been shown to be affected by changes in ionic conditions. The 68kD erythrocyte tau showed low affinity to MTs during temperature cycling and may be removed by the detergent mixture. The loss of tau protein during chicken MB isolation could be responsible for the sensitivity of the MTs in these MBs to disassembly by dilution, unlike dogfish MBs. Some tau protein still remained in isolated chicken MBs, as anti-tau antibody bound along its length, but this remaining tau may be in the tubulin molecular weight region. The major tau in dogfish (54kD) may have stronger MT affinity and could be sufficient for maintaining MB integrity.

Taxol stabilizes MTs by increasing their assembly rate whereas MT assembly is inhibited by increasing concentrations of salt (Olmstead and Borisy, 1975). Taxol-treated MT bundles diluted with PEM containing 1M NaCl and

taxol resulted in single MTs. Although taxol induces MT assembly, high salt conditions inhibit both tubulin assembly and MAP binding to MTs. This resulted in a lower amount of protein incorporated into polymers, although the turbidity at 350nm remained the same (Table II). This suggests that although the presence of salt increases MT disassembly and has an inhibitory effect on the net tubulin assembly, complete MT disassembly does not occur. Taxol retained its assembly promoting activity, thereby preventing total MT disassembly (Vallee, 1982).

In addition, the specific interaction of the MB/MS interface with the MTs of the MB contributes to the structural stability of the MB. Chicken erythrocyte MBs contain proportionally more outer MTs which may interact with the MB/MS interface as compared to dogfish erythrocytes. Because most of the tau in chicken MBs is lost during MB isolation, the remaining MB/MS interface remnants may play an important role in the stability of these isolated MBs. However, MT bundles are observed in areas devoid of these MS remnants suggesting that the presence of bundled MTs in chicken MBs is not dependent on the interaction of these MTs with the MB/MS region.

Although isolated chicken MBs are fairly stable in the isolation media, they were very sensitive to dilution (Fig.21), unlike the MTs of the MB in chicken cytoskeletons. The loss of the 68kD protein during MB isolation may be responsible for increased disassembly of MTs in isolated MBs

due to dilution. The effect of tau on MT stability during MT protein dilution has been shown previously to be dose dependent (Job et al., 1985).

MB tau protein

The tau protein in dogfish erythrocyte MBs exhibited extensive cross reactivity with monoclonal antibodies against epitopes which span the length of mammalian brain tau. Two dimensional electrophoretic separation of the isolated MB proteins showed the presence of proteins with pIs in the range expected for mamalian brain tau (Cleveland et al., 1977; Lee et al., 1989). The fact that only one pI was observed for the MB tau suggests that the protein bands with the different apparent molecular weights may be due to the presence of different SDS resistant secondary structures and not to an overall difference in their phosphorylation state which in turn would affect their pI. Additional properties of brain tau also observed in erythrocyte preparations included comparable molecular weight, recycling with tubulin, ability to form aggregates, and heat stability.

Although isolated dogfish MBs contained proteins in the 40-62kD region which bound antibodies against mammalian brain tau (Fig. 31), these bands also bound monoclonal antibodies (mAbs) 4F7 and 5F9 which are MAP-2 Abs with epitopes near the amino end, outside of the MT binding domain (Kosik et al., 1988). There are, however, reports in

the literature of antibodies against domains outside the MT binding triplet of MAP2 which cross-react with tau molecular weight proteins (Kosik et al., 1988; Yen et al., 1987). The 5F9 antibody is known to bind to paired helical filaments (PHFs) as shown by immunocytochemistry (Kosik et al., 1988). The 4F7 mAb bound a phosphorylation dependent epitope as shown by its decreased reactivity with MAP-2 in phosphatase treated immunoblots. The binding of 5F9, however, is not affected by the phosphorylation state of tau but seems to be conformation dependent as it does not label SDS extracted neurofibrillary tangles (NFTs). It is possible then, that these two antibodies (4F7 and 5F9) recognize conformation dependent epitopes found in dogfish MB tau isoforms even though they do not recognize the MAP2-like antigen in the erythrocyte cytoskeleton.

Tau protein has been described as a component of MT protein in preparations obtained by temperature cycling of chicken erythrocyte proteins extracted using cell sonication (Murphy et al., 1983b). This tau has not been sequenced or further examined, with respect to its immunological similarity to mammalian brain tau. Murphy and colleagues (1985) found that polyclonal sera against hog brain tau reacted with the chicken erythrocyte low molecular weight MAP but antibody against the chicken erythocyte tau did not bind to hog brain tau. These data suggested that brain and erythrocyte tau proteins differ in their respective major antigenic sites. The differences may be partly due to the

phosphorylation state of tau (Murphy and Wallis, 1985). As noted earlier, tau is a very heterogeneous protein resulting from modifications at the transcriptional and translational levels, as well as posttranslational modification such as phosphorylation (Lindwall and Cole, 1984b; Ksiezak-Reding et al., 1987). The different apparent molecular weights of tau protein in chicken MT protein in SDS-PAGE seem to be at least partially the result of their phosphorylation state, as indicated by its increased migration in SDS-PAGE after alkaline phosphatase treatment (Fig.40b). Tau protein has been described as having very little, if any, secondary structure, but the length of tau molecules has been reported to increase upon Ca^{+}/calmodulin mediated phosphorylation.

Ultimately, sequencing would be required in order to determine the true extent of homology between MB tau and brain tau, and to better understand its function in the mature erythrocyte MB and during MB biogenesis.

Tau protein aggregates

Mammalian brain tau has been shown to form aggregates (Lichtenberg-Kraag and Mandelkow, 1990). This property of tau is of particular interest in relation to the study of paired helical filaments (PHFs) found in the brain of patients with Alzheimer's disease. Tau protein has been shown to be the major component in PHFs, and therefore understanding of tau-tau interactions would bring insight to the formation of PHFs as well as to the presumed role of tau

as a MT cross-linker (Ksiezak-Reding and Yen, 1991; Willie, et al., 1992). A tau molecule would have to be able to bind two or more other tau molecules in order for aggregates to form. Among the tau isoforms described to date, the longest tau cDNA (Goedert et al., 1989, 1990) codes for a protein containing a "bipartite" distribution of charges which could be very important for its presumed MT cross-bridging activity and would allow the formation of "filaments" in the absence of microtubules (Fig.35). De Garcini and colleagues (1986) reported that porcine brain tau polymer formation was an enzyme-induced phenomenon resulting from a change in amino acids from glutamate to glutamic acid. Similarly, aggregates of both human brain and chicken erythrocyte tau were also observed (Montejo de Garcini and Avila, 1987; Lichtenberg-Kraag and Mandelkow, 1991). These tau aggregates were soluble in ionic detergents. In trying to understand the characteristics of the neurofibrillary tangle (NFT) tau two of their properties become important: their high state of phosphorylation, and their solubility state in certain detergents, such as SDS. Two different types of NFTs have been described so far: those that are soluble in ionic detergent and those that are insoluble (Lee et al., 1991). The PHF taus in soluble NFTs have been found to be highly phosphorylated. Most importantly these taus were found to contained a phosphorylated serine residue which is not phosphorylated in "normal" brain tau or non-PHF tau (Ueda et al., 1988).

Heat stable proteins obtained from salt extracts of dogfish cytoskeletons or from in vitro assembled MT bundles formed aggregates when desalted and concentrated. Formation of aggregates was inhibited by high salt conditions, which suggests that the nature of the tau molecular interaction during aggregate formation may be ionic. The inclusion of increasing concentrations of nucleotide triphosphates (GTP and ATP) during preparation of these heat stable proteins did not inhibit aggregate formation.

Phosphorylation has been shown to cause conformational changes in tau and could possibly result in epitope masking by the induction of SDS resistant domains (Hagestedt, et al., 1989). Whether phosphorylation of the partially purified tau protein took place during preparation remains to be determined.

Assembled MT bundles vs MBs

Some of the assembly properties of the MT protein from dogfish and chicken cytoskeletons were examined in relation to MT polymerization and bundling. In addition, the assembly properties studied involved tubulin protein from the polymerized pool only and thus the properties of MT protein in the MB were specifically examined as opposed to the whole cell MT protein. So far, this has been the only such study involving temperature cycling experiments with MT protein obtained from erythrocyte cytoskeletons. Other studies reported utilized MT protein obtained after

sonication of erythrocytes at low temperature and MT reassembly in the presence of the lysate (Murphy and Wallis, 1983 a,b; Centonze et al., 1986). The MT protein thus obtained contained proteins such as hemoglobin and was recycled several times to obtain a cleaner preparation. Those preparations contained total cell tubulin and associated proteins with very high affinity to the reassembled MTs. Such procedures do not allow identification of MT associated proteins which have low MT affinity during temperature-induced recycling and may be lost during several assembly-disassembly cycles. In contrast, the MT protein used in this study was obtained from cytoskeleton MBs and was essentially free of contaminants (Fig. 37,38,55).

The protein in low temperature extracts from dogfish cytoskeletons consisted primarily of tubulin plus several species cross-reacting on Western blots with antibodies to mammalian brain tau. The syncolin immunoreactive protein (290kD) remained in the cytoskeleton after low temperature disassembly of the MB. Some of the 290kD protein was extracted when 0.1M NaCl or 0.1M KCl was included during the low temperature incubation, but this protein was not required for MT bundle formation in vitro. Late gel filtration column fractions containing no 290kD were still able to form MT bundles (Fig.46). Rewarming of these MT protein preparations under conditions which allow tubulin polymerization induced MT assembly and the formation of

linear MT bundles. The assembled MT bundles consisted primarily of proteins in the 55 to 62kD region, mostly tubulin but also proteins of the tau family. Assembly of MT protein from dogfish cytoskeletons was followed by changes in turbidity at 350nm. In addition to MT assembly, bundling was examined by negative staining (TEM) of samples at different time intervals during temperature induced MT reassembly. MT bundles formed consisted of parallel MTs which resembled the MB. Both the mean MT length and number of MTs per bundle increased as a function of time. Conditions which affected MT assembly also had an effect on the extent of MT bundling. When polymerization conditions were optimal and MT assembly was the most rapid, thinner MT bundles were observed. The use of increasing concentrations of GTP during temperature-induced MT assembly resulted in a greater extent of polymerization, possibly by increasing the assembly competent tubulin pool. The MT bundles formed in such preparations appeared thinner than those resulting from MT assembly without the inclusion of GTP.

The presence of 0.1M NaCl during MT assembly had an inhibitory effect on nucleation. However, it did not seem to affect elongation as protein incorporation into polymers was fairly high in proportion to turbidity (350nm) compared with MT assembly in the absence of NaCl. In addition, the formation of MT bundles was not inhibited in the presence of 0.1M NaCl. It was observed that MT bundles formed even in the presence of 0.2M NaCl, indicating that the protein

involved in MT bundling has very high MT affinity. This is also suggested by the observation that MT protein temperature cycled three times was able to form MT bundles during reassembly.

The addition of antibodies against brain tau completely blocked MB microtubule protein polymerization. In contrast, antibodies against syncolin or actin had no effect on MT assembly or on bundle formation. The addition of taxol to MT protein after tau protein had been removed by immunoprecipitation resulted in single MTs, which suggests that tau plays a role not only in MT assembly but also in bundling. Controls for these experiments included antibodies pre-absorbed with protein A or with dogfish cytoskeleton preparations, and these had no effect on assembly or bundling.

MT bundles were seldom observed upon rewarming of chicken MT protein. The major tau protein obtained during low temperature extraction of chicken cytoskeletons was ~68kD. Since some MT bundling was observed at high protein concentrations, it is possible that MT preparations contained a very low concentration of the protein required for bundling. Thus, although the 68kD tau in chicken MT preparations has been shown to have MT assembly promoting activity, it did not show MT bundling activity (Murphy et al., 1986; Sanchez, unpub. obs.). The higher apparent molecular weight of the chicken tau (68kD) seems to be at least partially due to phosphorylation. Alkaline

phosphatase treatment of the cytoskeletons prior to low temperature extraction resulted in less, if any, 68kD in the low temperature extract as compared to the controls. However, a few bands of lower molecular weight (62-66kD) were seen in these phosphatase-treated samples. The shift in mobility of tau was not observed when acid phosphatase was used, suggesting that this phosphorylation may be site specific. The effect of phosphorylation on the migration of brain tau protein in SDS-PAGE gels has been studied by several laboratories (Steiner et al., 1990; Greenberg and Davies, 1990). Phosphorylation of tau at a serine residue in the carboxyl terminus is believed to result in SDS-resistant domains on tau, causing its slower migration in SDS-PAGE. No increase in bundling activity was observed after treatment with alkaline phosphatase in either dogfish or chicken MT protein preparations. That is, chicken MT protein was not induced to form bundles and the bundling activity of dogfish MT protein was not inhibited.

Detailed comparison of turbidity measurements of the assembly of dogfish and chicken MT protein preparations is complicated by the possible effects of bundling of MTs during or after assembly. Thus, the turbidity changes of chicken MT protein reflected only MT assembly while dogfish MT protein assembly included both MT assembly and bundling. Addition of MT seeds to chicken MT protein obtained by low temperature extraction of chicken cytoskeletons did not result in MT bundling, suggesting that although MTs were

present and MT assembly was taking place bundling of MTs was not induced.

The dogfish MT bundles assembled in vitro resembled MBs in some of their mechanical properties. For example, they showed flexibility in being able to bend and return to linearity without breaking when the coverslip was tapped (Fig. 45). This property would require both MT flexibility as well as a strong interaction between these MTs responsible for maintaining the structural integrity of the bundle. Physical interaction between MTs forming the bundles was also demonstrated by the MTs remaining bundled during flow, rigorous pipetting, or during dilution. MT bundles diluted to concentrations above their determined critical concentration retained the same turbidity at 350nm as well as the percent protein incorporated into polymers (Table II). This was particularly interesting as isolated MBs also show the same properties, i.e., the ability to remain intact when diluted. Although very few MT ends are observed in isolated MBs, the stability of MT bundles in which all the MT ends are exposed to the medium, strongly suggests that a stabilizing factor is bound to these MTs. As noted above, brain tau protein has been shown to have dose-dependent MT stabilizing properties, the mechanism of which seems to be the decrease of the MT disassembly rate (Job et al. 1985; Sloboda and Rosenbaum, 1979). The contribution of the bundled organization of MTs to stability of their MTs has not been examined to date in other

laboratories.

Tau role in MB structure

The structural and biochemical data obtained suggest that erythrocyte MB tau protein is involved in MT bundling in MBs, including cross-linking them. The spacing between MTs of the MB in whole cells is within the range of that reported by Hirokawa and colleagues (1988) for the mammalian brain MT bundles assembled in vitro and in MT bundles induced by tau cDNA transfection of fibroblasts and of SF9 cells (Hirokawa et al., 1989; Knops et al., 1991). The lack of visible MT cross-links in isolated MBs and in assembled MT bundles observed by conventional TEM would be expected if tau was present. As previously reported, low angle shadowing is necessary to visualize tau molecules due to their small diameter (Wille et al., 1992). In addition, partly purified dogfish MB tau binds to itself by forming filamentous aggregates. This has also been shown for brain tau and is one of the properties which would qualify it as a cross-linker, taking into consideration the length of tau (Montejo de Garcini and Avila 1987; Lichtenberg-Kraag and Mandelkow, 1990).

Limited subtilisin treatment of isolated MBs from dogfish and chicken erythrocytes resulted in the unbundling of MTs. The same was observed when the isolated MBs were treated with salt. Similarly, MT bundles assembled in vitro unbundled after exposure to high salt or subtilisin. The

unbundling of isolated MBs and of in vitro assembled MT bundles by exposure to high salt results from the removal of MAPs from MTs by different mechanisms. Subtilisin has been shown to cleave the COOH terminal of tubulin which is believed to be the tubulin domain to which tau binds (Serrano et al., 1985; Littauer et al., 1986; Maccioni et al., 1988). As mentioned in the Results section, MT unbundling also occurred in preparations not treated with taxol. In order to increase the stability of MTs after tau removal the preparations were treated with taxol. This MT stabilizing drug has been shown not to interfere with MAP binding to MTs (Vallee, 1982). In addition, the stabilizing activity of taxol is not inhibited by proteolysis of tubulin with subtilisin (Serrano et al., 1984). It has has been shown previously that salt can strip MAPs from taxol-stabilized MTs (Valle, 1982).

Exposure of dogfish isolated MBs to high salt resulted in unbundling of MTs and removal heat stable protein (~ 54kD) from MBs. This suggests that the 54kD may be involved in the bundled arrangement of MTs in the MB. Unbundling of MTs by exposure of MBs to salt or subtilisin resulted in relatively few MTs, which were very long. This suggests that MBs are be composed of a small number of MTs which encircle the circumference of the cell at least once. However, the specific number of MTs composing the MB is not clear according to these data (Fig.22), and may vary according to species and maybe even between different

erythrocytes of the same species.

Currently, there is controversy over the mechanisms by which MT bundles form in living cells. It has been proposed that MT bundling may occur either as a result of tau-induced MT stabilization or by actual physical cross-linking of MTs.

Tau proteins are thought to be essential during neurite outgrowth, with a role in MT polymerization and possibly also in establishing the polarity of axonal MTs (Heidemann et al., 1981; Drubin, et al., 1985; Caseres and Kosik 1990). Transfection of Sf9 cells with tau cDNA resulted in MT bundles with mostly uniform polarity (Knops et al., 1991; Baas et al., 1991). This indicates that there may be a very specific protein interaction if tau-induced bundling is due to tau-tau cross-bridging, as has been suggested (Hirokawa et al., 1988). A number of mechanisms have been proposed for MT cross-linking by tau. Several laboratories have tried to define tau domains responsible for MT bundling activity in living cells. Cowan et al., (1990) proposed that tau-tau interaction via the carboxy terminus is reponsible for MT bundling. In order to account for the observed spacing between MTs, either some of the repeat domain would have to be lifted away from the MT lattice or cross-bridging would require an additional protein interacting with MT bound tau.

Recently, the interaction of tau with MTs has been described as being mediated by tau MT binding domains having strong and weak MT affinity (Butner and Kirschner, 1991).

The first repeat has the strongest MT affinity as compared to the other repeats, thereby permitting the tau to pivot on the MT surface. These data on tau interaction with MTs would allow for MT cross-linking by tau-tau interaction at the tau carboxy terminus, as proposed by Lewis and Cowan (1989). However, transfection experiments with tau cDNAs containing three and four repeat resulted in MT bundles with the same spacing between MTs (Knops et al., 1991). Hirokawa and colleagues examined the spacing between MTs in Sf9 cells transfected with MAP2, MAP2C, and tau cDNA and observed that the spacing between MTs differed, indicating that the spacing is instead determined by the length of the projection domain (Chen et al., 1992). It is interesting to note that amino terminal fragments of tau synthesized by E. coli formed aggregates (Lee et al., 1989). This correlates with Hirokawa's proposal that the amino terminus of tau is essential for its MT bundling activity. Kanai et al., (1992) proposed that both the carboxy terminus and a neutral region near the amino terminus of tau are essential in MT bundling. Based on findings with their tau cDNA deletion mutants, they suggested that the carboxy terminus was involved in the stabilization of MTs while the amino terminus may be involved in cross-bridging. There is still controversy, however, on the contribution of MT stabilization to MT bundling. It has been proposed by Melki et al. (1991) that stabilization of MTs resulting by binding of tau fragments to MTs may result in MT bundling. They

proposed that the binding of factors such as MAPs results in the neutralization of those sites on the MT lattice thereby allowing ionic MT-MT interaction by bound and non-bound sites on the adjecent MT. Although the experiments by Kanai and colleagues suggested that the contribution of MT stabilization to MT bundling is very small, it has been very difficult to distinguish among MT stabilization, MT binding, and MT bundling activities, as all are properties of tau. Transfection experiments using tau cDNA with only the MT binding domain (without the projection domain) should produce MT binding and stabilization, and would help in determining the role of MT stabilization in bundling.

In this thesis, the specific mechanism by which bundling occurs in this system has not been determined. However, the data indicate that protein of the tau family is present in the assembled MT bundles and is an intrinsic component of the MBs. The immunoprecipitation and reconstitution experiments suggest that tau is not only involved in the assembly of these MTs but is also required for bundling. Reassembly of MT protein from dogfish cytoskeletons produced MT bundles in suspension only when tau was present during assembly. However, assembled single MTs incubated with tau did not form bundles. Thus, MT bundling seems to occur concommitant with assembly. This indicates that specific MT-MT interaction is required throughout assembly in order for tau mediated bundling to occur, which is also consistent with the fact that optimal

conditions for MT bundling involve slow MT assembly. Bundling may require not only physical proximity of MTs but also a specific parallel alignment. In this respect MT stability is not enough for MT bundle formation in suspension. This was also observed with taxol-treated preparations of assembled MTs from chicken MBs which remained as single MTs. As shown by Hirokawa et al. (1988), in vitro MT bundles assembled from phosphocellulose brain tubulin and tau in the presence and absence of 10μM taxol contained cross-linked parallel MTs (Hirokawa et al., 1988). However, when 20μM taxol was added during reassembly, cross-links were observed only on those MTs which ran parallel to each other, although tau seemed to have bound throughout the MTs. Possibly the rapid MT assembly due to a higher concentration of taxol resulted in less efficient bundling. The decrease in MT bundling observed with dogfish MT protein as the MT assembly rate increased correlates with this finding, indicating that MT assembly and tau induced MT bundling may be concurrent events. In addition, the lack of cross-bridges in non-parallel tau-saturated MTs observed by Hirokawa also stresses the specific physical MT-MT interaction required for bundling. In preparations in which tau was added to pre-assembled single dogfish MTs, fewer bundles would be expected to form if a specific parallel alignment of MTs was necessary, as was observed.

As shown in this study, the involvement of tau in MT bundling is concurrent with MT assembly, thereby

complicating the interpretation of studies by other laboratories examining the role of different tau isotypes in MT bundling in vivo (Kanai et al., 1991; Knops et al., 1991). Although an increase in tubulin polymerization in cells after tau cDNA transfection has been observed by several laboratories, the possible correlation between MT assembly and bundling activity of tau has not been considered. Thus, the study of the MT bundling activity of tau in these cells may be complicated by the MT assembly promoting activity of the tau introduced into cells by transfection.

Feick and coworkers (1991) obtained MT bundles from chicken erythrocyte taxol-assembled MTs and purified syncolin. In these preparations syncolin was obtained from taxol-treated MTs simply by incubation at low temperature, and its solubilization did not require MT disassembly. This suggests that in the whole cytoskeleton, syncolin binds to MTs with fairly low affinity, and its redistribution in the cytoskeleton during low temperature induced MB disassembly may be a result of altered binding properties during low temperature exposure. Unlike the dogfish MT bundles obtained in this study, the MT bundles obtained by Feick and co-workers did not seem to require concurrent MT assembly. In addition, in the present work, globular protein bridges between MTs were not observed in whole cytoskeletons, in isolated MBs, or in the in vitro assembled MT bundles from either dogfish or chicken

preparations. Rather, according to the data, syncolin is more likely present at the MB/MS interface and may possibly play a role in the consolidation of the MB at the plane of flattening during MB biogenesis. Studies on MT cross-linking factors in Bufo marinus erythrocytes revealed a high molecular weight protein, immunologically related to MAP-2, which induced the formation of MT networks, but not parallel bundled MTs (Centonze et al., 1986). Unlike previous studies, the dogfish MT bundles were assembled from MT protein obtained directly by low temperature disassembly of the MB. These MT bundles resemble the MB with respect to its mechanical properties and protein composition. Both the MT bundles and isolated MBs contain tubulin and protein of the tau family.

It has been shown previously that thin MBs are composed primariry of MTs with uniform polarity, while those MBs with many MTs contain two or more separate clusters of opposite polarities (Euteneuer et al., 1985). The data support the model of MB biogenesis proposed by Cohen and coworkers by suggesting that the MB forms from one or two MT bundles which encircle the circumference of the cell (Nemhauser et al., 1983). The existing data on tau-induced MT bundles in neurons, which includes ~20nm spacing between MTs, uniform MT polarity (Baas et al.,1991), and ability to induce morphological changes (Knops et al., 1991), are consistent with the structural and mechanical properties of erythrocyte MBs.

Summary of results and conclusions:

In this thesis I sought to examine the protein composition and structure of isolated MBs, and of MT bundles assembled in vitro, focusing on MAPs. The following are the major results and conclusions.

1) MBs can be isolated from nucleated erythrocytes of various species by a non-proteolytic method utilizing detergent mixtures.

2) The major protein components of isolated MBs are tubulin and protein of the tau family.

3) A high molecular weight protein, immunologically related to syncolin, is not an intrinsic component of the MB, and is present in a specialized "ring" at the MB/MS interface.

4) F-actin is also localized at the specialized MB/MS region and is not intrinsic to the MB.

5) The specialized "ring" containing actin, spectrin, syncolin (and probably ezrin) may be responsible for maintaining the elliptical shape of mature erythrocytes.

6) MB tau is immunologically and biochemically similar to mammalian brain tau.

7) MT protein obtained by low temperature extraction of cytoskeletons and isolated MBs form MT bundles upon rewarming.

8) The MT bundles assembled in vitro exhibited similar mechanical properties to those of MBs.

9) MB tau is necessary for MT assembly and bundle formation in vitro.

10) In vitro MT bundling of dogfish MB protein requires the presence of tau during MT assembly; MB tau does not induce bundling when added to single MTs.

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