

IMMUNOLOGICAL APPROACHES TO FLAGELLAR MOVEMENT

Thesis by

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This thesis is dedicated to My Parents, to whom I say:

You must remember this, a kiss is still a kiss,

A sigh is just a sigh;

The fundamental things apply, As time goes by.

--Herman Hupfeld

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ABSTRACT

This thesis summarizes attempts in our laboratory to use antibodies to study flagellar motility. After preliminary work on the effects of antibodies to intact dynein 1 and a tryptic fragment of dynein 1, a thorough study of antibodies against outer doublet tubulin has been made.

Both anti-dynein 1 and anti-fragment 1A inhibit movement-coupled ATP dephosphorylation and the movement of reactivated Strongylocentrotus purpuratus spermatozoa. Anti-fragment 1A, which also inhibits the ATPase activity of isolated dynein, inhibits the frequency and bend angle of the flagella. Anti-dynein 1 has a relatively smaller effect on beat frequency and is inhibitory of bend angle. These results suggest that binding to dynein is not, by itself, sufficient to strongly inhibit movement and that a more specific interference with ATPase activity produces more severe effects on movement.

Antibodies to tubulin were purified from rabbit sera by tubulin-affinity chromatography. Four induced anti-tubulins from immune sera and four spontaneous anti-tubulins from the corresponding preimmune sera were isolated and characterized. Both induced and spontaneous anti-tubulins are of the IgG class of antibody. Both are specific for tubulin when presented with a crude mixture of axonemal proteins. Only induced anti-tubulin precipitates tubulin in immunodiffusion assays. Both anti-tubulins as well as their monovalent Fab fragments bind to sea urchin axonemes in a radiobinding assay. Both stain demembranated sperm flagella in indirect immunofluorescence but only induced anti-tubulin decorates chick fibroblast cytoplasmic microtubules. I conclude that the affinity-purified antibodies are specific for tubulin and are able to bind sea urchin sperm flagella.

Induced anti-tubulins from four different immunizations all specifically reduce bend angle and symmetry of reactivated flagella without affecting beat

frequency. At identical concentrations, spontaneous anti-tubulins, and immune and preimmune Fab fragments have no effect on any of the parameters of flagellar movement. The ATP-mediated disintegration of elastase-digested axonemes is not prevented by concentrations of induced anti-tubulin which are more than sufficient to paralyze reactivated spermatozoa, but the ATP concentration threshold for sliding is raised. Under similar conditions, anti-fragment 1A has been shown to completely inhibit microtubule sliding. Induced anti-tubulin behaves very similarly to CO₂ in its movement-inhibitory activity.

These three antibodies all inhibit the bend angle of the reactivated flagella; they represent the first reports of amplitude inhibition by reagents known to be specific for the components responsible for active sliding. The specific bend angle inhibition and lack of effect on sliding by induced anti-tubulin is consistent with a microtubule conformational control of cross-bridge activity as a mechanism controlling the bending of the flagellum. The induced anti-tubulin effect is the first report of an antibody to tubulin having an inhibitory activity on microtubule-associated movement.

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".....I can start my paper with observation."

Doc bought a package of yellow pads and two dozen pencils. He laid them out on his desk, the pencils sharpened to needle points and lined up like yellow soldiers. At the top of a page he printed: OBSERVATIONS AND SPECULATIONS. His pencil point broke. He took up another and drew lace around the O and the B, made a block letter of the S and put fish hooks on each end. His ankle itched. He rolled down his sock and scratched, and that made his ear itch. "Someone's talking about me," he said and looked at the yellow pad. He wondered whether he had fed the cotton rats. It is easy to forget when you're thinking.

.....His pencil point broke. He took another, and it broke with a jerk, making a little tear in the paper. He read what he had written; dull, desiccated, he thought. Why should I presume that an animal so far removed from the human--perhaps I'm fooling myself.....

Doc put his face in the palm of his hand and pressed blackness on his eyes until specks of green and red light swarmed on his vision. And then he got up and went across the street for beer.

--John Steinbeck, Sweet Thursday

PREFACE

Perhaps the single, most striking property of living organisms is their ability to move. Motility takes on many aspects: many bacteria are able to propel themselves and respond chemotactically via rotating flagella; eukaryotic cilia and flagella move Paramecia and Chlamydomonas as well as bronchial fluids and spermatozoa; striated and smooth muscles perform their tasks by sliding filaments; eukaryotic chromosomes are pulled apart by the mitotic spindle; intracellular movement of organelles and granules is apparent in the orderly secretion of molecules as well as in cytoplasmic streaming; and many events on the surfaces of lymphocytes and other cell types are modulated by cytoskeletal, transmembrane-mediated movement.

In recent years at least three completely different mechanisms which operate to make different organisms move have been identified. Prokaryotic flagella are made of flagellin polymers forming stiff, helical propellers which are rotated, perhaps by an ion pump (1, 8). Muscle contraction is mediated by actin thin filaments interacting with myosin thick filaments and other proteins (6, 7). Eukaryotic flagella and cilia move via active microtubule sliding (3, 14). Microtubules also constitute at least the major structure in the mitotic spindle; chromosome movement may be a result of flagella-type microtubule sliding (10) or of some dynamic directional assembly-disassembly of the spindles (9). Circumstantial evidence indicates that microtubules are also involved in intracellular particle movement and secretion (11-13). Maintenance and alteration of cell shape, movement of surface molecules, and spreading of cells in culture (2, 4, 5, 15-18) apparently involve actin microfilaments, intermediate filaments, microtubules, and probably myosin. Despite the complete non-relatedness of flagellin, actin, and tubulin, it is interesting that all three motile systems employ proteinaceous

filamentous structures in conjunction with energy-transducing proteins (prokaryotic flagella "motor," myosin, and dynein) to effect movement.

This thesis represents an exploration of immunological approaches to understanding the motility of eukaryotic flagella. The first chapter will review microtubules and antibodies to tubulin. The second chapter will review flagellar movement, pointing out some of the questions which are still to be answered in order to understand flagellar motility.

Chapter 3 summarizes some information on dynein ATPases derived from biochemical and immunological studies. The major portion of Chapter 3 is taken from a published manuscript (Ogawa, K., D. J. Asai, and C. J. Brokaw, J. Cell Biol. **73**: 182-192, 1977) which reports our work with an antibody to intact dynein 1 and anti-fragment 1A to study flagellar movement as it relates to dynein ATPase activity.

After anti-dynein 1, attention was turned to making antibodies to sea urchin sperm flagellar tubulin. The isolation of tubulin, production of antisera, purification of anti-tubulins from immune sera as well as preimmune sera, the characterization of these antibodies, and the demonstration of their binding to axonemes are presented in Chapter 4. The effects of these preimmune spontaneous anti-tubulins and immune-induced anti-tubulins on reactivated spermatozoan motility and microtubule sliding are reported in Chapter 5.

Chapter 6 summarizes the results of my studies with antibodies to flagellar proteins and discusses these results in the context of flagellar mechanisms controlling motility.

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Chapter 1

ANTIBODIES TO TUBULIN: A Literature Review

Microtubules (98) are characteristic structural components of eukaryotic cells. The structure, function, and assembly of microtubules have been the subject of several reviews (17, 29, 52, 77, 84, 92, 99, 101, 128). Microtubules have been implicated in a variety of functions ranging from the determination of cell morphology and cell membrane specializations to motile phenomena such as axoplasmic transport and flagellar motility. These functions have not been shown to involve any active changes in microtubule conformation such as bending or contracting, and generally appear to involve either the processes of assembly and disassembly at the ends of microtubules or the action of accessory proteins such as the ciliary and flagellar dynein ATPases that are responsible for the sliding displacements between adjacent microtubules which drive the bending movements of cilia and flagella.

The common subunit protein of microtubules has been named tubulin (76); the functional subunit is actually a dimer of α - and β -tubulins which are related but not identical (4, 18, 22, 33). In spite of the great variety of functions of microtubules in eukaryotic cells, there is remarkably little diversity in microtubule substructure or in the amino acid compositions and primary amino acid sequences of tubulins from various sources (67). Tubulin was apparently present very early in the evolution of eukaryotic cells and has been highly conserved. However, the study of tubulin diversity is not yet well-developed. Nothing is yet known about the possible functional significance of the minor sequence variations which have been reported, the actual number of tubulin genes available in a given organism, or the mechanisms which control the disposition of tubulin variants within an individual cell.

Several techniques have been of particular importance in providing our present knowledge of microtubules. Microtubules were initially identified by

electron microscopy, and, with continually improving techniques, this has been an important source of information about the existence, localization, and detailed structure of microtubules. This method is particularly useful to study microtubules in concentrated arrays or well-defined structures such as cilia and flagella, but is less useful for understanding the role of less-ordered microtubules in the cytoplasm of cells.

Major advances in tubulin biochemistry followed the discovery that the mitotic poisons--drugs such as colchicine which cause the disruption of microtubules--bind strongly to tubulin subunits in solution, so that radioactively labeled colchicine can be used to identify and quantify tubulin in cell extracts (11, 104, 125, 126). Subsequently, as tubulin became more familiar, its appearance in a characteristic position following SDS polyacrylamide gel electrophoresis (SDS PAGE) has become a common method for preliminary identification of tubulin.

Procedures have been developed for the controlled in vitro polymerization and depolymerization of microtubules, first from material from tubulin-rich tissue such as fetal brain and, more recently, from other sources such as cultured cells, platelets, and sperm flagella (10, 34, 35, 62, 69, 70, 96, 110, 111, 122). These in vitro techniques have defined several important factors inducing assembly or disassembly, have provided a rapid purification procedure for tubulin, and have identified several microtubule-associated proteins (MAPs) which may be involved in the polymerization process. The ability for cellular material to co-assemble with microtubule fragments has served to identify tubulin (7, 9, 61, 68). It remains unclear as to how in vitro polymerization relates to the events in situ and the real functions of the MAPs remain to be determined.

The development of these procedures for tubulin purification has made possible another, newer method for the identification and localization of microtubules

and tubulin, i.e., the use of antibodies directed against tubulin. If properly prepared, anti-tubulins are potentially more specific than the drugs which bind to tubulin (38, 65, 75, 100, 105), and will interact with stable microtubules such as flagellar outer doublet microtubules, which do not interact with colchicine. Anti-tubulins can also be incorporated into immunofluorescent methodology which provides a much more sensitive detection of microtubules in cell cytoplasm than traditional transmission electron microscopy. These advantages have prompted a dramatic proliferation of work with antibodies against tubulin, which I will attempt to review in this Chapter.

Antibodies to tubulin

Since 1970 (through 1978), at least 82 reports have been published in which antibodies to tubulin were utilized. Since that first report in 1970 (81), the 82 papers represent, to my best deduction, 46 different antisera. Information about these antisera is summarized in Table 1-1. The popularity of this technique has been explosive--of the 82 papers, 20 were published between 1970-1975, 17 in 1976, 19 in 1977, and 26 in 1978.

The attractiveness of this technique is easy to understand. With appropriate tricks, the mammalian immune system can be exploited to obtain a specific probe for virtually any antigen. Assuming antibody purity and specificity, small amounts of anti-tubulin can be used without serious erroneous interpretation to identify structure, assign function, determine cross-reactivity, and measure synthesis of tubulin and the assembly of microtubules. In order to ensure specificity, two approaches can be taken, often in tandem: the antigen must be pure and the resulting antiserum can be fractionated for its anti-tubulin activity. As Table 1-1 shows, the literature reports a wide range of preparations, and some of the anti-tubulins are probably more specific than others.

Tubulin as an antigen

The source of tubulin most prevalently used as antigen has been brain tissue, both mammalian (29 antisera) and avian (5 antisera). Brain tubulin has commonly been purified by repeated cycles of in vitro polymerization and depolymerization usually followed by sieving chromatography to remove the high molecular weight MAPs. Brain tubulin has also been purified by the DEAE cellulose batch procedure (32, 123). One could argue that the repeated polymerization procedure is preferable because it yields "functional" tubulin capable of assembly.

Even though a substantial portion of the protein of brain tissue is tubulin (106), other molecules may co-purify with tubulin. These contaminants might include proteins which naturally associate with microtubules, such as the MAPs, as well as proteins which share tubulin's biochemical properties. Some of the high molecular weight contaminants might be removed if sieving chromatography is employed but the lower molecular weight MAPs (including tau) as well as actin--another ubiquitous eukaryotic protein, which is involved in a multiplicity of interactions with other proteins (47)--might remain with the tubulin. Therefore, the additional step of preparative SDS PAGE has often been employed to purify tubulins from brain as well as other sources. Typically, a partially purified tubulin preparation is one-dimensionally electrophoresed, the gels are stained for protein, and the bands corresponding to tubulin cut out. Elution in high yields from the acrylamide can be accomplished electrophoretically or by homogenization and extraction (63). This procedure should allow for the removal of MAPs, actin, and other contaminants. Furthermore, it is possible to electrophoretically separate α - and β -tubulins and immunize with the purified monomers as Piperno and Luck succeeded in doing (A-25, ref. 91). Examples of antisera which employed careful electrophoretic purification of tubulin antigen as well as affinity-purification of the resulting

antisera include A-15, 26, 36, and A-43. Several other antisera resulted from immunization with electrophoretically purified tubulin; these are noted in Table 1-1.

Vinblastine paracrystallization of tubulin has also been used as a purification step for tubulin from brain and other tissue. This yields insoluble aggregates which are immunogenic. However, vinblastine has also been shown to precipitate other, non-tubulin, molecules. One of the earliest reports of anti-tubulin antibodies (A-1, ref. 81) employed as an antigen vinblastine precipitates which not only had colchicine-binding activity but also contained ATPase activity. Vinblastine paracrystallization is probably not sufficient to satisfactorily purify tubulins but could be used in conjunction with other steps to ensure antigen purity, or with later antiserum absorptions.

Besides brain tissue, four antisera have been raised against mammalian tissue culture cell tubulin and mammalian platelet tubulin. The platelet tubulins were purified by polymerization cycles (54). In addition, several "lower" species have been utilized as sources of tubulins; one antiserum has been prepared against sea urchin egg cytoplasmic tubulin, and seven antisera have been prepared against tubulin from cilia and flagella from Tetrahymena, Naegleria, and sea urchin spermatozoa. Several of these studies employed SDS PAGE as a purification step.

Immunization procedures

Many immunization protocols, regardless of the antigen, attempt to irritate the recipient animal's immune system, usually using adjuvants and by injecting in places directly accessible to the lymphatic system. Emulsification with adjuvant until an extremely viscous suspension is obtained is usually recommended. All of the tubulin immunization schemes employ this strategy of irritation.

In addition, many workers attempting to elicit an anti-tubulin response have gone to extra steps to increase antigenicity. These extra steps are considered

necessary because of the ubiquity of tubulin and the high degree of structural conservation among tubulins from a wide range of species. Unfortunately, reports of unsuccessful immunizations are not often published, so the necessity of these extra steps is not really known. Many of the successful immunizations have used mammalian tubulin injected into another mammal, even though it would be expected that their tubulins are virtually identical. Despite assumptions about immunogenicity, no systematic study has been noted in the literature to investigate the necessary degree of dissimilarity for a positive anti-tubulin response.

The most common method of increasing antigenicity has been to denature the tubulin either with SDS or by cross-linking it with glutaraldehyde or by both methods together. When SDS is used, it is difficult to obtain a good emulsion with adjuvant; however, partially emulsified material elicits an immune response. A common way of introducing SDS denaturation is by purifying tubulin on SDS PAGE and eluting the tubulin from the acrylamide in SDS. Most of the acrylamide is assumed to be removed prior to immunization, as antibodies to acrylamide would possibly complicate subsequent interpretations (82).

Despite denaturation of antigen prior to immunization, the resulting antisera yield anti-tubulins capable of recognizing tubulin in its native conformation. This is similar to other situations where SDS apparently enhances antigenicity of already exposed determinants in the native conformation (63).

Tubulin has also been conjugated to alum or to methylated BSA or injected in a slurry with DEAE cellulose beads or has been paracrystallized with vinblastine. All of these procedures have worked to give a positive response but vinblastine precipitation by itself, as already noted, is not sufficient to assure specificity. The various treatments of tubulins prior to immunization are included in Table 1-1.

Gozes et al. (49) attempted a modest study employing a microhemagglutination technique using formalinized sheep red blood cells in order to test the antigenicity of different tubulin preparations. They found that the rabbit sera titers were not significantly different among those elicited (all at 2 mg/ml, repeated injections) with calf brain tubulin plus adjuvant, calf brain tubulin conjugated with methylated BSA plus adjuvant, and calf brain tubulin conjugated with DNP plus adjuvant. Although it is difficult to control this sort of study (since different rabbits respond differently), these results suggest that the altered conformation of the tubulins, either by conjugating tubulin to an immunogenic carrier protein such as BSA or by conjugating an immunogenic hapten, DNP, to tubulin, does not appreciably increase its antigenicity.

In most cases rabbits have been used as recipients. Two antisera produced in goats and one in sheep have also been reported--they do not appear to be different from rabbit antisera in terms of their anti-tubulin activity. Most immunizations utilized complete Freund's adjuvant initially and many procedures have also used complete or incomplete Freund's in the booster injections. Many workers opted for large quantities of antigen (e.g., A-24, ref. 129); some have used up to 50 mg of tubulin in a single injection. Anti-tubulin antibodies have also been elicited via small quantities of immunogen (a few hundred micrograms). There is no apparent correlation between success of immunization and the protocols used, nor is it possible to evaluate different procedures in terms of the resulting anti-tubulin activities.

Affinity purification of antisera

Several workers (A-10, 15, 18, 26, 30, 34-36, 43, 45) have employed affinity chromatography as a method of purifying anti-tubulin antibodies. Regardless of the apparent purity of the antigen, it is often useful to maximize purity of the antiserum by absorption.

The technique of affinity chromatography depends on a subpopulation of antibodies recognizing the immobilized antigen, and allows one set of antibodies of interest to be isolated from a mixture of antibodies recognizing several distinct antigens. This technique is dependent on the quantity and purity of the tubulin that is coupled to the column and is usually unable to retain all of the anti-tubulin specificities contained in the serum (e.g., SDS-denatured tubulin used as an antigen might elicit responses to determinants not present in the native conformation tubulin coupled to the column). A drawback to this method is that only a small fraction of the antibody is absorbed to the column, so the yield of purified antibody is low.

A few reports refer to "monospecific" anti-tubulins (e.g., A-10, ref. 43) produced by affinity chromatography. The term "monospecific" in these cases might be misleading in that there is no guarantee that the purified antibodies all recognize only one determinant. A theoretical treatment of multi-specific antiserum fractionation by affinity chromatography (31) reveals that, despite absolute absorbent purity, multiple specificities of antibody can be absorbed and subsequently eluted. True monospecificity is more likely to be achieved by elution with specific tubulin peptides or by developing monoclonal anti-tubulin antibodies.

Characteristics of anti-tubulins

Many of the anti-tubulin antibodies have been shown to precipitate tubulin in immunodiffusion or in crossed immunoelectrophoresis assays. In some cases, anti-tubulins were detected by immunofluorescence (A-36, ref. 30) or by radio-immune assay (A-29, ref. 80), but did not precipitate tubulin. Lack of the ability to precipitate tubulin could be due to an insufficient titer of antibody or to the production of a non-precipitating class of immunoglobulin. All indications, from those papers in which the appropriate experiment was performed, point to the

elicitation of IgG as at least part of the anti-tubulin response--the presence of IgG anti-tubulin is implied from the successful use of anti-IgG as a second antibody in radioimmune assays and in indirect immunofluorescence, the ability of Staphylococcus aureus protein A to precipitate anti-tubulin activity, and the direct demonstration of IgG by immunoelectrophoresis and diffusion against the appropriate antisera. Generally, the immune response to tubulin has been reported as low when compared to the response commonly elicited against other antigens; most workers have not, however, reported an actual anti-tubulin titer of their sera.

The anti-tubulins elicited against one species' tubulin always, in those reports which tested this, cross-reacted with at least some other species' tubulins. Rarely has it been reported that an anti-tubulin did not cross-react with every tubulin presented it; the rare example is A-2 which was shown by Ouchterlony precipitation assays to react with sperm flagella tubulin from five sea urchin species and a sand dollar, but did not react with starfish sperm flagella tubulin (44). Cross-reaction has been demonstrated by immunodiffusion, immunofluorescence, and radioimmune assays. The inter-species cross-reactivity is often phylogenetically extensive and seems to provide evidence for the highly conserved structure of tubulins. For example, A-1 produced against mouse cytoplasmic tubulin cross-reacted with vinblastine-precipitated tubulins from HeLa cells, chicken, monkey, mouse, turtle, iguana, gekko, viper, fish, mosquito, and moth. A-18 produced against porcine brain tubulin also reacted, by immunofluorescence, with mouse 3T3 cells, bull sperm, PtK2 cells, HeLa cells, human sperm, rat sperm, newt sperm, urchin sperm, and Tetrahymena cilia. A-21 made against sea urchin egg cytoplasmic tubulin cross-reacted with tubulins from PtK2 cells, salamander, sea star, and mouse. A-26, produced in a rabbit against rat brain tubulin, reacted with brain tubulins from pig and rabbit.

A different view of the response to tubulin

In one sense successful immunization with tubulin is paradoxical. Tubulins from different species are structurally highly conserved; bovine brain tubulin, for example, would be expected to be virtually identical to rabbit tubulin. Nevertheless, mammalian brain tubulins, as well as tubulins from non-mammalian sources, elicit positive immune responses in rabbits. Why should the rabbit respond to a substance presumably nearly identical to its own tubulin? Why isn't it tolerant to tubulin? Although tubulin is often denatured prior to immunization, the rabbit nevertheless makes antibody recognizing native conformations of tubulin. This general phenomenon has been widely recognized in the field of immunology for a number of years (71a).

This anomaly is furthered by the extensive cross-reactivity of the elicited anti-tubulin antibodies. Cross-reactivity is another argument for the highly conserved structure of tubulins; however, cross-reactivity also means that the antibody is able to recognize a wide range of tubulins and again one wonders why the rabbit is capable of response at all. In fact, as mentioned above, rabbit anti-serum to rat brain tubulin was shown to also react with rabbit brain tubulin (A-26, refs. 55, 56).

None of the reports of anti-tubulins noted any autoimmune-like dysfunction caused by the immunization. In my hands, all five rabbits successfully immunized with tubulin continued to behave normally for more than two years after immunization and after repeated booster injections.

The ability of a rabbit to respond immunologically to tubulin might be partially explained if the rabbit's microtubules and soluble tubulin pools are never exposed to its immune system and could therefore be regarded as immunological "privileged sites." In contrast to experimental autoimmune myasthenia gravis

which can be induced in rats and rabbits by injection of non-homologous acetylcholine receptors (2, 51), tubulin immunization does not result in any apparent breakdown of microtubule-containing tissue (e.g., peripheral neuronal tissue) in the recipient animal.

Further insight is provided by the finding that normal, nonimmune sera from several different mammals (rabbit, pig, cattle, human) contain low levels of non-precipitating anti-tubulin antibodies (57). These antibodies can be isolated by affinity chromatography and have been shown to be of the IgG class. They are able to recognize mammalian tubulins. The natural presence of these antibodies may be related to the general difficulty in raising high titers of anti-tubulins, if the animals are already making a secondary response to tubulin. Also, with these spontaneous anti-tubulins already circulating, perhaps there is a mechanism to clear any exposed tubulin resulting from tissue damage, thereby circumventing an autoimmune dysfunction upon immunization. This would imply that all tubulins, even the rabbit's tubulin, would remain "foreign" and antigenic and could explain why the rabbit makes anti-tubulins capable of cross-reacting with rabbit tubulin without any apparent autoimmune problem. It remains to be determined how the animal is initially exposed to tubulin and why the spontaneous antibodies appear to be a low-level secondary response.

Applications of anti-tubulin antibodies

The most prevalent application of anti-tubulin antibodies has been to visualize microtubule structures in a large variety of cells. Immunofluorescence of fixed cells using fluorescein-conjugated anti-tubulins or untreated anti-tubulins followed by conjugated anti-IgG, and electron microscopy utilizing ferritin-conjugated anti-tubulins or peroxidase-anti-peroxidase techniques have been used in this application. These studies and others with anti-actin antibodies have shown arrays

of filaments in the cytoplasm and have revolutionized the conception of the cytoskeleton. Several reports compared the organization of cytoplasmic microtubules in normal and transformed cells. Depending on the cell line used, microtubules are either disrupted or unaffected by transformation. Other reports appear to be mainly a cataloguing of cell types and the presence and appearance of microtubules. Antisera used in the visualization of microtubules include: A-1, 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 32, 33, 36, 44, 45, 46.

The second application of these antibodies has been to assay for the presence of tubulin-like material in several organisms. Competitive radioimmune assays, immunofluorescence, and immunodiffusion assays have been used to identify cross-reacting material. Margulis et al. used anti-tubulins to argue for the presence of microtubules in spirochetes residing in the termite hindgut (A-10 and A-21, ref. 71). Antibodies to tubulin have been used to detect the presence of tubulin in pigeon erythrocyte membranes (A-24, ref. 129), Dictyostelium (A-37, ref. 20), yeast extracts (A-38, ref. 3) and soybean seedlings (A-39, ref. 58). Bibring and Baxandall used A-5 (8) to show that meralluride extracts the mitotic apparatus, and Twomey and Samson identified tubulin associated with synaptic vesicles and synaptosomes (A-4, ref. 109).

A third and related application of antibodies to tubulin has been the development of immunoassays (radioimmune assays, complement fixation, hemagglutination of red blood cells, and immunofluorescence) to compare the tubulins from a range of cells and organisms. The general cross-reactivity of these sera has been discussed earlier. Studies of this sort have shown that flagellar, cytoplasmic, and spindle tubulins are similar (e.g., A-18). Competitive radioimmune assays (A-29, ref. 80) showed that tubulins from lamb, mouse, and chick brains

could be immunologically distinguished, arguing for different densities of cross-reactive determinants on each species' tubulin and a unique determinant on chick tubulin.

A fourth application of anti-tubulin antibody has been elucidating some of the mechanisms of lymphocyte capping. Yahara and Kakimoto-Sameshima (A-43, ref. 127) and Gabbiani et al. (A-30, ref. 46) have demonstrated by immunofluorescence that in lymphocyte capping induced by anti-surface immunoglobulin, cytoplasmic microtubules are concomitantly rearranged. Despite possible artifactual problems inherent in fixation and immunofluorescence, this approach of directly visualizing cytoplasmic elements during surface modulation is promising and avoids some of the problems of non-specificity in experiments relying on drug or temperature effects.

A fifth application has been the study of the synthesis of tubulin during stages of cellular development. Fulton et al. showed by radioimmune assay and pulse-chase experiments that during flagellar differentiation in Naegleria, 97% of the tubulin appears to be newly synthesized (A-8, ref. 45). Piperno and Luck used "rocket" crossed immunoelectrophoresis to measure cytoplasmic tubulin pools during the Chlamydomonas cell cycle (A-25, ref. 91). Tamura measured by immunofluorescence and immunoprecipitation the synthesis and assembly of tubulin in Tetrahymena organelles (A-3, ref. 103).

A sixth use of these antibodies has been to probe the assembly of microtubules. Meier and Jørgensen made antibodies to assembled microtubules and showed by crossed immunoelectrophoresis that their antiserum contained a mixture of antibodies to three determinants--one apparently against a high molecular weight MAP, one recognizing an apparent tau MAP, and the third reacting with tubulin (A-31, ref. 73). Morgan et al. found that their antibody to tubulin inhibited

microtubule polymerization and that this inhibition was independent of MAP concentration (A-42, ref. 79). This result implies that the critical step in microtubule polymerization is the coming together of tubulin subunits without the involvement of MAPs.

Especially in light of the recent observations of spontaneous anti-tubulin antibodies (57), it is unfortunate that most of the studies using anti-tubulins failed to use preimmune serum controls, and that in the few cases where preimmune serum was included, the spontaneous antibodies to tubulin were not purified prior to their use. The inclusion of properly prepared preimmune antibodies is especially useful to control immunofluorescent visualization and radioimmune assays.

At first glance, it may seem strange that in spite of the many situations where microtubules are known to participate in motile phenomena, anti-tubulins have never been used to investigate these phenomena. However, an alteration of motility by anti-tubulin primarily indicates the involvement of microtubules in the motile mechanism, and in most cases this is already known. Of greater value would be ways to use anti-tubulins to provide information about how microtubules function in motility. This type of study will require more detailed measurement of the way in which motility is altered in the presence of anti-tubulins.

In summary, the bulk of the literature on the applications of antibodies to tubulin has dealt with the visualization of microtubules. This application has been of some interest in the comparison of normal and transformed phenotypes and in the elucidation of cytoskeletal elements. Perhaps of more interest has been the use of these antibodies to study the phylogenetic structural relationships of tubulins and the synthesis and assembly of microtubules during development. The use of antibodies to tubulin will continue to develop into a powerful technique taking advantage of the potential specificity of the antibodies.

Table 1-1. Summary of published work with antibodies to tubulin. Abbreviations used: alum, tubulin is mixed with alum prior to injection; batch, purification of brain tubulin without using polymerization; colch bind, colchicine binding assay; DEAE, tubulin is mixed with DEAE slurry prior to injection; EM-Fe, immunoferritin electron microscopy; EM-PAP, peroxidase-anti-peroxidase electron microscopy; glut, tubulin is cross-linked with glutaraldehyde prior to injection; IEP, immunoelectrophoresis; If, immunofluorescence; Immcept, immunoprecipitation; MeBSA, tubulin is conjugated to methylated bovine serum albumin prior to injection; micro C', microcomplement fixation assay; OD, outer doublets; ouch, Ouchterlony immunodiffusion assay; polym, tubulin is purified by cycles of polymerization-depolymerization; RIA, radioimmune assay; SDS, tubulin is dissolved in sodium dodecyl sulfate prior to injection; SDS-PAGE, tubulin is purified by SDS gel electrophoresis; SU, sea urchin; tub, tubulin; vin, tubulin is paracrystallized with vinblastine prior to injection.

Table 1-1

Summary of Published Work with Antibodies to Tubulin

Antiserum	Antigen source	Procedures	Applications	Notes	References
<u>1970</u>					
A-1	mouse L cells	vin	If, EM-Fe		23, 24, 81
<u>1971</u>					
A-2	SU sperm OD		ouch		44
A-3	<u>Tetrahymena</u> cilia	SDS-PAGE	If, Immpept		102, 103
A-4	bovine brain	batch, Vin	micro C'		94, 109
A-5	SU sperm flagella		If, ouch, IEP		8, 107, 118
<u>1972</u>					
A-6	mouse tumor		ouch		28
A-7	rat brain	batch	ouch		28
<u>1974</u>					
A-8	<u>Naegleria</u> flagella		RIA		45, 59, 60
A-9	rat brain	batch, vin	micro C'		108

1975

A-10 bovine brain polym, glut If affinity purified 5, 6, 12
13, 14, 15,
40, 42, 43, 72

A-11 SU sperm OD glut If 116, 118

A-12 bovine brain polym If 21

A-13 porcine platelet polym If 21

1976

A-14 porcine brain polym, SDS-PAGE If 19, 63

A-15 rat brain polym, SDS-PAGE EM-PAP affinity purified 25, 26, 27
39, 93, 121 22

A-16 porcine brain polym If 16

A-17 chick brain polym, SDS-PAGE If

A-18 porcine brain polym, glut If, EM affinity purified 37, 83, 85,
86, 87, 88,
89, 90, 114,
115, 117, 119,
120

A-19 mouse brain polym, SDS-PAGE If, micro C' 124

A-20 chick brain batch, SDS-PAGE coleh bind, IF 1, 36

A-21 SU egg vin If 41, 95, 113

A-22 porcine brain polym, glut, DEAE If, EM-PAP 74

A-23 human platelet polym tub purif. 54

129

RIA

polym, SDS

bovine brain

A-24

1977

91

crossed IEP

SDS-PAGE

Chlamydomonas flagella

A-25

55, 56

RIA

polym, SDS-PAGE

rat brain

A-26

49

RIA

batch

calf brain

A-27

64

RIA

polym

mouse brain

A-28

78, 80

RIA

polym, glut

lamb brain

A-29

46

If

polym, alum

porcine brain

A-30

73

IEP, assembly

polym

rat brain

A-31

48

If, EM-PAP

polym, SDS-PAGE

rat brain

A-32

48

If, EM-PAP

polym

porcine brain

A-33

57

If

polym, glut

porcine brain

A-34

1978

53

RIA

polym

chick brain

A-35

30

If

polym, SDS-PAGE, glut

porcine brain

A-36

20

ouch, crossed IEP

polym

porcine brain

A-37

3

RIA

polym, SDS-PAGE glut, SDS

porcine brain

A-38

A-39	bovine brain	polym, SDS-PAGE	RIA	58
A-40	porcine brain	polym	RIA	112
A-41	<u>Tetrahymena cilia</u>	SDS-PAGE	RIA	112
A-42	chick brain	polym, glut	assembly	79
A-43	porcine brain	polym, SDS-PAGE	If	127
A-44	chick brain	polym, MeBSA	If	50
A-45	bovine brain	polym, glut	If	66
A-46	bovine brain	polym	If	97
			in goat	
			affinity purified	
			affinity purified	
			in goat	

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Chapter 2

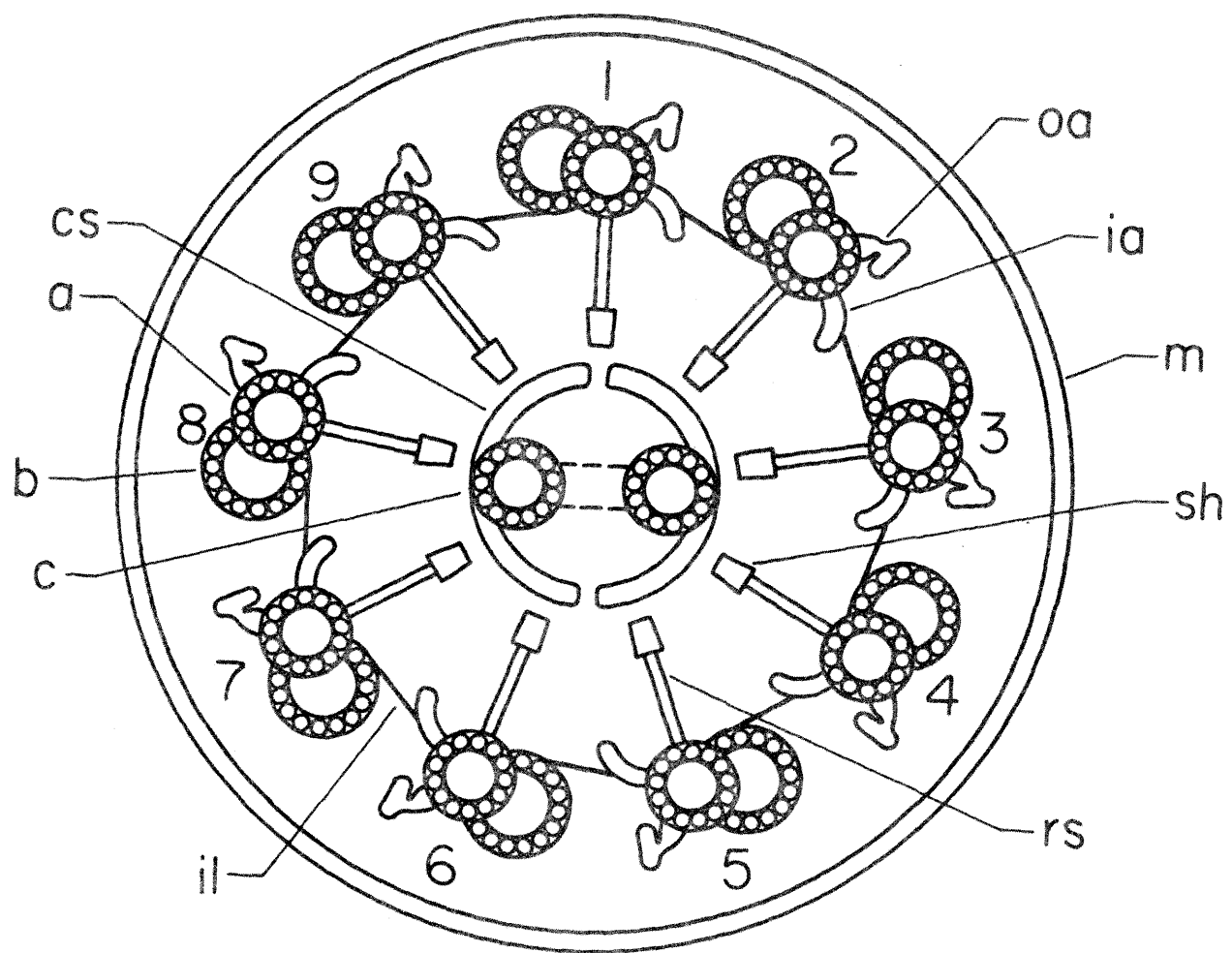
FLAGELLAR MOTILITY

The movements of cilia and flagella are extremely diverse. Although sea urchin sperm flagella have regular, almost symmetrical, planar waves, others do not. Many cilia have asymmetrical three-dimensional patterns with distinctive effective and recovery strokes. Flagella of Chlamydomonas beat with a ciliary type of movement when swimming forward but appear to change to a sea urchin flagella-type beat when the organism reverses itself. This thesis concerns itself with spermatozoa from the sea urchin, Strongylocentrotus purpuratus, which propel themselves through the seawater by means of their flagella (approximately 40 microns long) which beat with regular, two-dimensional waves. This flagellar movement is quite symmetrical and is driven by ATP diffusing from mitochondria at the basal end of the flagellum.

The components of flagella and cilia are very similar among different species. Figure 2-1 shows the classic "9 + 2" cross-sectional arrangement of microtubules which is basic to the flagellar design. Other structures are arranged within this framework as accessories. The nine outer doublet tubules are the moving units of the flagellum. Each outer doublet is almost two microtubules fused together along their long axes--the A subfiber is a complete tubule composed of 13 protofilaments and the B subfiber is an 11-protofilament partial tubule sharing 5 more protofilaments with the A tubule. Between each pair of outer doublets extend dynein side arms which produce cyclic cross-bridge activity to induce outer doublet sliding. The dynein arms reach from each A tubule toward the B tubule of the adjacent outer doublet; two such arms per outer doublet are seen in cross-sections.

Besides the components which generate sliding, there are the central pair microtubules running through the center of the flagellum, nexin links which probably are elastic structures which hold the outer doublets together but allow the flagellum to bend, and the radial spokes which radiate from the central pair to each A subfiber of the outer doublets.

Figure 2-1. Schematic representation of a transverse section of flagellum or cilium. (m) membrane, (a) and (b) A and B tubule of outer doublet, (oa) and (ia) outer and inner arm, (il) interdoublet nexin link, (rs) radial spoke, (sh) spoke head, (cs) central sheath, (c) central tubule. (From: Mohri, H. 1976. The function of tubulin in motile systems. Biochim. Biophys. Acta 456: 85-127.)



The "9 + 2" arrangement is surprisingly conserved among different species' cilia and flagella which behave very differently. Instead of evolving more specialized structures for different kinds of wave forms, nature has apparently opted to maintain the "9 + 2" structure as a very adaptable structure for many beating patterns.

Some work (15, 24, 25) with Chlamydomonas mutants has suggested that lesions in any of the major components strongly affects the function of the flagellum (the mutants are selected by their immotile or altered motile behavior). It is difficult to assign any absolute role to a particular structure simply by these mutant correlations because a loss of function may be due to the altered structure or to other, undetected mutations, or to a combination of these.

Conditions for reactivation of demembranated spermatozoa have been worked out for several species' sperm, including S. purpuratus (4, 7). This technique allows careful study of the movement parameters because the demembration makes the axoneme dependent on exogenously supplied ATP and sensitive to different reagents. Reactivated flagella can generate bending waves which appear completely normal (7).

In order to produce the orderly, symmetrical bends which begin at the basal end of the flagellum and are actively propagated distally, two mechanisms must be acting within the flagellum: first, dynein cross-bridge activity must create ATP-dependent active sliding of the outer doublet tubules relative to each other; and second, there must be a mechanism(s) to produce localized control of sliding, in the right places and at the right times, to initiate and propagate bends. Several advances have been made to develop understanding of these processes. This Chapter will discuss the demonstration of active sliding, the measurement of tubule displacement relative to each other, and partial characterization of cross-bridges.

The "9 + 2" arrangement of microtubules was shown as early as 1954 by Fawcett and Porter (6). And in that year, Huxley and Hanson (14) published their sliding filament model for muscle movement. It is somewhat surprising, then, that a contracting microtubule model for flagellar movement (12) persisted for so long. This model was somewhat dispelled by Satir's reports in 1965 and 1968 (18, 19) which showed that, after fixation for electron microscopy, ciliary bending was accompanied by a displacement of microtubules at the tip of the cilium. The amount of displacement for a given bend argued for an unchanging microtubule length. However, the contracting tubule idea remained a viable alternative until 1971.

In that year, Summers and Gibbons (20, 21, 22) showed that microtubules in flagellar fragments slide against each other under reactivation conditions. They took advantage of brief trypsin digestion to eliminate the nexin links and the spoke heads, added ATP, and observed disintegration by tubule extrusion. This extrusion paralleled flagellar beating in nucleotide (ATP) and cation (Mg^{2+}) requirements. Electron microscopy revealed that the brief trypsin digestion left intact the "9 + 2" arrangement of microtubules, including the dynein side arms. Addition of ATP left randomly scattered doublets with the dynein still attached. These experiments demonstrated the ability of the doublets to slide relative to one another under motile conditions. From theoretical work (3), it can be seen that controlled sliding between certain doublets at a particular time can produce bends; this process can continue as long as the right cross-bridges are turned on and off at the right times. Also, Gibbons (10) showed that the velocity of sliding was dependent on ATP concentration and that the rate of sliding was approximately equal to that predicted by a sliding filament model for the propagation of normal waves.

Further work, by Sale and Satir (17), allowed the electron microscopic visualization of the results of active sliding. Tetrahymena ciliary axonemes were applied to a polylysine-coated grid, and induced to slide by adding a drop of ATP directly to the grid. The samples, after several seconds, were fixed with glutaraldehyde, and negatively stained with uranyl acetate. The authors were able to distinguish the distal tips of the outer doublets by a number of markers including the non-even spoke group spacing. They observed "telescoping" of the outer doublets; these extruded tubules were of the same length before and after sliding. Because dynein side arms are attached only to the A subfiber of each doublet and because of the previously mentioned polarity markers, it was possible for Sale and Satir, by examining high magnifications of many isolated pairs (where only two doublets, after sliding, were interacting by some overlap), to determine the direction of sliding. The doublet which was transiently bridged with an adjacent doublet's dynein was moved distally relative to its partner. In other words, if the doublet containing the interacting dynein arms attached to its A subfiber is imagined to be stationary, these dynein cross-bridges, by cyclic attachment and detachment, "walk" along the adjacent doublet towards the sperm head. These results showed that sliding, after trypsinization, has a single polarity. The experiment does not show how the axoneme recovers for the next stroke after this polar sliding nor does it show what controls the process so that only certain doublets slide at a given time.

Implicit in these sliding experiments is the function of the dynein arms as cycling cross-bridges. Gibbons and Gibbons (8) showed that the rate of beating was directly dependent on the number of dynein arms left on the axoneme. They accomplished this by extracting the intact axonemes with 0.5 M KCl, which solubilized the outer arms. If all of the outer arms were removed, beat frequency

was reduced to half. This implies that both the inner and outer dynein arms are functional and that they are responsible for sliding the tubules.

The Gibbons went on to demonstrate that, by abruptly removing the ATP from axonemes which had been reactivated in low concentrations (10-20 μM) of ATP, the flagella assumed a "rigor" state in which the flagella remained bent in waveforms similar to beating sperm (9). Upon re-addition of small amounts (3 μM) of ATP, the rigor flagella slowly (10 min) relax by propagating their bends distally and ending in a straight configuration; at higher ATP (greater than 10 μM) they proceed to beat normally (11). The maintenance of rigor waves implies that, in the absence of ATP some cross-bridges remain attached and their attachment is sufficient to hold the flagellum in the bent form, overcoming the elastic forces which would have the flagellum naturally straighten out. Relaxation of the rigor sperm suggests that the small quantity of ATP allows the cross-bridges to detach but that there is insufficient ATP to drive the dynein arms through subsequent cycles of attachment and detachment.

Warner (23) was able to show selective attachment of dynein arms under certain conditions by electron microscopy of cross-sections of Unio gill cilia. Isolated cilia were fixed either in the presence of Mg^{2+} , ATP, or MgATP^{2-} . Mg^{2+} induces the attached cross-bridged state; the absence of Mg^{2+} produces the relaxed state. Axonemes fixed in the presence of only ATP showed no cross-bridge attachment, axonemes fixed in the presence of Mg^{2+} showed a uniform and high (87% mean frequency) amount of attachment, and axonemes fixed in the presence of MgATP^{2-} showed a characteristic pattern of attachment (48% mean frequency) --doublet pairs 9-1, 1-2, 4-5, 5-6, and 6-7 retained a greater number of cross-bridges than doublet pairs 2-3, 3-4, 7-8, and 8-9. Predicted values of sliding based on the plane of bend (which passes through the 5-6 pair) argue that doublet pairs

3-4 and 7-8 exhibit maximal sliding and pairs 9-1, 1-2, and 5-6 should show minimal displacement. These are in exact inverse to Warner's data and suggest that MgATP^{2-} induces all the cross-bridges to attach, then ATP hydrolysis generates sliding force and the release of the cross-bridges involved in movement. This model would have as its control a mechanism which determined which cross-bridges are to become functional and detach.

To summarize the preceding descriptions: flagellar movement is produced by active sliding between adjacent outer doublet tubules. Sliding is a direct result of dynein cross-bridge activity. Both the inner and the outer dynein arms appear to be able to function as cross-bridges. The attachment of arms seems to be induced by the presence of Mg^{2+} ; attachment is a separate step from ATP hydrolysis-driven tubule displacement and cross-bridge detachment. The energy produced by ATP drives the sliding process; the frequency of beating is directly related to the number of functional cross-bridges and to the concentration of ATP.

But flagella do not propel themselves by extrusion. They bend. What causes the sliding to be translated into bending? And what controls the sliding to produce repeated, propagated bending?

Trypsin allows the tubules to continue sliding until they have extruded. Trypsin-sensitive structures, such as the nexin links and the radial spoke heads, probably play an important role in translating sliding to bending by preventing sliding from proceeding past a point and providing elastic force to help cause the outer doublets to return to their pre-displacement positions. Also, by connecting all the doublets in a bundle, nexin links and the spokes cause the axoneme to behave as a unit--in a symmetrical bend, a displacement of tubules on one side of the flagellum will be accompanied by an equal but opposite displacement on the other

side of the flagellum. Nevertheless, a clear understanding of the process of translating sliding into bending remains to be determined.

Control of sliding to produce repeated and propagated bends also is not well understood. Mathematical models (2) provide for a feedback mechanism so that, at a certain bend curvature, the cross-bridge behavior changes to decrease that bend and allow for a reverse bend to grow. Since bends begin at the proximal end and are regularly propagated, without loss of amplitude, cyclic attachment and detachment of cross-bridges must proceed down the flagellum in rhythmic waves. It is possible to build into a model this sort of feedback mechanism which would allow the propagated bend to grow to a given fullness before dissipating. The nature of the controlling events allowing repeated bending and the nature of this feedback mechanism remain to be determined.

To explore these problems, it is useful to find chemical probes which can be added to reactivated flagella and which have specific effects on motility. For instance, beat frequency appears to be specifically determined by the concentration of ATP or competitive inhibitor such as ADP, while the amplitude remains constant (1, 13). CO_2 specifically inhibits amplitude while the frequency remains constant (5). Magnesium ion causes amplitude and frequency to vary inversely, so that sliding velocity remains constant (16). These observations are indicative of interesting and sophisticated control mechanisms operating within the flagellum.

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Chapter 3

DYNEINS AND ANTI-DYNEINS

As discussed in the previous chapter, flagella move by an active sliding process among the outer doublets--this process is controlled so that the axonemes are caused to propagate regular, repeated waves of bending. Between each pair of adjacent outer doublets are series of inner and outer arms which appear to be securely attached to the A tubule and reach toward the B tubule of the adjacent doublet. Under conditions of rigor, these arms are seen to actually bridge the interdoublet space. These side arms are responsible for the active sliding of doublets and presumably act as cycling cross-bridges to effect sliding.

The most prominent component of the side arms is dynein ("dyne + protein"), first termed by Gibbons in 1965 (15). Dynein is a large, oligomeric protein with Mg^{2+}/Ca^{2+} -dependent ATPase activity. If axonemes are briefly extracted with 0.5 M KCl (or NaCl), 65% of the ATPase activity can be solubilized. Gibbons and Gibbons (8) performed this extraction and determined by electron microscopy that the outer arms had been selectively removed. If all of the outer arms were extracted, the beat frequency of the axonemes, upon reactivation, was approximately half-normal. They determined that the rate of loss of arms was paralleled by reduction of frequency until all the outer arms had been removed. Interestingly, Gibbons and Gibbons (9) were also able to add back up to 90% of the initially solubilized dynein by diluting out the KCl; addition of arms was accompanied by a gradual increase of beat frequency to almost normal. Addition occurred in the presence or absence of ATP, although ATP inhibited addition slightly.

In Tetrahymena cilia, dynein can be extracted in "monomeric" (14s) form and an oligomeric (30s) form. Takahashi and Tonomura (29) were able to add back to extracted axonemes 30s dynein and investigate by electron microscopy the nature of recombination. The outer arms added back in normal spacing and normal angles. In the absence of ATP, the arms were attached to both the A and B

tubules; however, in the presence of ATP, there was a specific dissociation of the dynein from B tubules. Upon ATP hydrolysis, the arms rebound to the B tubules forming cross-bridges. Other experiments have shown that soluble tubulin (from porcine brain as well as from Tetrahymena) is able to stimulate the ATPase activity of dynein (16, 27). The ability to add back the outer arms producing increased beat frequency and the demonstration that the dynein is specifically recombining with tubulin and microtubules indicate that dynein is the molecule responsible for inducing active sliding.

Careful work, largely reported from Gibbons' laboratory (12, 17, 18), has biochemically characterized the extracted dyneins from sea urchin sperm flagella. Low percentage acrylamide gels revealed at least four bands--C, A, D, and B--of which two, A and D, have been shown to have ATPase activities. A band ATPase has been termed dynein 1 and is found in the outer arms. It is the principle ATPase of the axoneme. Under certain extraction conditions, dynein 1 can be solubilized with low activity (latent activity dynein, LAD-1) which is restorable by reassociation with tubules (10, 13, 14). This is reminiscent of the effects of mechanochemical "uncouplers" such as thiourea which have the property of raising the in vitro ATPase activity of immotile spermatozoa (4). LAD-1 may represent a "coupled" form of dynein, and may be similar to "30s" dynein from Tetrahymena cilia.

Another ATPase from sea urchin sperm flagella, dynein 2, has been characterized (14, 22). It is represented by D band material and is found in smaller quantities than dynein 1. The location of dynein 2 and whether or not it has a latent form remain to be determined. Table 3-1 summarizes some of the biochemical data of dyneins.

Table 3-1

Summary of Biochemical Properties of Dynein 1 and

Dynein 2 from Tripneustes

	Dynein 1	Dynein 2
electrophoretic	A Band	D Band
native size	750,000 \pm 100,000 daltons	720,000 \pm 50,000 daltons
monomeric size by SDS PAGE	330,000 \pm 40,000 daltons	325,000 \pm 40,000 daltons
sedimentation coefficient	12 \pm 1 s	12 \pm 1 s
Stokes radius	14.6 \pm 1 nm	12.8 \pm .4 nm
latent form	yes, LAD-1 can be stimulated 10x	not known
tryptic fragment	Fragment 1A 400,000 daltons ATPase active	Fragment 2A 360,000 daltons ATPase active
effect of anti- Fragment 1A	precipitates dynein 1 precipitates Fragment 1A inhibits 80% ATPase activity	no precipitation of dynein 2 no precipitation of Fragment 2A no inhibition of ATPase activity

The use of antibodies to study motility was pioneered by Ogawa and Mohri who raised an antibody to a tryptic Fragment 1A from Anthocidaris spermatozoan dynein 1 (24). This Fragment 1A was approximately 400,000 daltons and retained the ATPase activity. The rabbit antiserum precipitated Anthocidaris dynein 1 and Fragment 1A as well as dyneins from other sea urchin species: Pseudocentrotus and Hemicentrotus from Japan, Colobocentrotus from Hawaii, and Stronglyocentrotus from California.

Anti-Fragment 1A was able to inhibit the ATPase activity of solubilized dynein 1 as well as of demembranated spermatozoa. This antiserum was shown by Gibbons et al. (11) to inhibit the beat frequency of reactivated Colobocentrotus spermatozoa and by Okuno et al. (26) to inhibit the motility of reactivated spermatozoa from both Anthocidaris and Hemicentrotus. Work by Ogawa et al. (25) with indirect ferritin-electron microscopy using anti-Fragment 1A showed that the antiserum labeled the distal ends of the outer dynein arms, did not label the inner arms, and did not label the outer arm between the 5-6 doublets. This anti-Fragment 1A was also used by Masuda et al. (19) to show that the ATP-driven extrusion of microtubules from trypsin-digested axonemes, like their ATPase activities, could be inhibited by the antiserum. Finally, two papers from Mohri's laboratory (20, 28) used the anti-Fragment 1A to immunofluorescently localize dynein-like material in cleaving sea urchin eggs and to inhibit the motion of the chromosomes by microinjection of sea urchin and starfish eggs (anti-starfish myosin had no inhibitory effects on chromosome movement). Anti-Fragment 1A has proven to be a very useful reagent in the study of microtubule-mediated motility.

Work in our laboratory furthered the use of anti-Fragment 1A and produced the first antibody to intact dynein 1. The remainder of this Chapter is taken from

a paper (21) which compared the properties of the two antisera by their effects on ATPase activity and their properties on reactivated spermatozoa.

Properties of an antiserum against dynein 1

MATERIALS AND METHODS

Materials

Concentrated spermatozoa were collected from the aboral surface of the sea urchin, Strongylocentrotus purpuratus, after injection of 0.6 M KCl to induce shedding. For experiments with reactivated spermatozoa, a stock sperm suspension was prepared by dilution with 1-2 vol of cold 0.5 M NaCl. The concentration of the sperm suspension was adjusted until a 10 μ l portion, diluted with 5.0 ml of 0.5 M NaCl, produced an optical density reading of 0.24-0.26 at 550 nm (6). For preparations of axonemes and axonemal proteins, the spermatozoa were washed once with seawater, and then either used at once for preparation of axonemes, or stored with 50% glycerol at -15°C (23).

Preparation of axonemes

Axonemes were isolated from freshly shed or glycerinated spermatozoa by procedures based on those used by Gibbons and Fronk (12), modified for treatment of 50-100 ml of packed spermatozoa. The spermatozoa were demembrated with 150 ml of a 1% solution of Triton X-100 in solution A: 0.1 M KCl, 5 mM MgSO_4 , 0.2 mM EDTA, and 1 mM dithiothreitol (DTT) buffered with 10 mM tris and 10 mM NaH_2PO_4 at a pH of 7.8. All steps were carried out at 0-4°C. The suspension was homogenized gently with 7 strokes of a Teflon homogenizer, and then centrifuged at 800 x g for 5 min. The supernatant (I) was saved and the pellet (I) was suspended in 150 ml of Triton X-100 in solution A. Supernatant (I) was centrifuged at 13,000 x g for 10 min, and the pellet (II) was saved. The suspension of pellet (I)

was centrifuged at 800 x g for 5 min, and the supernatant (II) was combined with pellet (II), homogenized gently to disperse the pellet, and centrifuged at 13,000 x g for 10 min. The resulting pellet consists of a pinkish axoneme layer on top of a white head layer. A spatula and pipette were used to remove only the axoneme layer, which was then suspended in 100 ml of solution A and centrifuged at 13,000 x g for 10 min. This process was repeated two more times to yield a pellet of axonemes with negligible head contamination.

Preparation of dyneins and tryptic fragments

Dynein 1 was prepared from axonemes obtained from freshly shed spermatozoa by low ionic strength extraction, followed by purification by column chromatography on Sepharose 4B and hydroxyapatite, as described by Ogawa and Mohri (23). The result of SDS gel electrophoresis of a purified dynein 1 preparation is shown in Fig. 3-1A.

Dynein 2 was prepared from axonemes previously extracted with 0.6 M KCl to remove dynein 1, by low ionic strength extraction, followed by purification by column chromatography on Sepharose 4B and hydroxyapatite, as described by Ogawa and Gibbons (22). The result of SDS gel electrophoresis of a dynein 2 preparation is shown in Fig. 3-1B.

Fragment A used in these experiments was prepared previously by trypsin-digestion of dynein from spermatozoa of the Japanese sea urchin, Anthocidaris crassispina (24). Following identification of dyneins 1 and 2, this fragment is now referred to as fragment 1A (22). The result of SDS gel electrophoresis of fragment 1A is shown in Fig. 3-5H.

A tryptic fragment of dynein 1 from S. purpuratus spermatozoa was prepared by mixing 16 ml of a concentrated crude low ionic strength dynein 1 extract containing 15 mg ml⁻¹ protein with 2 ml of 3.5 mg ml⁻¹ trypsin (Sigma

Chemical Co., T8003). After digestion for 2 h at 16°C, 2 ml of 5 mg ml⁻¹ soybean trypsin inhibitor (Calbiochem) was added. The digest was applied to a 2.4 x 90 cm Sepharose 4B column equilibrated with Tris-EDTA solution (10 mM Tris-HCl buffer, pH 8.3, containing 0.2 mM EDTA and 0.1% β-mercaptoethanol), and eluted with the same solution. Fractions of 3.75 ml volume were collected and aliquots were assayed for ATPase activity using Fiske-Subbarow determination of inorganic phosphate. Results are shown in Figure 3-4. Fractions 55-66 were pooled and concentrated by collodion bag ultrafiltration; this preparation of unpurified tryptic fragment (or fragments) will be temporarily designated as fragment 1AS. Its band pattern on SDS gel electrophoresis is shown in Fig. 3-5D.

When necessary, these preparations were mixed with glycerol to give a glycerol concentration of 50%, and stored at -15°C.

Preparation of antisera

Purified dynein 1 from S. purpuratus at a concentration of approximately 2 mg ml⁻¹ was emulsified with an equal volume of complete Freund's adjuvant (Difco Co.). In the initial immunization, the rabbit received 11 mg of dynein 1, divided among the foot pads, femoral muscles, and the back of the neck. After 7 weeks, a booster injection of 4.6 mg of dynein 1 without adjuvant was given intraperitoneally. One week after this booster, a serum sample showed ability to precipitate dynein 1. Serum collected from this rabbit 4 weeks after the booster injection was used in the experiments described in this paper. After addition of 1% NaN₃, the serum was stored at 4°C until use.

Antiserum against fragment 1A from Anthocidaris was that previously described by Ogawa and Mohri (24) and used for experiments with reactivated spermatozoa (11, 14, 26). Serum obtained from a non-immunized rabbit from the same group as the rabbit immunized with dynein 1 was used for a control.

Before use in these experiments, a partially purified antibody preparation was obtained from each of the sera. Ten ml of serum was mixed with 10 ml of saturated $(\text{NH}_4)_2\text{SO}_4$ at 0°C . The precipitate was collected and dissolved in 8 ml of the buffer solution appropriate for a particular experiment, and dialyzed against 1 liter of the buffer solution, which was changed twice during a 16 h period of dialysis at 4°C . The dialyzed solution was then centrifuged at $13,000 \times g$ for 20 min and the supernatant was used as the purified preparation. The preparation obtained from the non-immune serum will be designated NI. The preparation obtained from the anti-fragment 1A serum will be designated AF-1A. The preparation obtained by ammonium sulfate purification of the anti-dynein 1 serum absorbed with impurity protein, as described in the next section, was designated AD-1. These preparations were stored at 4°C and used within 2-3 days.

Preparation of impurity antigen and purification of anti-dynein 1

A second precipitation band was regularly found when the anti-dynein 1 serum was tested by immunodiffusion against the low ionic strength extract used to obtain dynein 1, and was usually visible when the serum was tested against purified dynein 1. This impurity band was much more prominent when the anti-dynein 1 serum was tested against the axonemal extracts obtained in the course of purifying dynein 2. We found that the impurity antigens in these extracts could be readily separated from dyneins 1 and 2 by Sepharose 4B chromatography. Two methods were used: For method I, the 0.6 M KCl extract used to remove dynein 1 before extraction of dynein 2 was used. The extract was dialyzed against Tris-EDTA solution and concentrated with an Amicon membrane filter (XM 100 A). Fifteen ml of this concentrate was applied to a 2.4×90 cm Sepharose 4B column equilibrated with Tris-EDTA solution, eluted with the same solution, and 3.75 ml fractions were collected. When the void volume was at fraction 34, dynein 1 ATPase

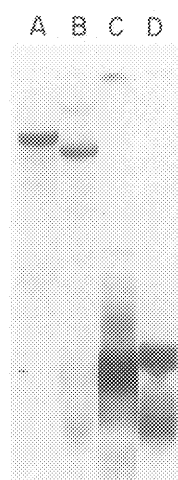
activity was eluted at fractions 40-64, as in Fig. 3-4, and the impurity antigen detected by immunodiffusion was eluted at fractions 64-80. Fractions 74 to 80 were pooled, concentrated by collodion bag ultrafiltration, and stored in 50% glycerol at -15°C . Approximately 3 ml, containing 12 mg ml^{-1} protein, was obtained. For method II, a similar procedure was used, except that the starting material was the low ionic strength extract of dynein 2, and 0.1 M KCl was added to the Tris-EDTA solution. In this case, dynein 2 ATPase activity appeared in fractions 58-70 and the impurity antigen was found in fractions 78-88, which were pooled and concentrated. Approximately 3 ml, containing 16 mg ml^{-1} protein, was obtained. Figure 3-1C shows the results of SDS gel electrophoresis of a sample of impurity antigen prepared by method II.

Impurity antibody was removed from the anti-dynein 1 serum by mixing 10 ml of the antiserum with 2.0 ml of the concentrated impurity in 50% glycerol prepared by method II. After 3 days at 4°C , the mixture was centrifuged at $13,000 \times g$ for 20 min to remove the antigen-antibody precipitate (see Fig. 3-1D), and the supernatant was purified by ammonium sulfate precipitation as described in the preceding section, to obtain the antibody preparation, AD-1, used for experiments.

Observation of motility of reactivated spermatozoa

Demembranated spermatozoa of S. purpuratus were prepared and reactivated using extraction and reactivation solutions with the same compositions used for previous experiments with Lytechinus spermatozoa (1) with an ATP concentration of 0.2 mM in the reactivation solution. A 10 μl portion of a standardized stock sperm suspension was mixed into 1.0 ml of extraction solution. After 60 sec, a 10 μl portion of extracted spermatozoa was added to 1.0 ml of reactivation solution containing either AD-1, AF-1A, or NI, and gently mixed.

Figure 3-1. SDS gel electrophoresis using 3.5% polyacrylamide gels. Gel A is a purified dynein 1 preparation (20 μ gm protein). Gel B is a purified dynein 2 preparation (15 μ gm protein). Gel C is a preparation of impurity antigen prepared by method II (158 μ gm protein). Gel D is precipitated antigen-antibody complex from a mixture of impurity antigen prepared by method II and anti-dynein 1 serum under the conditions described in Table 3-2 and Figure 3-5; except that in this case the washed precipitate was dissolved in 1.5 ml of SDS buffer solution.



A drop of this mixture was placed in a well slide, covered with a cover glass, and examined with dark-field illumination using a 40x oil immersion objective. Stroboscopic illumination was used, with the flash frequency adjusted to exactly 4 times the beat frequency of the spermatozoon which was being observed. A spermatozoon beating with its head attached to the bottom surface of the well slide and with its beat plane parallel to the surface was selected as quickly as possible, and photographed at intervals until its movement stopped. The time and flash frequency corresponding to each photograph was recorded. All work was carried out in a room maintained at 16°C.

The photographs were printed to give a final magnification of 2000x, and these prints were used for measurement of bend angles with a protractor. Since the bend angle may decrease near the distal end of the flagellum, especially in spermatozoa exposed to antisera, the bend angle measurement was routinely made as near as possible to halfway along the length of the flagellum.

Measurements of ATP dephosphorylation

Measurements of ATP dephosphorylation by suspensions of demembranated spermatozoa were made using standard pH-stat techniques for S. purpuratus spermatozoa (7), except for the addition of antibody preparations to the reactivation solution at the beginning of an experiment. An ATP concentration of 0.33 mM and a MgSO_4 concentration of 1.3 mM were used for these experiments.

Similar methods were used for measurements on axonemes, except that the axonemal samples were 10 μl aliquots containing 15 mg ml^{-1} axonemal protein.

Measurements of the effect of antisera on dynein ATPase activities were also carried out with the same pH-stat techniques, but in these experiments a simpler assay solution was used which contained 0.05 M KCl, 1.3 mM MgSO_4 , 0.33 mM ATP, 0.2 mM EGTA and 1 mM thioglycollate. The measurements were

made at pH 8.4 and 16°C. The reaction was started by the addition of a 0.4 ml sample of the enzyme pretreated with antibody preparations, as described in Table 3-2.

Other procedures

Methods used for double diffusion in agar (Ouchterlony's test), SDS polyacrylamide gel electrophoresis, determination of protein by the Lowry method, and assay of ATPase activity by measurement of inorganic phosphate were standard methods which have been referenced in previous work (24).

RESULTS

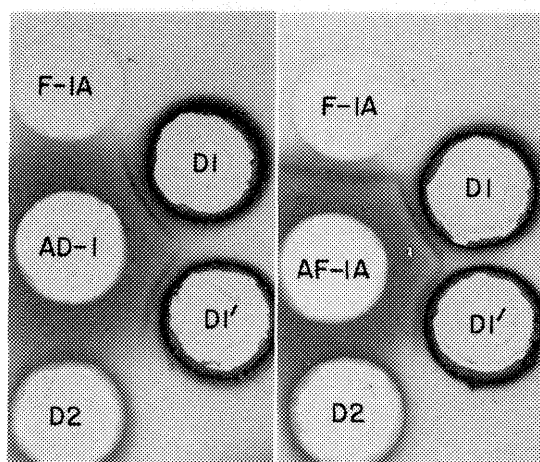
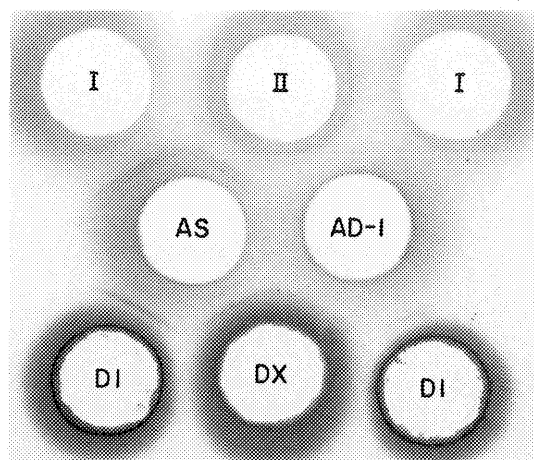
Immunodiffusion

Figure 3-2 shows that the unpurified anti-dynein 1 serum forms two precipitation bands against purified dynein 1 and against the low ionic strength extract obtained in the first step of the dynein 1 preparation. The multiple reactivity of the anti-dynein 1 serum was also confirmed by immunoelectrophoretic analysis. Two closely-spaced bands can be seen between the anti-dynein 1 serum and the impurity protein preparation obtained by method I and by method II. After absorption of the anti-dynein 1 serum with the impurity protein preparation obtained by method II, there is no precipitation with either impurity preparation, confirming the identity of the preparations obtained by these two methods. The absorption-purified anti-dynein 1 serum, which will now be referred to as AD-1, shows a single precipitation band against both the purified dynein 1 and the low ionic strength dynein 1 extract.

The reactivities of AD-1 and AF-1A, the antibody preparation obtained from the anti-fragment 1A serum, are compared in Fig. 3-3. As previously reported, AF-1A precipitates with dynein 1 and with fragment 1A, but not with

Figure 3-2. Immunodiffusion tests. AS is antiserum obtained from the rabbit immunized with dynein 1. AD-1 is the antiserum following absorption with impurity protein prepared by method II [before $(\text{NH}_4)_2\text{SO}_4$ fractionation]. I is impurity protein prepared by method I (0.3 mg protein). II is impurity protein prepared by method II (0.39 mg protein). D1 is purified dynein 1 (0.053 mg protein). DX is a low ionic strength extract of axonemes, used to prepare D1 (0.17 mg protein). Antisera wells in this figure and in Figure 3-3 received 25 μl antisera.

Figure 3-3. Immunodiffusion tests. AF-1A is the anti-fragment 1A serum. AD-1 is the anti-dynein 1 serum absorbed with impurity protein prepared by method II. F-1A is fragment 1A (0.008 mg protein). D1 is purified dynein 1 from a low ionic strength extract of axonemes (0.053 mg protein). D1' is purified dynein 1 from a 0.5 M KCl extract of axonemes (0.06 mg protein).



dynein 2. AD-1 is also specific for dynein 1, showing no precipitation with dynein 2, but, in contrast to AF-1A, it fails to precipitate with the fragment 1A preparation from Anthocidaris spermatozoa.

Effects on ATPase activities of purified dyneins

The ability of AD-1 and AF-1A to inhibit and precipitate the ATPase activity of dynein 1, dynein 2, and fragment 1A was compared by the experiments described in Table 3-2. Under the conditions of these experiments, there was no detectable inhibition of ATPase activity of any of the enzymes by AD-1. The ATPase activity measurements indicate that AD-1 precipitated about 70% of the dynein 1 and none of the dynein 2 or fragment 1A, in agreement with the results in Fig. 3-3. In contrast, AF-1A, which also precipitates about 70% of the dynein 1, inhibits about 30% of the dynein 1 ATPase activity and almost all of the fragment 1A ATPase activity. The 30% inhibition of dynein 1 ATPase activity by AF-1A is considerably lower than was obtained in previous measurements of the effect of AF-1A on dynein 1 from spermatozoa of Anthocidaris and Tripluostes (22, 24).

A tryptic fragment of dynein 1 from S. purpuratus spermatozoa

A single peak of ATPase activity was found by Sepharose 4B chromatography of dynein 1 from S. purpuratus spermatozoa after digestion with trypsin (Fig. 3-4). The preparation obtained by pooling fractions 55-66 from the Sepharose 4B column is assumed to contain a single ATPase-active species, which will be referred to as fragment 1AS. Other tryptic digestion products may also be present in this preparation. As seen by comparing gels D and H in Fig. 3-5, SDS gel electrophoresis of the fragment 1AS preparation produces a band pattern significantly different from that obtained with the original fragment 1A from Anthocidaris sperm dynein, which was obtained after digestion of the dynein with trypsin obtained

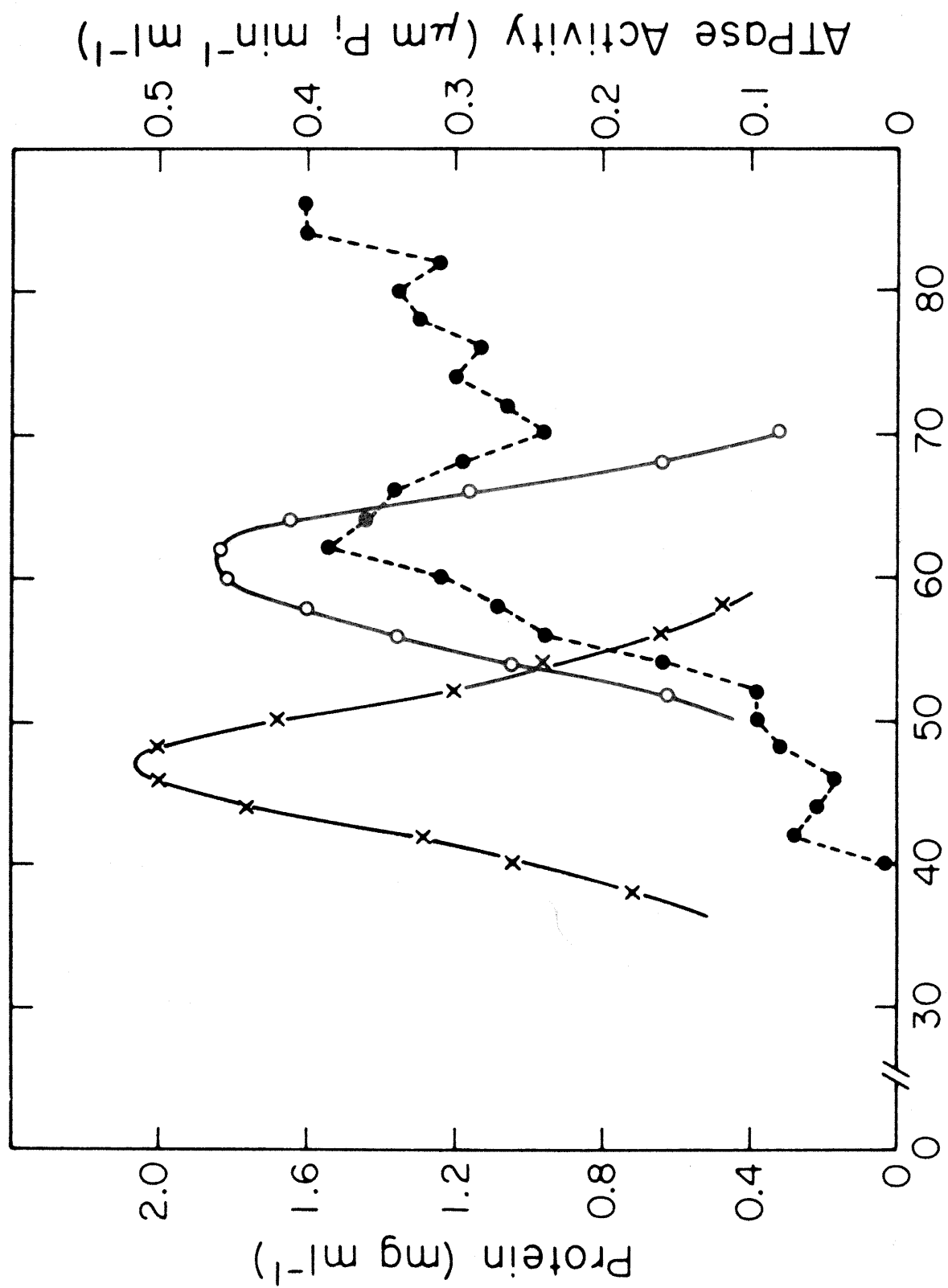
Table 3-2

Effects of Antibody Preparations on ATPase Activity

	Mixture			Supernatant		
	NI	AD-1	AF-1A	NI	AD-1	AF-1A
Dynein 1	30	31	24	31	9	9
Dynein 2	13	13	12	13	13	12
Fragment 1A	41	41	2	41	41	2

1.0 ml of purified preparations of anti-dynein 1 serum (AD-1), anti-fragment A serum (AF-1A), or non-immune serum (NI) was mixed with 0.1 ml of dynein 1 (2.1 mg ml^{-1}), 0.2 ml of dynein 2 (1.5 mg ml^{-1}), or 0.1 ml of fragment 1A (0.3 mg ml^{-1}). Duplicate mixtures were prepared and incubated for 6 hr at 4°C . One of the mixtures was then centrifuged at $1300 \times g$ for 20 min, and the ATPase activity of 0.4 ml portions of the supernatant was assayed. Similar ATPase measurements were performed on 0.4 ml portions of the uncentrifuged mixtures, after uniformly resuspending any antigen-antibody precipitate. The values represent the mean of two ATPase measurements, in units of nM min^{-1} .

Figure 3-4. Sepharose 4B column chromatography of dynein 1 and its tryptic fragment. Measurements of protein and ATPase activity of fractions obtained after applying a tryptic digest of dynein 1 to the column, as described in the methods section, are shown by the circles. The open circles represent ATPase activity and the solid circles represent protein. The x's show results obtained from another experiment with the same column, in which 15 ml of a low ionic strength extract of axonemes, containing 13.2 mg ml^{-1} protein, was applied to the column. In this case, the ATPase peak represents intact dynein 1. Fraction 34 was the void volume, and the tubulin peak, located by SDS gel electrophoresis of the fractions was at fraction 68.



from Worthington Biochemical Co. Further work will be required to determine whether the difference between fragment 1AS and fragment 1A is the result of a species difference or differences in the conditions for trypsin-digestion.

Using a procedure similar to that used for the experiments in Table 3-2, neither AD-1 nor AF-1A inhibited or precipitated the ATPase activity of the fragment 1AS preparation, although under the same conditions, AF-1A almost completely inhibited the ATPase activity of the original fragment 1A. However, some precipitation was observed when the fragment 1AS preparations were mixed with AD-1 or AF-1A. These precipitates were collected by centrifugation, redissolved, and analyzed by SDS gel electrophoresis. The results are shown in Fig. 3-5. Comparison of gels D and G in Fig. 3-5 shows that at least one of the bands obtained with the fragment 1AS preparation, corresponding to the largest peptide chain, is not precipitated by AF-1A, and may therefore be derived from the fragment 1AS retaining ATPase activity. This band is not found in the SDS gel of fragment 1A (Fig. 3-5H). These results suggest tentatively that fragment 1AS is a smaller tryptic fragment than the original fragment 1A, which lacks the principal antigenic determinants of fragment 1A but retains the active ATPase site.

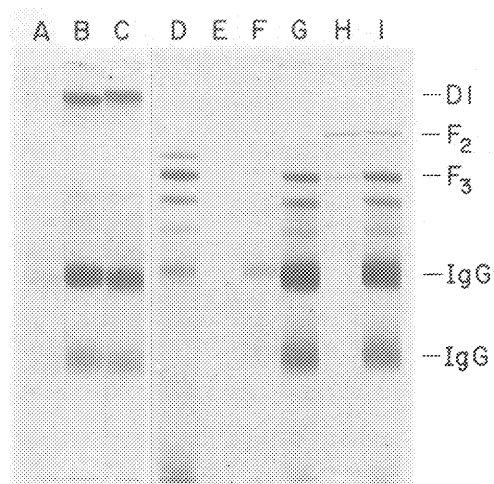
Effects on ATP dephosphorylation by demembranated sperm suspensions

After addition of demembranated spermatozoa to reactivation solution containing 2 or 5 $\mu\text{l ml}^{-1}$ of AD-1 or AF-1A, a gradually decreasing rate of ATP dephosphorylation was observed. When the reactivation solution contained 10, 20, or 30 $\mu\text{l ml}^{-1}$ of AD-1 or AF-1A, the rate of ATP dephosphorylation decreased during the first 5 min, and then leveled off to give a reasonably constant rate, which was only slightly lower at the higher antiserum concentrations. Microscopic observations on samples taken from these sperm suspensions during the period of steady dephosphorylation rate showed that with 20 μl of AD-1, most of the

Figure 3-5. SDS gel electrophoresis using 5% polyacrylamide gels. Gel A is the precipitate of dynein 1 and NI. Gel B is the precipitate of dynein 1 and AD-1. Gel C is the precipitate of dynein 1 and AF-1A. Gel D is a sample of the fragment 1-AS preparation (18 μ gm protein). Gel E is the precipitate of fragment 1-AS and NI. Gel F is the precipitate of fragment 1-AS and AD-1. Gel G is the precipitate of fragment 1-AS and AF-1A. Gel H is a sample of fragment 1A (1.6 μ gm protein). Gel I is a mixture containing both G and H.

D1 indicates a subunit polypeptide of dynein 1; F_2 and F_3 indicate the principal component polypeptide subunits of fragment 1A with molecular weights of 190,000 and 135,000, respectively (24). IgG indicates the immunoglobulin bands from serum, with molecular weights of approximately 60,000 and 20,000.

Antigen-antibody precipitates were obtained from the experiments described in Table 3-2, and similar experiments with a fragment 1-AS preparation containing 1.8 mg ml⁻¹ protein. Each precipitate was washed with 3 ml of 0.85% NaCl buffered with 10 mM sodium phosphate at pH 7.0 and containing 0.1% sodium azide. The precipitate was then dissolved in 1.0 ml of SDS-buffer solution (24), and 50 μ l was applied to the gel.



spermatozoa showed erratic, low amplitude beating. Only a few showed motility that was regular enough to measure a beat frequency; in those cases the frequency was in the neighborhood of 10 Hz, about half the frequency obtained in control experiments. With 20 μ l AF-1A, the amount of motility appeared less, and was usually limited to low-frequency (≤ 1 Hz) bending near the basal end of the flagellum.

Table 3-3 compares the effects of the antibody preparations on motile demembranated spermatozoa, non-motile broken spermatozoa, and axonemes. Both AD-1 and AF-1A inhibit the movement-coupled ATP dephosphorylation, if this is defined as the difference between the rates of ATP dephosphorylation by unbroken and broken sperm preparations (3). In addition, AF-1A, but not AD-1, appears to inhibit a significant portion of the ATP dephosphorylation by broken spermatozoa and by flagellar axonemes.

Effects on the motility of reactivated spermatozoa

Figures 3-6 and 3-7 show the results of measurements of beat frequency and bend angle of spermatozoa exposed to AD-1, AF-1A, or NI. Examples of the photographs used to obtain these measurements are shown in Fig. 3-8. Both antibody preparations inhibit both the frequency and amplitude of flagellar beating, in contrast to earlier observations of the effect of AF-1A on reactivated spermatozoa from another sea urchin, Colobocentrotus atratus, where inhibition of beat frequency with little change in amplitude was obtained (11). During the first 2 min of exposure to 20 μ l ml⁻¹ of AD-1, both the beat frequency and bend angle decrease by about 10%. After this initial decrease, there is only a very slow decrease in beat frequency, while the bend angle continues to decrease until it is about 30-40% less than the original value. The movements then become very erratic and the flagellum soon sticks to the surface of the glass slide, terminating

Table 3-3

Effects of Antibody Preparations on ATP Dephosphorylation
by Demembranated Spermatozoa and Axonemes

	NI	AD-1	AF-1A
Unbroken spermatozoa	54 \pm 2.2	23 \pm 4	14 \pm 4.9
Broken spermatozoa	20 \pm 2.2	18 \pm 1.6	13.5 \pm 3.6
Axonemes	23 \pm 0.5	23 \pm 1.4	15 \pm 0.2

Each value represents the mean of 4-6 pH-stat measurements, and the standard deviation is indicated. For axonemes and for unbroken spermatozoa, the reactivation solution contained 20 $\mu\text{l ml}^{-1}$ of antibody preparation. For the broken spermatozoa, 10 $\mu\text{l ml}^{-1}$ was used. Units are nM min^{-1} .

Figure 3-6. Effects of antibody preparations on the beat frequency of individual reactivated spermatozoa. The curves labeled AD-1 were obtained with reactivation solution containing $20 \mu\text{l ml}^{-1}$ of the purified anti-dynein serum, AD-1. The curves labeled AF-1A were obtained with reactivation solution containing $5 \mu\text{l ml}^{-1}$ of the purified anti-fragment 1A serum, AF-1A. The demembrated spermatozoa were added to the reactivation solution at time 0.

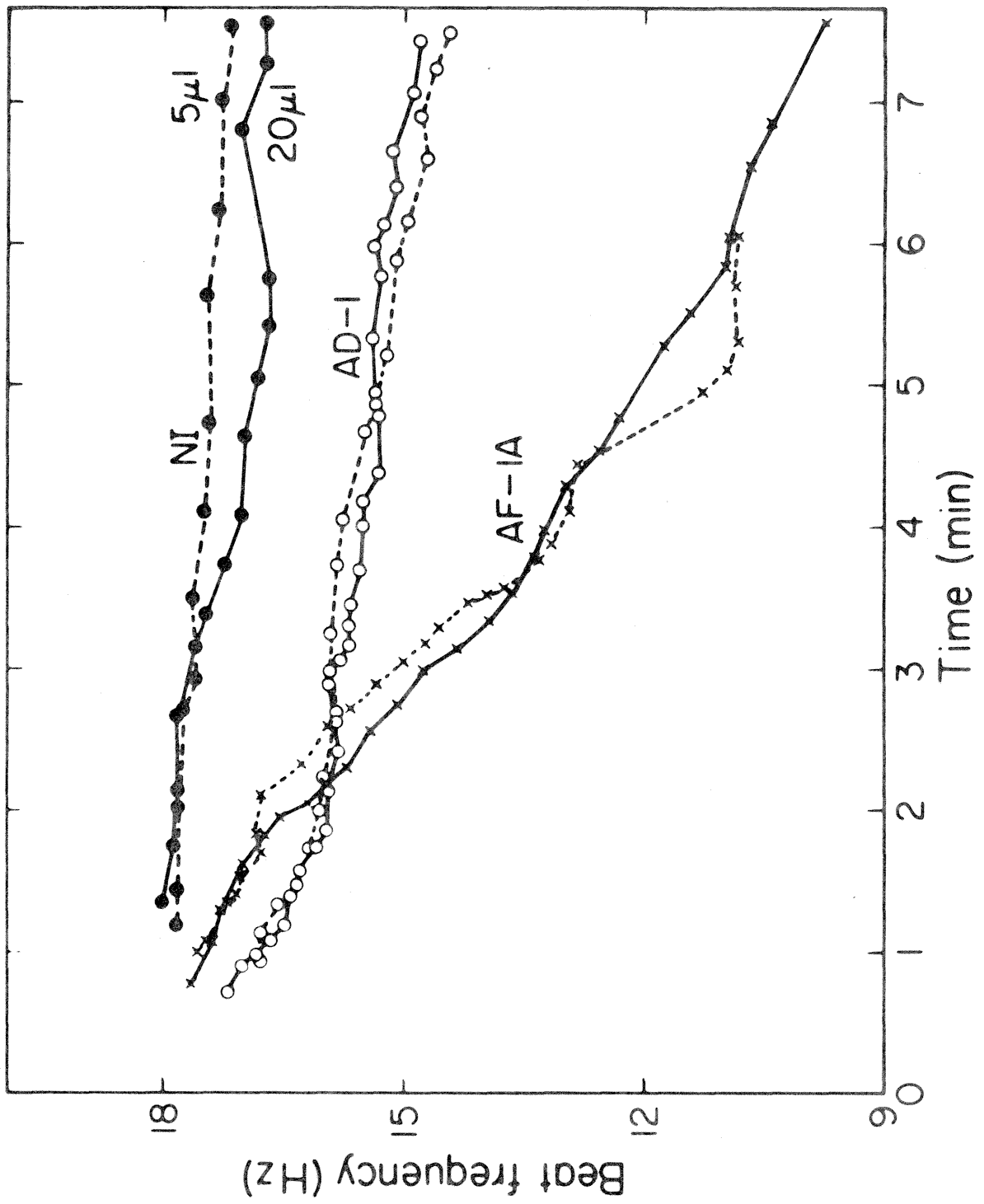


Figure 3-7. Effects of antibody preparations on the bend angles of the same reactivated spermatozoa studied in Figure 3-6. The curves are labeled as in Figure 3-6, and dashed and solid lines are used as in Figure 3-6 to identify the two spermatozoa studied with each antibody preparation.

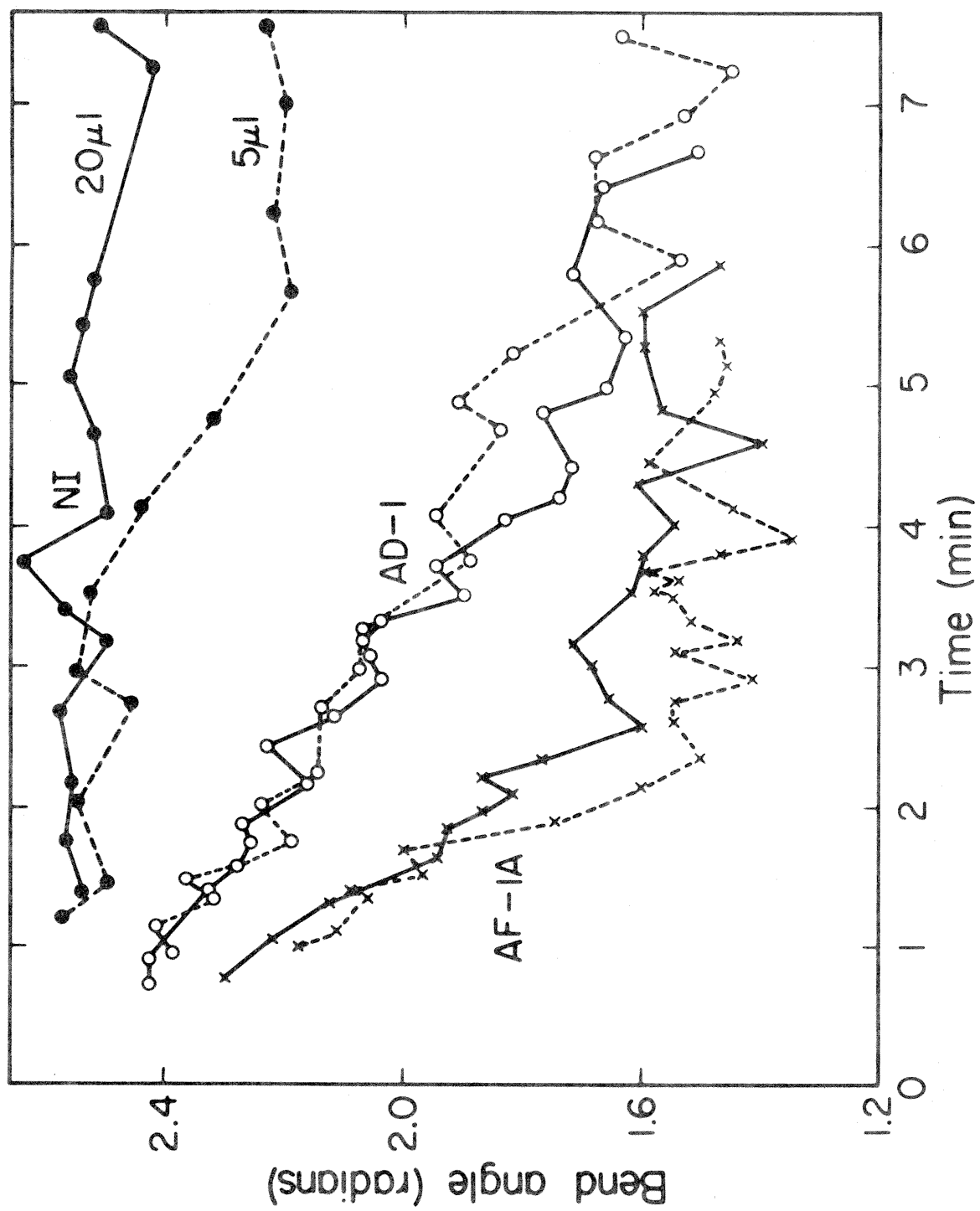
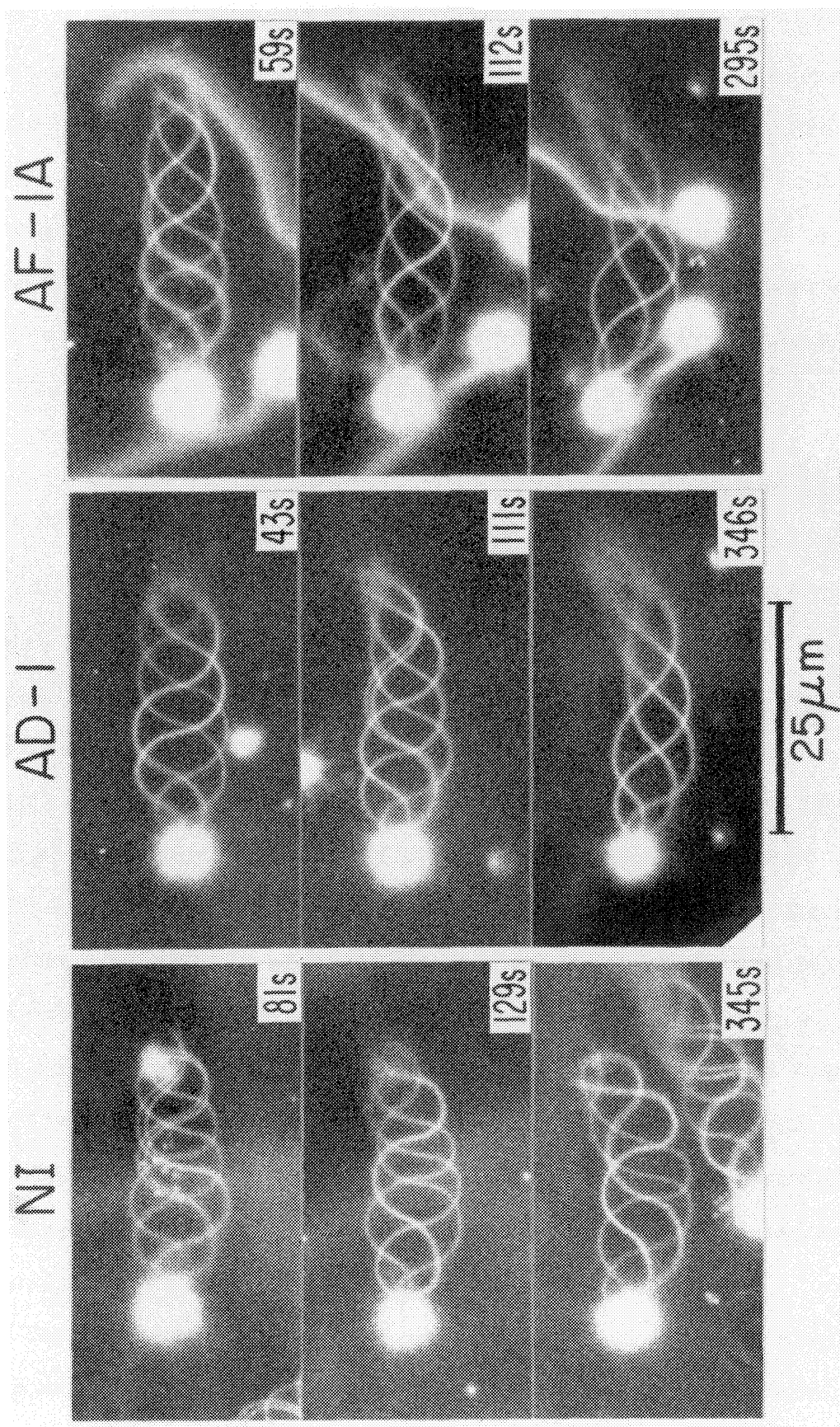


Figure 3-8. Samples of the photographs used to obtain the bend angle measurements shown in Figure 3-7. NI represents the spermatozoon exposed to $20 \mu\text{l ml}^{-1}$ of the non-immune serum preparation, NI. AD-1 represents the spermatozoon exposed to AD-1 and represented by the open circles with solid lines in Figures 3-6 and 3-7. AF-1A represents the spermatozoon exposed to AF-1A and represented by the x's with dashed lines in Figures 3-6 and 3-7.



the experiment. With $5 \mu\text{l ml}^{-1}$ of AF-1A, the beat frequency continued to decrease and reached a value about 40% less than the original frequency by the time the movements became so erratic that the flagellum stuck to the surface. In terms of movement-inhibitory activity for a given concentration, AD-1 appears to be almost an order of magnitude weaker than AF-1A, except for the initial decrease in beat frequency, where the effects of AD-1 are relatively greater and appear to correspond to about one-half the activity of AF-1A.

DISCUSSION

Antigenic determinants

AD-1 and AF-1A have approximately equal abilities to precipitate dynein 1, but AD-1 has much less effect on motility, and its ability to precipitate fragment 1A and to inhibit the ATPase activity of dynein 1 or fragment 1A was too low for us to detect. Presumably most of the antigenic determinants for AD-1 on the dynein 1 molecule are too far from the active ATPase site to induce the formation of antibody molecules which modify the function of the active ATPase site. With the smaller tryptic fragment of dynein 1, fragment 1A, a larger fraction of the antigenic sites must lie sufficiently close to the ATPase site so that they induce antibodies which modify the function of the ATPase site. However, the ability of dynein 1 to give rise to another tryptic fragment (fragment 1AS), which retains ATPase activity but which was not precipitated or inhibited by AF-1A indicates that the major antigenic sites on fragment 1A do not include the active ATPase site itself, which may be a highly conserved and poorly antigenic region of the dynein 1 molecule.

The much lower inhibition of movement which was obtained with AD-1, compared to AF-1A, indicates that antibody binding by dynein 1 is not, by itself,

sufficient to give a strong inhibition of movement. Inhibition of movement may require a more specific interference with functionally active sites on the dynein molecule. However, this conclusion must be accepted only tentatively, as we have not excluded the alternative explanation that the principal antigenic sites on the isolated dynein molecule are not exposed when dynein is in situ in the axoneme.

Some species specificity has previously been found for the effect of AF-1A on the movement of reactivated spermatozoa (26). Complete inhibition of the movement of reactivated spermatozoa by AF-1A occurred only in the same species used to obtain the antigen for preparation of AF-1A. In other species, including S. purpuratus, low frequency (≤ 1 Hz) bending near the basal end of the flagellum continues even after extended exposure to high concentrations of AF-1A. This species specificity may be related to the differing capabilities of AF-1A to inhibit the ATPase activity of dynein 1 from various species, although little difference was found between the three Japanese species studied by Ogawa and Mohri (24). Such a correlation would strengthen the conclusion that inhibition of movement requires interference with the ATPase active sites on the dynein 1 molecule.

If allowance for the effects of species specificity is made, the relative effectiveness of an anti-fragment 1A preparation and an anti-dynein 1 preparation for inhibition of movement would show an even greater difference than is indicated by our results (Figs. 3-6 and 3-7) comparing the effects of AD-1 on the homologous species and AF-1A on a heterologous species.

Inhibition of movement-coupled ATP dephosphorylation

AD-1 can be added to the list of agents which inhibit the movement-coupled

ATP dephosphorylation of suspensions of reactivated spermatozoa, but do not directly inhibit dynein ATPase activity. If movement-coupled ATP-dephosphorylation is considered to be measured by the difference between the rates of ATP dephosphorylation by motile and broken sperm preparations (3), both AD-1 and AF-1A appear to inhibit the movement-coupled ATP dephosphorylation. The additional inhibition of the ATP dephosphorylation of broken spermatozoa by AF-1A but not by AD-1 may then be associated with the ability of AF-1A to directly inhibit some part of the ATPase activity of dynein 1.

The movement-coupled ATP dephosphorylation represents an activation of the ATPase activity of flagellar dynein by tubule interactions which occur during movement. When the motility of a reactivated sperm suspension is eliminated by 0.1 M NaHCO_3 (6) or by trypsin-distintegration (7), a larger fraction (77% or 82%) of the ATP-dephosphorylation is inhibited, compared to the 67% inhibition obtained by sperm breakage. Since these agents, like sperm breakage, do not directly inhibit dynein ATPase activity, these results indicate that some part of the ATP-dephosphorylation measured with broken spermatozoa represents activation of dynein ATPase by interaction with flagellar tubules, and should perhaps be included in the movement-coupled ATP dephosphorylation (6, 7). In this case, our results (Table 3-3) with the antibody preparations indicate that AF-1A inhibits a larger fraction of the tubule-activated or movement-coupled ATP dephosphorylation than does AD-1, which is consistent with our observations on the inhibition of motility by these preparations.

Comparison with Colobocentrotus

Our observation that AD-1 and AF-1A inhibit the amplitude of flagellar bending, as well as and sometimes more strongly than, the beat frequency, contrasts

with observations on Colobocentrotus spermatozoa, where AF-1A could reduce the beat frequency by more than 90% with little change in waveform (11). We have made some preliminary observations on reactivated spermatozoa from another sea urchin, Lytechinus pictus, which also indicate a strong inhibition of bend amplitude by AF-1A. The response of Colobocentrotus spermatozoa may be a relatively unique feature of this species. Colobocentrotus spermatozoa have previously been found to be unique in their insensitivity to calcium ion concentrations (5). Their response to KCl extraction of the outer dynein arms by a reduction in beat frequency with no change in waveform (8), which is similar to their responses to anti-fragment A serum, may also be unique, as attempts to obtain this result with spermatozoa from several other sea urchin species have been unsuccessful.¹ The mechanisms which regulate bend angle and symmetry appear to be less easily disrupted in Colobocentrotus spermatozoa than in other species.

Inhibition of bend angle by anti-dynein

The inhibition of bend angle by AD-1 and AF-1A is a significant new result. Previously, the bend angle, but not the beat frequency, of reactivated spermatozoa was found to be inhibited by CO₂ (6). Flagellar bending was completely inhibited in solutions containing 0.1 M NaHCO₃, while the active sliding of tubules from trypsinized axonemes and the ATPase activity of dynein 1 were not inhibited. It was concluded that CO₂ acted on some part of the axoneme which is involved in converting active sliding into flagellar bending. This conclusion agreed well with the observations on Colobocentrotus spermatozoa where the frequency, but not the amplitude, of beating was reduced by anti-fragment 1A and by extraction

¹ Unpublished observations by C. J. Brokaw (Lytechinus pictus); I. R. Gibbons (Tripneustes gratilla); and M. Okumo (Hemicentrotus pulcherrimus).

of dynein 1. It supports a model in which the dynein arms produce active sliding, and other structures, such as the radial spokes and their connections to the central pair of tubules which have been described in detail by Warner and Satir (30), convert active sliding into oscillatory bending and regulate the amplitude of bending. This conclusion and model must now be reexamined, since we have found that bend angle can be inhibited by AD-1 and AF-1A, which are expected to act only on dynein 1 of the flagella. Figures 3-6 and 3-7 show, in fact, that a large decrease in bend angle can be caused by AD-1 with little change in beat frequency, and the results are not very different from similar measurements on spermatozoa exposed to NaHCO_3 (2).

The possibility that the amplitude of flagellar bending may be determined in part by the activity of the dynein arms is strengthened by the observation that AF-1A, which inhibits ATPase activity of dynein, is more effective than AD-1 at inhibiting the bend angle. However, further supporting evidence is required before this conclusion can be fully accepted. The exact mechanism of the inhibitory effects of AF-1A and AD-1 is not known, nor is it known with certainty that the dynein arms which generate active sliding are the only locus of proteins which bind these antibodies. Additional information might be gained by preparing antibodies to a smaller fragment of the dynein molecule containing the active ATPase site, such as fragment 1AS, and by electron microscope examination of axonemes which have been incubated with ferritin-labeled antibody (25).

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Chapter 4

INDUCED AND SPONTANEOUS ANTIBODIES TO TUBULIN

To continue the exploration of antibodies as probes of flagellar movement, I undertook a study of anti-tubulin antibodies. Tubulin-affinity chromatography was used as a one-step purification of these antibodies. In the course of this study, I detected, by affinity chromatography, low levels of spontaneous anti-tubulin antibodies in normal rabbit sera. These observations of spontaneous anti-tubulins were confirmed by Karsenti et al. (9) who found low-level, nonprecipitating anti-tubulins in a variety of nonimmune mammalian sera. These spontaneous anti-tubulins in indirect immunofluorescence were able to stain vinblastine-induced paracrystals but gave only diffuse and indistinct staining of cultured mouse cells under conditions where induced anti-tubulins gave distinct staining of cytoplasmic microtubules. Spontaneous antibodies to another important and ubiquitous structural protein, a constituent of (100 Å) intermediate filaments, have been found in rabbit and human normal sera (2, 7, 10, 18).

This fourth Chapter reports the preparation and purification of induced anti-tubulin antisera as well as the purification from preimmune sera of spontaneous antibodies to tubulin. The properties of these antibodies are characterized with respect to their class, their specificity for tubulin, and their binding to axonemes. Chapter 5 will present the effects of these antibodies to tubulin on flagellar motility and microtubule sliding.

MATERIALS AND METHODS

Concentrated spermatozoa from Strongylocentrotus purpuratus were obtained as described previously (15). For preparations of axonemes and axonemal proteins, the spermatozoa were washed once with seawater, and then either used at once for preparation of axonemes or stored with 50% glycerol at -15°C (16). Dynein 1 was the same as that used in our previous experiments (15).

Preparation of tubulins for antigens and immunoabsorbent

Outer doublet microtubule protein was obtained from axonemes by the sequential solubilization procedure of Stephens (21). First, axonemes were extracted twice with 0.5 M KCl, 10 mM Tris-Cl, 0.1 mM DTT,¹ at pH 8.0, at room temperature for 30 min to remove most of the dynein and central pair tubulin. The pellets after a 30 min, 20,000 g centrifugation were then extracted at room temperature with 10 mM Tris-Cl at pH 8.0 for 30 min to remove minor components, and centrifuged for 30 min at 20,000 g. The resulting pellets were then extracted with 1 mM Tris-Cl at pH 8.0 at 37°C for 30 min and centrifuged at 20,000 g; the pellet was re-extracted with the same buffer at 45°C and centrifuged. The supernatants from both of these 1 mM Tris-Cl extractions contained outer doublet tubulin. A third tubulin preparation was obtained from the pellet remaining after the second outer doublet extraction. This pellet was suspended in distilled water, dialyzed against water, and lyophilized; it was labeled Residue tubulin. The three preparations were shown to have ATPase activities less than 0.01, as compared to an activity of 1.48 (μmol phosphate liberated per mg protein per min) for the combined 0.6 M KCl extracts. All three tubulin preparations were dialyzed against distilled water, lyophilized, and shown to be pure by SDS polyacrylamide electrophoresis which showed only tubulin, and no dynein bands. For some of the immunodiffusion assays, outer doublet tubulin was alkylated by carboxymethylation (3) with recrystallized iodoacetic acid.

¹ Abbreviations used in this Chapter: ATP, adenosine triphosphate; BSA, bovine serum albumin (fraction V); DTT, dithiothreitol; Fab, antigen binding fragment of immunoglobulin; Fc, crystallizable fragment of immunoglobulin; GTP, guanosine triphosphate; EGTA, ethyleneglycol-bis-(β -aminoethyl ether) N,N'-tetraacetic acid; IgG, immunoglobulin; MES, 2(N-morpholino)ethane sulfonic acid; PBS, phosphate buffered saline; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; and TCA, trichloroacetic acid.

Tubulin was further purified by preparative gel electrophoresis. A total of approximately 12.5 mg outer doublet tubulin was electrophoresed in 6% polyacrylamide SDS gels (a total of 36 tube gels were run). One gel from each electrophoresis run was stained to determine the relative mobility of the tubulin band. Tubulin was sliced out of each gel, the gel homogenized in 8 M urea, 0.36 M Tris-Cl, pH 8.6, and extracted at 37°C overnight. The acrylamide was pelleted by centrifuging the mixture at 20,000 g for 30 min, the supernatant was saved and concentrated. The supernatant contained only tubulin. Similarly purified tubulin was also used as an immunoabsorbent. Results of gel electrophoresis of this electrophoretically purified tubulin are shown in Fig. 4-1 (gel a).

Immunizations

Since tubulin is a highly conserved protein in a wide range of eukaryotes (14), sodium dodecyl sulfate (SDS) was routinely used to dissolve the antigen in an attempt to provide a maximum of denatured, "new" determinants to the rabbits' immune system. The use of SDS during immunization was similar to procedures of Lazarides (12). Complete Freund's (Difco, Detroit, Michigan) was used as an adjuvant. Table 4-1 summarizes the injection schedules for the five rabbits used in this study. Rabbits 1 and 2 were injected with electrophoretically purified outer doublet tubulin; rabbits 3 and 4 were injected with Residue tubulin; and rabbit 5 was injected with whole axonemes that had been extracted twice with 0.6 M KCl and extracted once in low ionic strength buffer (16) in order to remove most of the dynein. Figure 4-1 (gel b) shows that this material consisted mainly of tubulin.

Anti-dynein 1 serum was the same as that used in our previous experiments (15). Anti-fragment A serum was the gift of Dr. Kazuo Ogawa (17).

All sera were routinely stored at 4°C in 1% NaN_3 .

Figure 4-1. 6% polyacrylamide SDS gels showing examples of tubulins used in this study. Gel a: electrophoretically purified outer doublet tubulin (60 μ g) used to inject rabbits 1 and 2 and as an immunoabsorbent. Gel b: extracted axonemes (50 μ g) used to inject rabbit 5.

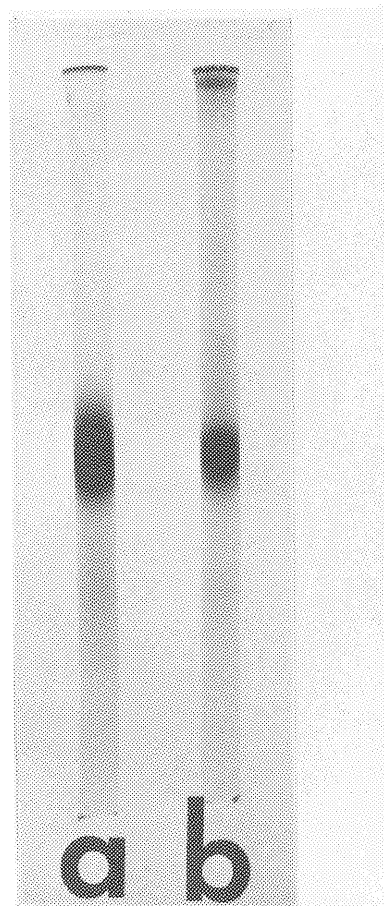


Table 4-1

Injection Schedules for the Rabbit Sera

Rabbit	Antigen preparation	1st injection	2nd injection	3rd injection	4th injection	Bleeding	Ouchterlony
1	OD tubulin SDS PAGE	day 0 0.48 mg 0.1% SDS CF tp,im,sc	day 16 0.31 mg 0.1% SDS CF sc	day 50 0.48 mg 0.1% SDS no adjuvant im	day 78 0.36 mg 0.1% SDS no adjuvant im	days 85,92,100	negative
2	OD tubulin SDS PAGE	day 0 0.72 mg 0.1% SDS CF tp,im,sc	day 16 0.31 mg 0.1% SDS CF sc	day 33 0.31 mg 0.1% SDS no adjuvant sc	day 50 0.18 mg 0.1% SDS no adjuvant im	days 65,71,78	positive
3	Residue tubulin	day 0 5.0 mg 0.05% SDS CF tp,im,sc	day 24 4.1 mg 0.1% SDS no adjuvant ip	—	—	day 38	positive
4	Residue tubulin	day 0 6.0 mg 0.1% SDS CF tp,im,sc	day 14 1.8 mg 0.1% SDS no adjuvant sc	day 31 3.0 mg 0.1% SDS no adjuvant im	day 59 1.5 mg 0.1% SDS no adjuvant im	days 66,73	positive
5	Axonemes, extracted	day 0 20.0 mg 0.1% SDS CF tp, im,sc	day 14 8.0 mg 0.1% SDS no adjuvant sc	day 31 3.0 mg 0.1% SDS no adjuvant im	—	days 45,62	positive

Table 4-1 (continued)

Adult female New Zealand White rabbits were used in all cases. Ouchterlony immunodiffusion was performed with whole sera against purified alkylated outer doublet tubulin. The following abbreviations are used: OD, outer doublet; SDS PAGE, tubulin eluted from SDS polyacrylamide gel slices; CF, complete Freund's adjuvant; tp, rear toepad; im, femoral intramuscular; sc, subcutaneous along the back; ip, intraperitoneal.

Purification of immunoglobulins

IgG was purified from rabbit sera by the method described by Garvey et al. (5). Purity was confirmed by 12% polyacrylamide SDS gel electrophoresis which revealed only the expected bands for the heavy and light chains of IgG.

Preparation and purification of Fab fragments

Monovalent Fab fragments were obtained from immune and preimmune sera (from rabbit 2) by a limited papain cleavage (5). Proteolysis was accomplished with approximately 1 mg papain (Sigma, St. Louis, Missouri, 2X recrystallized) per 100 mg IgG at 37°C for 12 hr. The reaction was stopped with an excess of cysteine. Fraction I Fab fragments were purified by chromatography over a carboxymethylcellulose column using a step-gradient of acetate. Figure 4-2 shows the elution patterns of immune and preimmune samples. The peak eluting at 0.01 M acetate was concentrated and shown to not contain any Fc activity as assayed by goat anti-rabbit IgG heavy chain sera (Cappel Laboratories, Cochranville, Pennsylvania) but was precipitated by another antiserum specific for Fab (Cappel), as shown in Fig. 4-3.

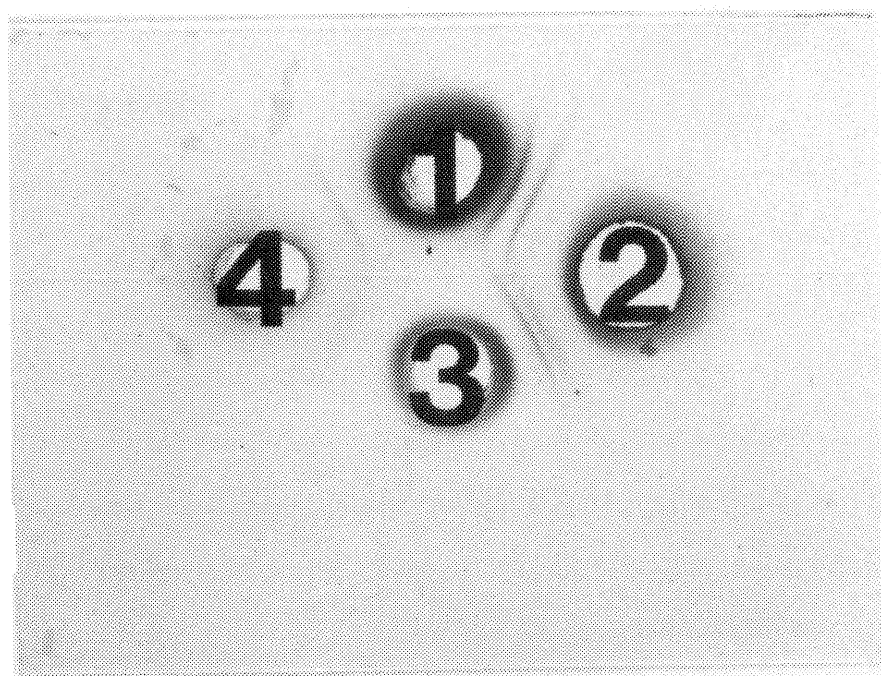
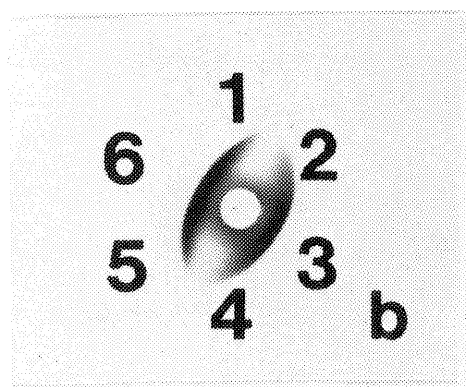
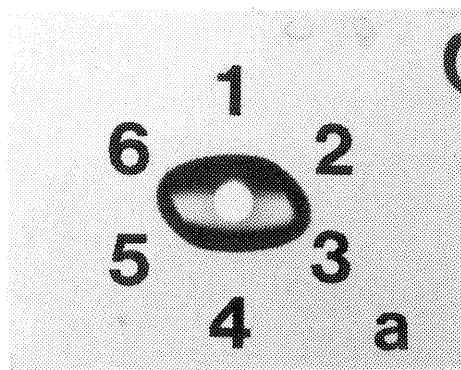
Tubulin-affinity chromatography

The procedure used to couple electrophoretically purified outer doublet tubulin to Sepharose was similar to that recommended by Pharmacia (20). Cyanogen bromide activated Sepharose 4B (Pharmacia, Uppsala, Sweden, and Sigma, St. Louis, Missouri) was hydrated in 1 mM HCl. To this was added electrophoretically purified outer doublet tubulin in 0.1 M carbonate/bicarbonate, 0.5 M NaCl, pH 9.2 buffer. Coupling was accomplished at 4°C for 24 hr. The slurry was washed with the carbonate-bicarbonate buffer. Unreacted active groups were blocked by treatment with an excess of 0.5 M ethanolamine, pH 9.2, at room temperature

Figure 4-2. Carboxymethylcellulose chromatography of papain digested immunoglobulins from immune (solid circles) and preimmune (open circles) sera from rabbit 2. A step gradient of acetate was used to elute the different fractions.

Figure 4-3. Immunodiffusion of carboxymethylcellulose-separated fractions of immune and preimmune IgGs (see Fig. 4-2). Samples were allowed to diffuse for 38 hr at room temperature after which they were dialyzed with PBS and stained with Coomassie Blue. Fig. 4-3a: well #1, fraction I from preimmune serum; #2, preimmune fraction II; #3, preimmune fraction IV; #4, immune fraction I; #5, immune fraction II; #6, immune fraction IV; center well, goat anti-rabbit IgG (Fab)₂ (Cappel Laboratories). Fig. 4-3b: wells #1-6, same as Fig. 4-3a; center well, goat anti-rabbit IgG heavy chain (Cappel Laboratories).

Figure 4-4. Immunodiffusion assay. Samples were allowed to diffuse 45 hr at room temperature after which they were dialyzed with PBS and stained with Coomassie Blue. Well #1, outer doublet flagellar tubulin from Strongylocentrotus purpuratus; #2 and #4, whole anti-tubulin sera from two different bleedings; #3, outer doublet flagellar tubulin from Lytechinus pictus.



for 3 hr. The product was alternately washed with pH 9 borate and pH 4 acetate buffers; both buffers also contained 0.5 M KCl. Typically, ammonium sulfate precipitated samples were dialyzed against buffer I containing 0.1 M NaCl, 20 mM Tris-Cl, 2 mM EGTA, 0.01% NaN_3 , pH 7.5, and were slowly run into the columns in the same buffer. The columns were continued in buffer I until the major, non-retained absorbance (at 280 nm) peak was fully eluted. The columns were then washed with several column volumes of buffer II containing 0.6 M NaCl, 20 mM Tris-Cl, 2 mM EGTA, 0.01% NaN_3 , pH 7.5, in order to wash off any other non-specifically aggregating material. Final elution was effected by a single pH step-down with buffer III which was buffer II brought to pH 2.2 with HCl.

Recovered antibody fractions were pooled and concentrated by ultra-filtration over an Amicon PM-10 membrane (Amicon, Lexington, Massachusetts), dialyzed against appropriate buffers for subsequent experiments, and stored in 0.01% NaN_3 at 4°C. Special care was taken throughout so that no cross-contamination between immune and preimmune samples occurred, i.e., two separate, identical affinity columns and two different Amicon membranes were employed.

Radioiodine binding assays

Tubulin-affinity purified antibodies--intact antibodies and Fab fragments from rabbit 2--and anti-BSA immunoglobulins were radioiodinated (New England Nuclear, Boston, Massachusetts) in isotonic phosphate using the chloramine T method (8). Free iodine was removed by desalting the samples over Sephadex G-25. Equivalent quantities of the immune and preimmune anti-tubulins (0.042 μg each), Fab fragments (0.028 μg each), or anti-BSA (0.150 μg) were mixed with varying dilutions of axonemes in PBS. Unlabeled BSA (2 mg/ml final concentration) was used as a carrier protein. The final volume per reaction tube was adjusted to 250 μl and the tubes were incubated overnight at 4°C with slow end-over-end

agitation. After incubation the tubes were centrifuged at 1400 g. Supernatants were separated from the axoneme pellets, and both were counted on a Nuclear Chicago gamma counter. The zero level binding was determined by measurements on blank preparations containing no axonemes. Maximum possible levels of binding were determined by mixing the labeled antibodies with 10% (final concentration) TCA and BSA (3.3 mg/ml, final concentration) and incubating at 4°C overnight. The pellets after centrifugation were counted; these represented 100% TCA precipitable counts.

Immunofluorescence

Chick embryo fibroblast cells were fixed in formaldehyde, incubated with equivalent quantities of immune or preimmune affinity-purified anti-tubulins, and observed by indirect immunofluorescence (11) in the laboratory of Dr. Elias Lazarides. Whole spermatozoa from S.purpuratus were allowed to settle on coverslips, demembrated with 1% Triton X-100, stabilized in 20 mM MES, 4% PEG, 70 mM NaCl, 1 mM EGTA, 0.5 mM MgCl₂, 2.5 mM GTP, pH 6.9, incubated with equivalent quantities of tubulin-affinity purified antibodies, and observed by indirect immunofluorescence.

Other procedures

Standard methods included: Ouchterlony immunodiffusion (19) and immunoelectrophoresis (5), SDS polyacrylamide gel electrophoresis (22) as modified by Gibbons et al. (6), determination of protein by the method of Lowry (1, 13), and assay for ATPase activity by measurement of inorganic phosphate (4).

RESULTS

Immunizations

Sera from the five rabbits injected with tubulins (summarized in Table

4-1) were assayed for anti-tubulin activity by immunodiffusion against outer doublet tubulin. Rabbits 2-5 all produced tubulin-precipitating sera. An example of precipitation by anti-tubulin antibody of tubulin from S. purpuratus and L. pictus is shown in Fig. 4-4. Not all injections were successful: over four years I have attempted various sea urchin tubulin immunizations of 17 rabbits; of these only 5 (rabbits 2-5 and one other) tested positive. The negative immunizations included several rabbits injected with electrophoretically purified tubulin (a procedure which was also successful once) and various preparations of α - and β -tubulins (three each).

Purification of anti-tubulins by affinity chromatography

Figure 4-5a shows an example of purification of anti-tubulin antibodies by tubulin-affinity chromatography of equivalent volumes of whole, untreated immune and preimmune sera. Note that the preimmune serum yielded a relatively small amount of protein. However, if the same preimmune serum was preconcentrated threefold and equivalent volumes of the two samples again chromatographed, as shown in Fig. 4-5b, a quantity of preimmune antibody comparable to the immune antibody was eluted. When equivalent amounts of the immune and preimmune ammonium sulfate precipitates from each of the four rabbits (rabbits 2-5) were chromatographed, approximately equivalent quantities of anti-tubulins were collected. Similar elution patterns were also obtained with immune and preimmune monovalent Fab fragments. Examples of these are shown in Figs. 4-6a and 4-6b.

The material recovered from tubulin-affinity chromatography is IgG

Immunoelectrophoresis of equivalent quantities of affinity-purified immune and preimmune material was shown to be exclusively IgG as shown in Fig. 4-7. No class of antibody other than IgG was detected by specific goat anti-rabbit antibodies to IgG, IgM, and IgA (Cappel Laboratories, Cochranville, Pennsylvania).

Figure 4-5. Tubulin-affinity chromatography of 2 ml samples of sera. Fifty drop fractions were collected and absorbance was measured at 280 nm. Fig. 4-5a: 2 ml of whole immune serum compared with 2 ml of whole preimmune serum. Fig. 4-5b: 2 ml of whole immune serum compared with 2 ml of preimmune serum that had been preconcentrated approximately threefold with an Amicon PM-10 membrane.

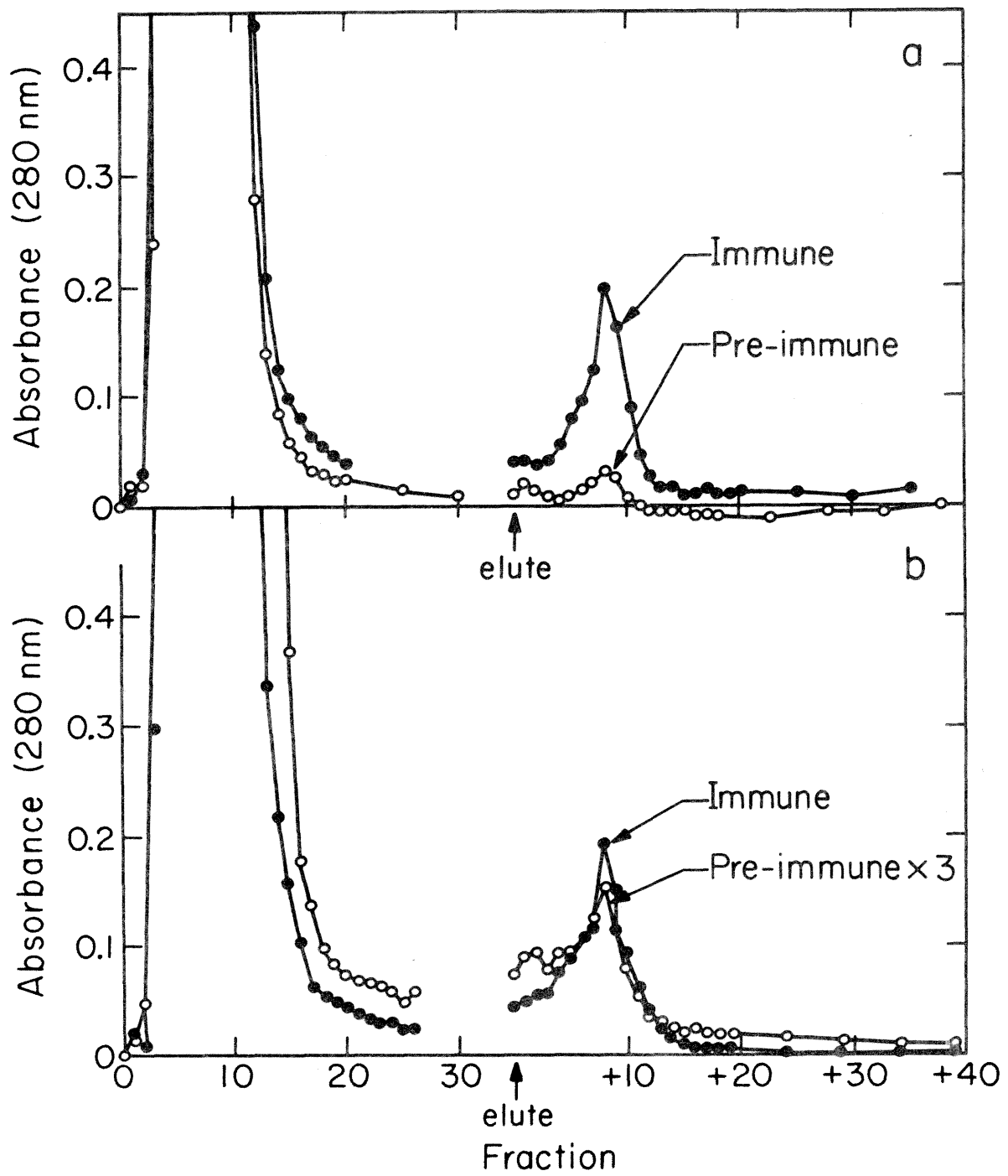


Figure 4-6. Tubulin affinity chromatography of ammonium sulfate precipitated IgGs (Fig. 4-6a) and of monovalent Fab fragments (Fig. 4-6b) from immune (solid circles) and preimmune (open circles) sera from rabbit 2. The columns were washed with high salt and then samples were eluted with a pH stepdown (see Methods).

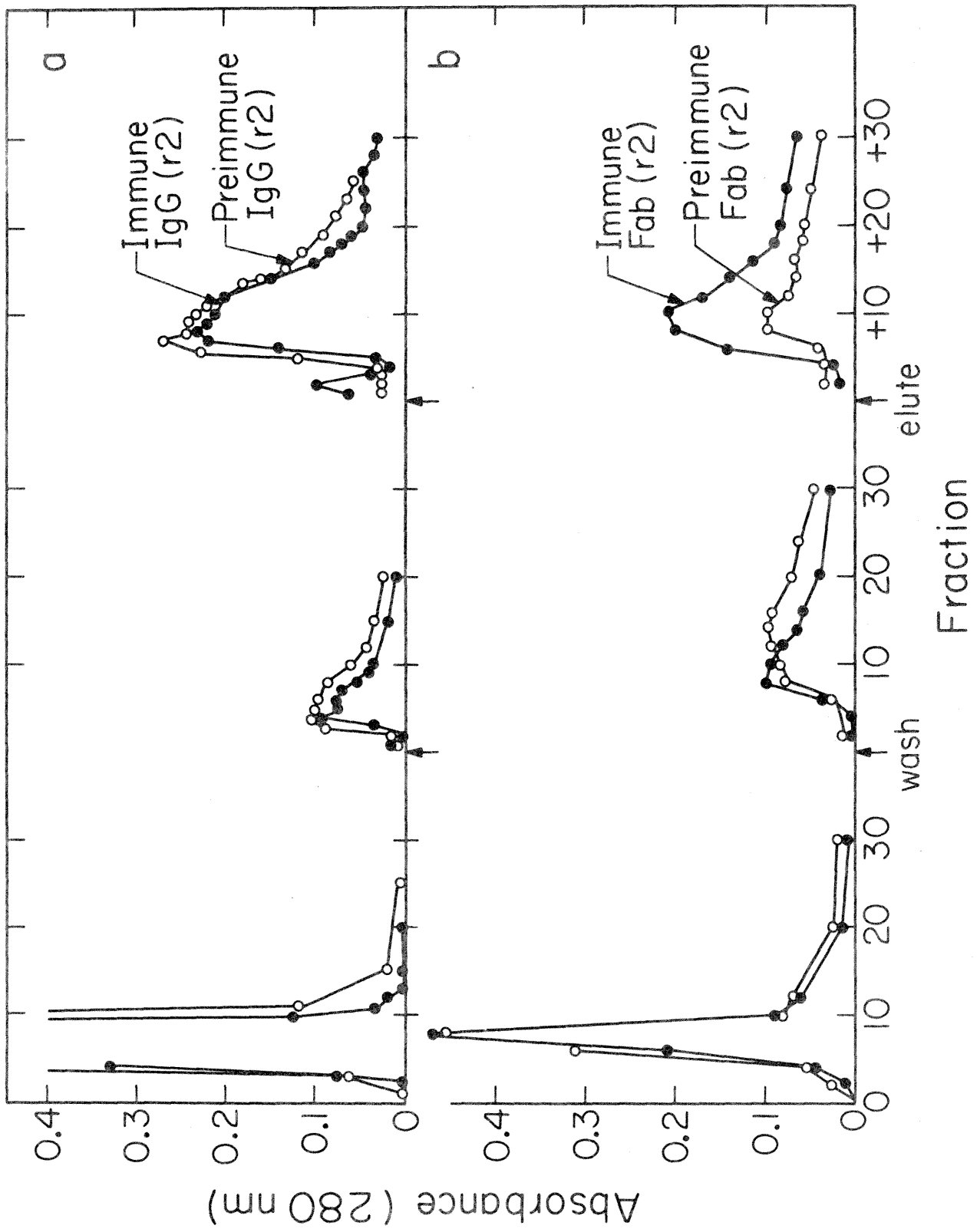
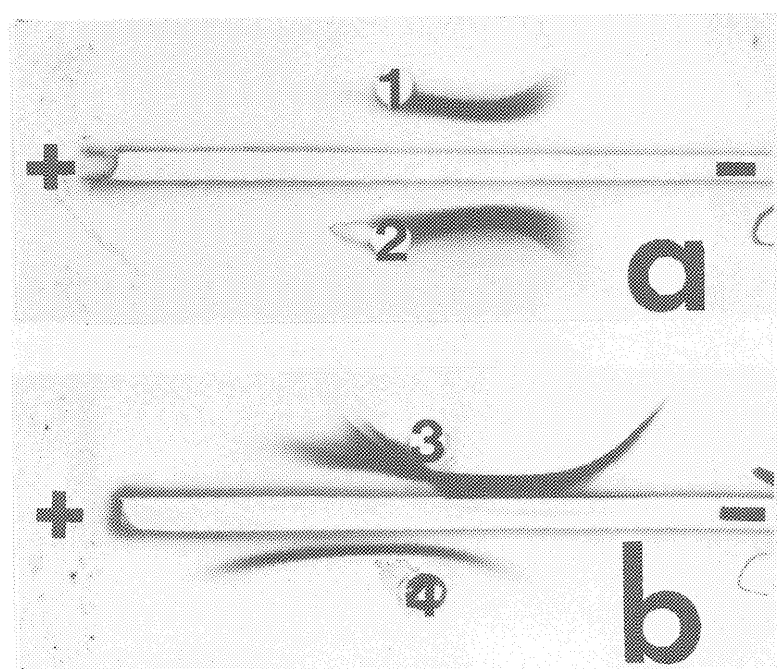


Figure 4-7. Immunoelectrophoresis of serum samples. Samples were electrophoresed at 6 mA/sample for 3 hr followed by diffusion for 48 hr at room temperature against goat anti-rabbit IgG, IgM, and IgA (Cappel Laboratories), center trough. Plates were then dialyzed with PBS and stained with Coomassie Blue. Plate a: affinity-purified anti-tubulins from preimmune (1) and immune (2) sera, both at 0.56 mg/ml. Plate b: whole rabbit serum (3) and rabbit IgA (4).



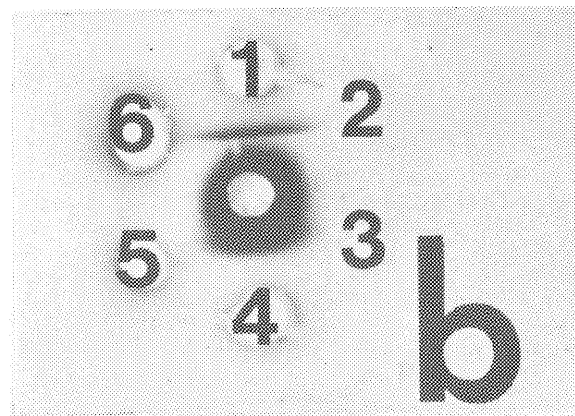
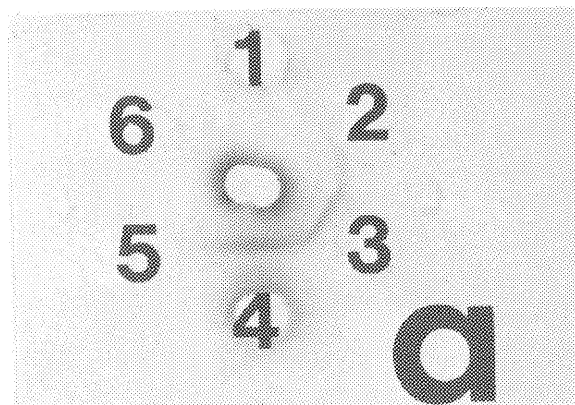
The homogeneity of the purified material was demonstrated further by electrophoresis of samples on 12% polyacrylamide SDS gels. The material retained on the affinity columns electrophoresed as two bands which run identically to IgG heavy and light chains (results not shown).

The immunoglobulins are specific for tubulin

Ouchterlony immunodiffusion assays were performed to test the ability of serum preparations to precipitate different axonemal proteins. Figure 4-8a shows that whole anti-tubulin immune serum formed two detectable precipitin lines against a solution of alkylated tubulin (similar results were obtained with other preparations of tubulin, alkylated or native; lyophilized alkylated outer doublet tubulin redissolved at 3 mg/ml in 0.5 M KCl was used in this study because of its homogeneity and its solubility). Affinity-purified anti-tubulin formed a precipitin line which was identical to the main line formed by the whole immune serum. Neither the preimmune serum nor the affinity-purified preimmune anti-tubulin (at the same protein concentration as the immune samples) formed a detectable precipitin line against tubulin under these conditions. The immunodiffusion shown in Fig. 4-8b indicates that neither of the affinity-purified anti-tubulins precipitated dynein 1. No spurs in the precipitin lines formed by anti-dynein 1 and by anti-fragment A occurred towards the anti-tubulins. This further indicates that the anti-tubulins used in these experiments did not cross-react with dynein 1.

Immunoglobulin-affinity chromatography was used to obtain further evidence that the immunoglobulins were specific for tubulin. Whole gamma globulin fractions were prepared from immune and preimmune sera. These preparations were not further fractionated. Each of these immunoglobulin preparations was coupled to CNBr Sepharose 4B (see Methods). Whole sea urchin sperm axonemes

Figure 4-8. Ouchterlony immunodiffusion assays. Samples were allowed to diffuse 48 hr at room temperature after which they were dialyzed with PBS and stained with Coomassie Blue. Fig. 4-8a: well #1, whole preimmune serum; #2 and #3, affinity-purified anti-tubulin from immune serum (0.56 mg/ml); #4, whole immune serum; #5 and #6, affinity-purified anti-tubulin from preimmune serum (0.56 mg/ml); center well, alkylated outer doublet tubulin (3 mg/ml). Fig. 4-8b: #1, anti-dynein 1; #2 and #3, affinity-purified anti-tubulin from immune serum (0.56 mg/ml); #4, anti-Fragment 1A; #5 and #6, affinity-purified anti-tubulin from preimmune serum (0.56 mg/ml); center well, dynein 1.



were extracted for 30 min at room temperature with a solution containing 0.5 M KCl, 10 mM Tris-Cl, 4 mM MgSO₄, 1 mM CaCl₂, 0.1 mM DTT, at pH 8.0. Gel electrophoresis showed that the 31,000 g supernatant of this extraction was enriched in dynein and also contained tubulin and several other axonemal proteins. This 0.5 M KCl extract was dialyzed overnight at 4°C against a buffer containing 0.5 M NaCl, 0.2 M glycine, at pH 8.0 and portions of it were chromatographed over the immune IgG and preimmune IgG affinity columns in the same buffer; material binding to the columns was eluted by a single pH stepdown to pH 2.8. Fractions were pooled, dialyzed against distilled water, lyophilized, and redissolved for electrophoresis. Figure 4-9 shows optical scans after electrophoresis on 3.5% polyacrylamide SDS gels and Coomassie staining. These low percentage gels were heavily overloaded in order to maximize detection of any dynein and other non-tubulin components. The material which did not bind when passed over either of the columns contained dyneins, tubulin, and at least four other intermediately-banding proteins. The bound protein eluted by the pH stepdown appears to be only tubulin. Based on relative amounts of protein recovered and loaded on the gels, approximately 4% of the tubulin in the sample was retained on the columns. These results suggest that not only the immune immunoglobulins, but also the immunoglobulins from preimmune serum, show antibody affinity for tubulin but for no other proteins in the axonemal extract.

Anti-tubulins bind to axonemes

In order to test whether the affinity-purified anti-tubulins from immune and preimmune sera are able to bind axonemes, a simple radio-binding assay was devised taking advantage of the easily sedimented axonemes. Results in Fig. 4-10 are expressed as percentages of the TCA precipitable counts of ¹²⁵I in the pellet after subtracting nonspecific backgrounds. All of the anti-tubulins (from rabbit 2),

Figure 4-9. Optical scans of 3.5% polyacrylamide SDS gels stained with Coomassie Blue. Gel a: material from 0.5 M KCl axoneme extract which did not bind to preimmune IgG-affinity column (200 μ g). An identical pattern was obtained with material which did not bind to immune IgG column. Gel b: material which bound to immune IgG column (approx. 120 μ g). Gel c: material which bound to preimmune IgG column (approx. 100 μ g).

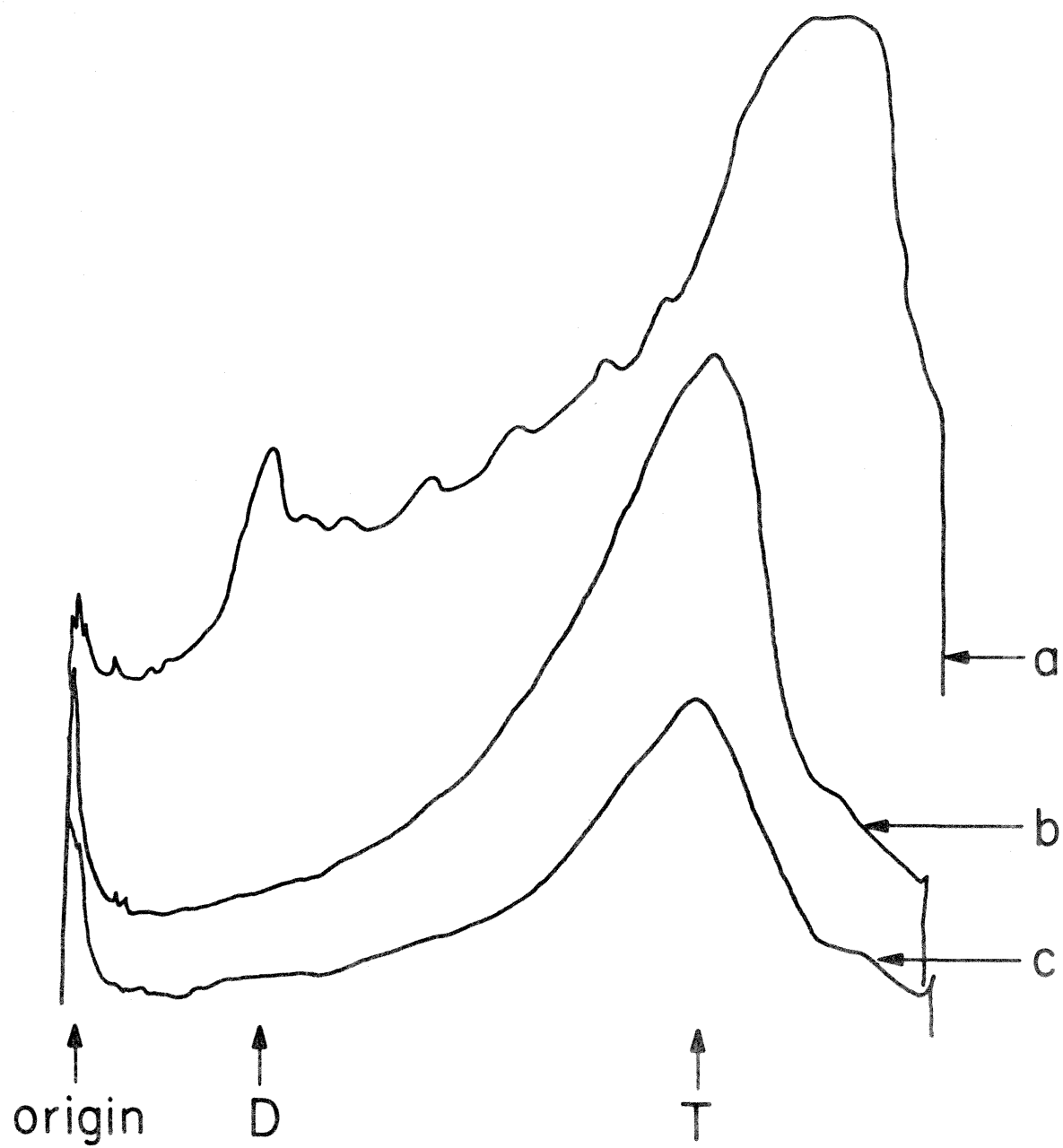
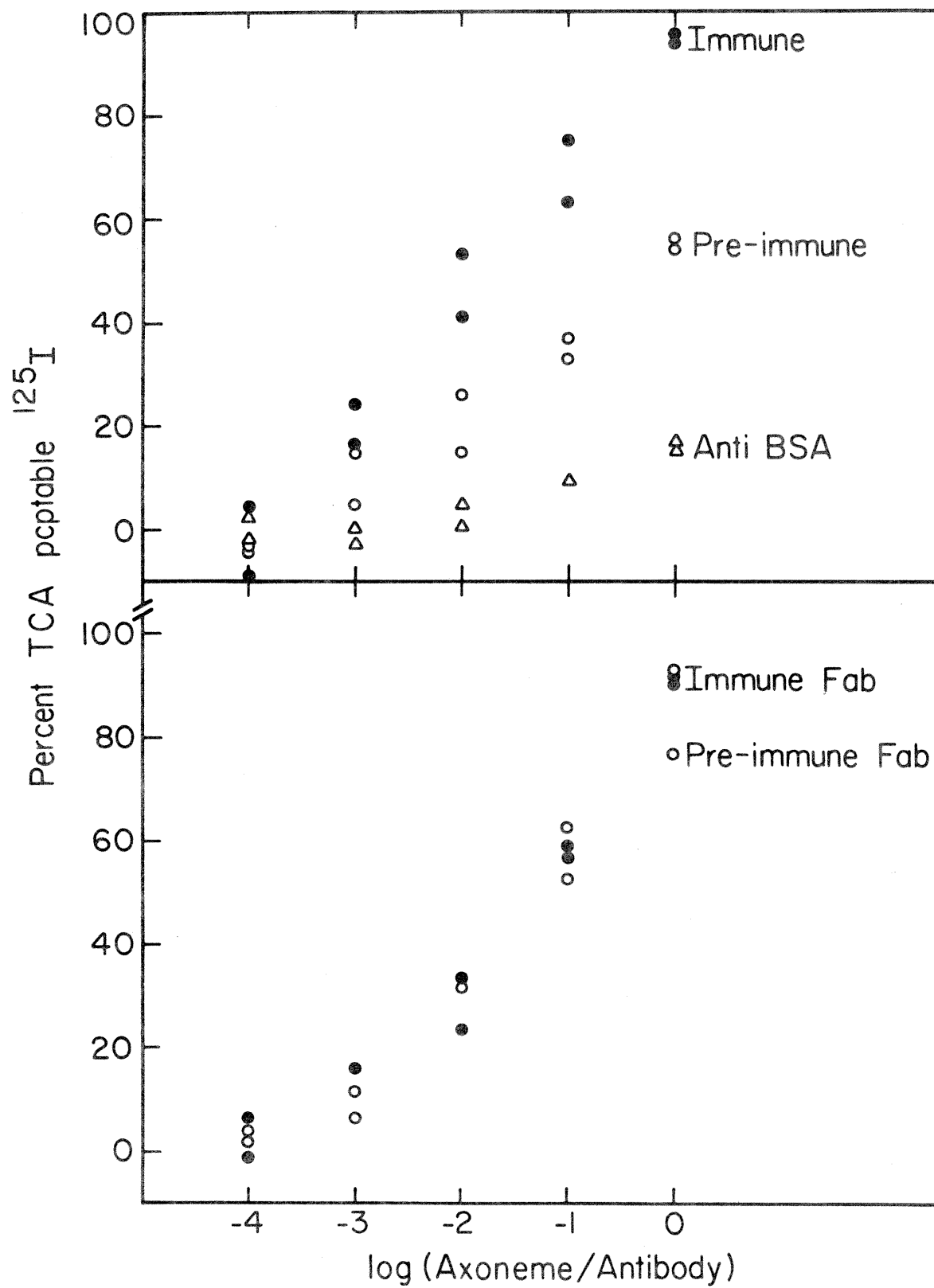


Figure 4-10. Radiobinding assay. Fig. 4-10a: binding to axonemes of affinity-purified intact anti-tubulins from immune and preimmune sera (0.042 μ g each). Precipitation of ^{125}I anti-BSA is also shown to demonstrate that nonspecific trapping of antibodies by the pelleting axonemes was minimal. Fig. 4-10b: binding to axonemes of affinity-purified Fab fragments from immune and preimmune sera (0.028 μ g each). In both figures, results are shown as percentages of TCA-precipitable counts ^{125}I in pellets versus the log of the dilution of axonemes (arbitrary units).



both intact antibodies and Fab fragments from both immune and preimmune sera, bound to axonemes. Although BSA was present as an unlabeled carrier, minimal trapping of ^{125}I anti-BSA was observed. The difference in binding between the whole immune antibodies and the whole preimmune antibodies is significant and may reflect an approximate 100-fold lower affinity for the axonemes by the preimmune anti-tubulins. The ^{125}I Fab fragments were assayed for binding at a different protein concentration than the intact antibodies, therefore amounts of binding of the intact antibodies and by the Fab fragments cannot be compared. No significant difference between the binding of immune Fab fragments and pre-immune Fab fragments was shown by this assay.

Immunofluorescence observations of anti-tubulins on chick fibroblasts and sea urchin spermatozoa

Indirect immunofluorescence was performed in order to further compare the two affinity-purified anti-tubulins. Figure 4-11 compares the fluorescent staining of chick fibroblasts by immune and preimmune anti-tubulins. The affinity-purified immune anti-tubulin gave the characteristic striking visualization of cytoplasmic and spindle microtubules while the affinity-purified preimmune anti-tubulin, at the same concentration, gave a weak, diffuse staining pattern. However, when the anti-tubulins were used to visualize demembranated spermatozoa, as shown in Fig. 4-12, both the immune and the preimmune anti-tubulins stained the flagella but not the heads. It appeared that the immune anti-tubulin more brightly stained the flagella than an equivalent amount of preimmune anti-tubulin.

Figure 4-11. Phase contrast (top) and indirect immunofluorescence (bottom) of chick embryo fibroblasts. Fig. 4-11a: cells incubated with affinity-purified anti-tubulins from immune serum (0.56 mg/ml). Fig. 4-11b: cells incubated with affinity-purified anti-tubulins from preimmune serum (0.56 mg/ml). Photographs were taken at the same exposure and developed under the same conditions for immune and preimmune samples.

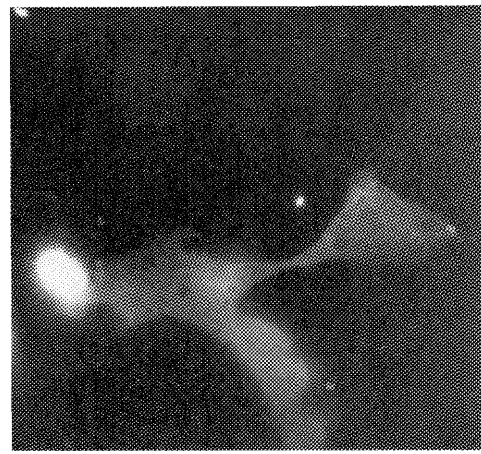
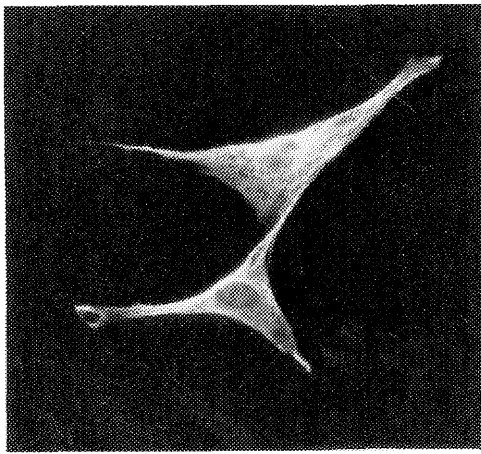
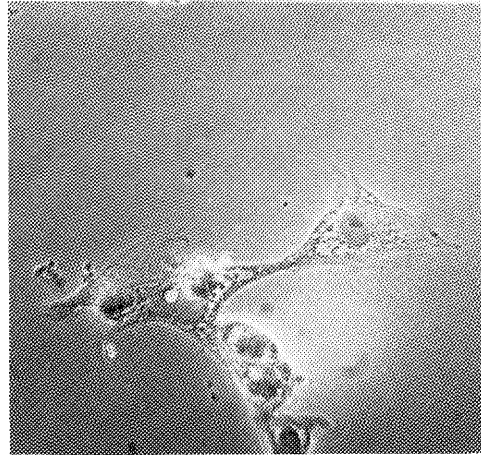
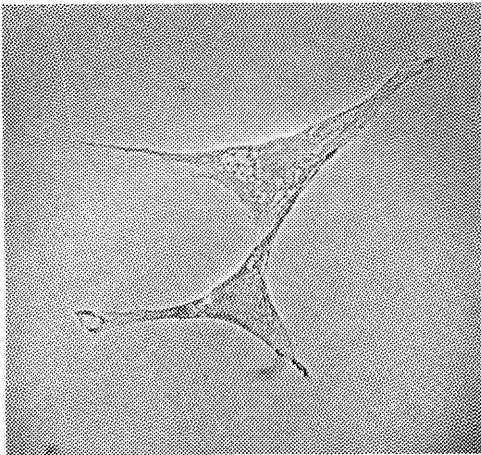
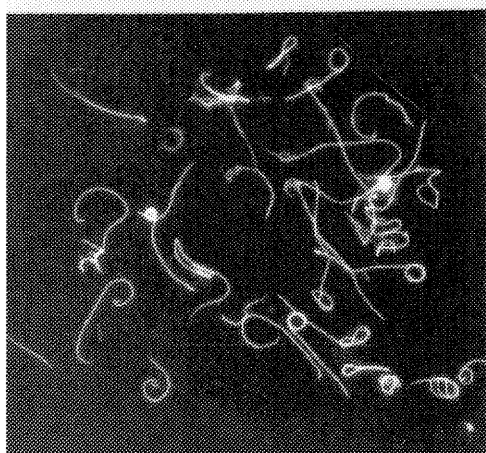
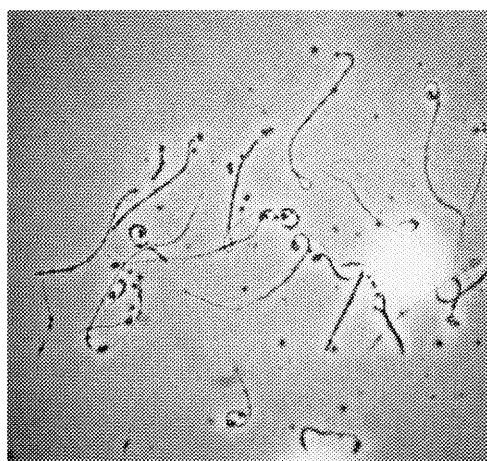
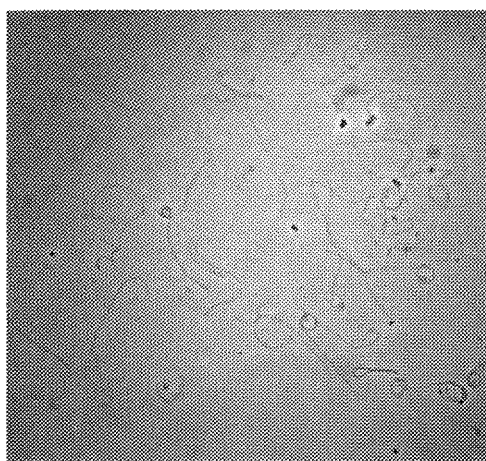
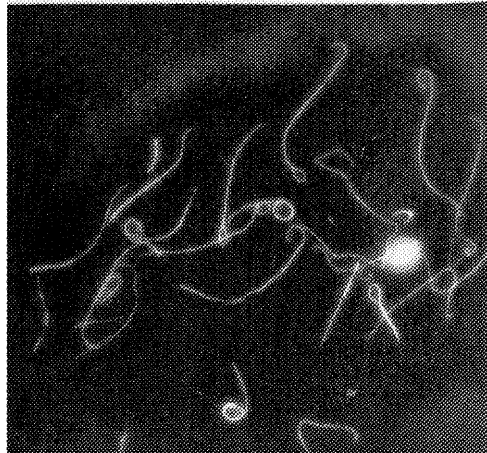
**a****b**

Figure 4-12. Phase contrast (top) and indirect immunofluorescence (bottom) of S. purpuratus sperm that had been demembranated with 1% Triton X-100 and then stabilized (see Methods). Fig. 4-12a: sperm incubated with affinity-purified anti-tubulin from immune serum (0.25 mg/ml). Fig. 4-12b: sperm incubated with affinity-purified anti-tubulin from preimmune serum (0.25 mg/ml). Photographs were taken at the same exposure and developed under the same conditions for immune and preimmune samples.

**a****b**

Summary

Antibodies binding to sea urchin flagellar outer doublet tubulin were purified from rabbit sera by affinity chromatography--"induced" anti-tubulins from rabbits immunized with SDS-denatured preparations of tubulin and "spontaneous" anti-tubulins from unimmunized rabbits. Spontaneous anti-tubulin is found in normal serum at approximately one-third the concentration of induced anti-tubulin. Both preparations are exclusively of IgG class antibodies. Both are specific for tubulin when presented with a crude mixture of flagellar proteins. Only induced anti-tubulin precipitates tubulin in an immunodiffusion assay. Both anti-tubulins and their monovalent Fab fragments bind to sea urchin axonemes in a simple radiobinding assay. Only induced anti-tubulin stains chick fibroblast cytoplasmic microtubules by indirect immunofluorescence; however, both induced and spontaneous anti-tubulins immunofluorescently stain demembranated sea urchin sperm flagella.

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Chapter 5

EFFECTS OF ANTI-TUBULINS ON FLAGELLAR MOTILITY AND MICROTUBULE SLIDING

The preceding chapter characterized the anti-tubulins obtained from immune and preimmune rabbit sera. I now turn my attention to the effects of these antibodies on sea urchin sperm flagella. The first portion of this chapter deals with the effects of these anti-tubulins on the movement of reactivated spermatozoa by measuring beat frequencies, bend angles, and symmetries. The second portion reports the effect of induced anti-tubulin on ATP-driven microtubule sliding of elastase-digested axonemes. These results are compared with the effect of anti-Fragment 1A which inhibits sliding (6).

Part 1. Effects of induced and spontaneous antibodies against tubulin on the movement of reactivated sea urchin sperm flagella.

MATERIALS AND METHODS

Materials

Concentrated spermatozoa from Strongylocentrotus purpuratus were obtained as described previously (7). For experiments with reactivated spermatozoa, a stock sperm suspension was prepared by dilution with 1-2 vol of cold 0.5 M NaCl. The concentration of the sperm suspension was adjusted until a 10 μ l portion, diluted into 5.0 ml of 0.5 M NaCl, produced an optical density reading of 0.24-0.26 at 550 nm (5).

Antibodies to tubulin, both from immune and preimmune sera, were purified by affinity chromatography as described in the previous chapter. Five different pairs of anti-tubulins were used in these studies; Table 4-1 summarizes the production of each immune serum.

Observations of motility of reactivated spermatozoa

Demembranated spermatozoa of S. purpuratus were prepared and reactivated

using extraction and reactivation solutions with the same compositions as those used previously (2), with an ATP concentration of 0.15 mM in the reactivation solution. A 10 μ l portion of a standardized stock sperm suspension was mixed into 1.0 ml of extraction solution. After 60 sec a 10 μ l portion of extracted spermatozoa was added to 1.0 ml of reactivation solution containing an aliquot of antibody preparation and gently mixed. Observations, frequency measurements, and photographs were made as described previously (7). All work was carried out at 18°C.

Bend angles were measured by protractor from the negatives on the screen of a microfilm reader. The bend angle measurement was routinely made as near as possible to halfway along the length of the flagellum, averaging two bend angles directly opposite to each other along the flagellum.

RESULTS

The effects of the motility of reactivated flagella were examined by briefly demembranating spermatozoa, mixing an aliquot of the demembranated spermatozoa into a reactivation solution containing 0.15 mM ATP and a known quantity of the affinity-purified immune or preimmune anti-tubulin, and examining the spermatozoa by dark-field microscopy. Since I observed a rapid initial inhibition of beat frequency by azide at concentrations used in these experiments (1% inhibition for experiments in Fig. 5-1 and 13% inhibition for experiments in Fig. 5-4), controls treated with dilution buffer alone were also measured. Fig. 5-1 shows the effects of affinity-purified immune and preimmune anti-tubulins (rabbit 4) on the frequencies and bend angles of individual spermatozoa as a function of time. Both of the antibodies were at a final concentration of 2.5 μ g/ml reactivation solution. Neither of the antibodies affected the beat frequencies when compared to the effects of the dilution buffer alone. However, the immune anti-tubulin

Figure 5-1. Effects of buffer, affinity-purified preimmune anti-tubulin, and affinity-purified immune anti-tubulin (from rabbit 4) on beat frequencies (open circles) and bend angles (solid circles) of individual reactivated spermatozoa as a function of time. Both anti-tubulins were added to reactivation solutions to a final concentration of 2.5 $\mu\text{g/ml}$. An equivalent volume (5 μl) of buffer was added to 1 ml reactivation solution to obtain the first set of curves. Each curve represents one spermatozoon.

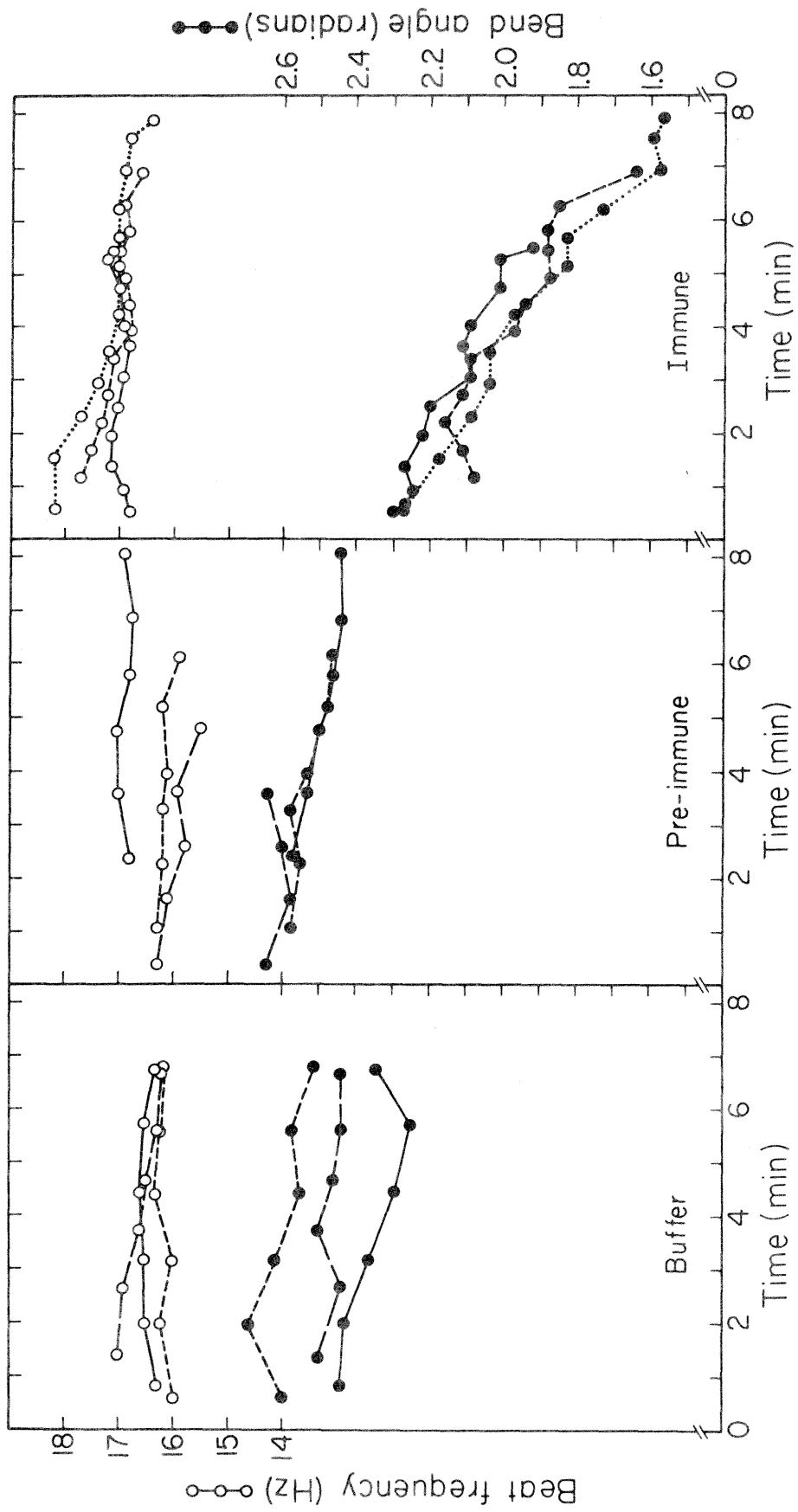
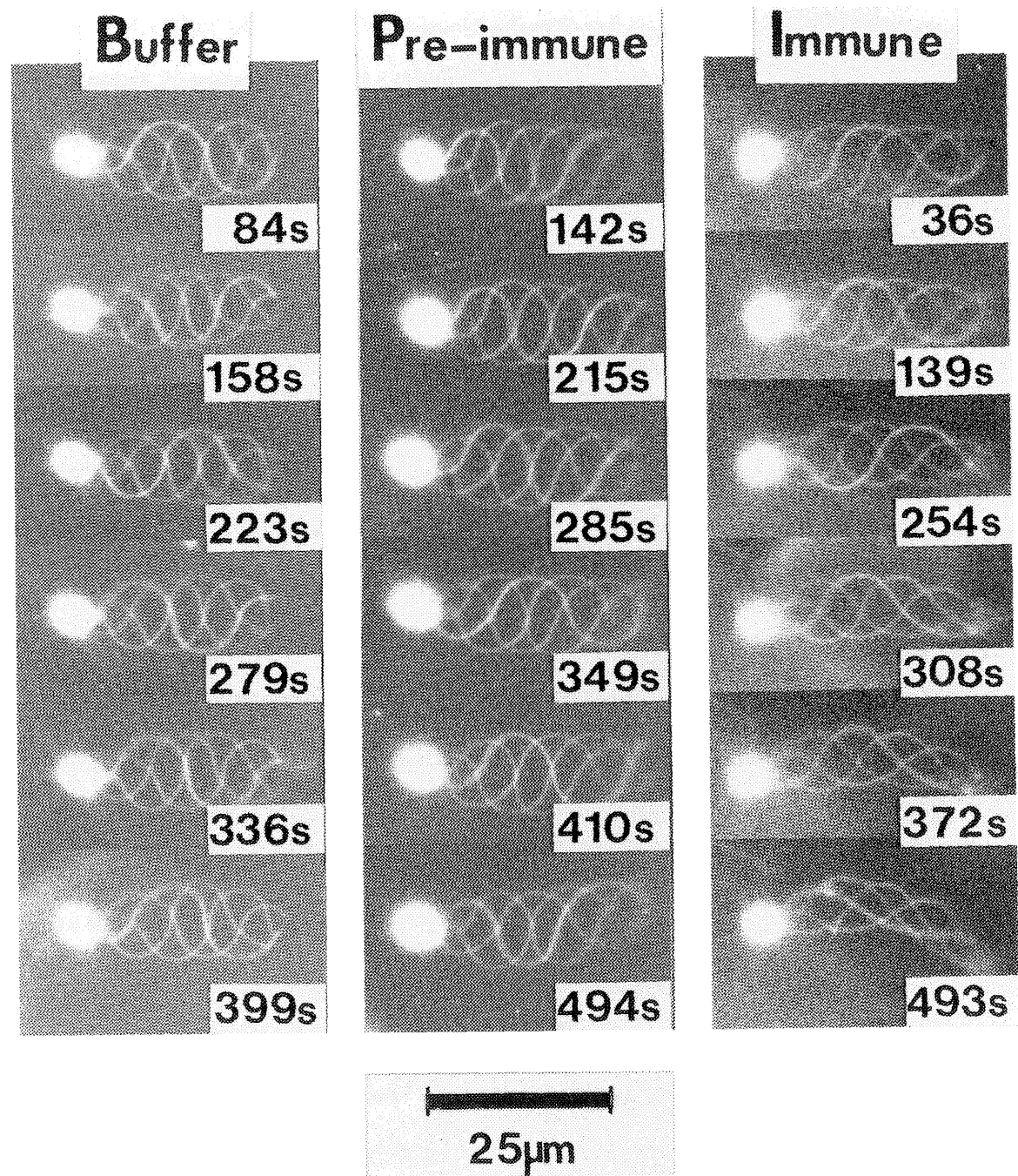


Figure 5-2. Examples of photomicrographs of spermatozoa measured in Fig. 5-1. The spermatozoon treated with buffer is plotted with a dashed line in Fig. 5-1, the preimmune-treated spermatozoon by a solid line in Fig. 5-1, and the immune-treated spermatozoon by a dotted line in Fig. 5-1.



selectively inhibited the bend angle of the flagella while the preimmune antibodies did not affect the amplitude. The effects of these two anti-tubulins are also shown in the photographs in Fig. 5-2.

Another pair of affinity-purified anti-tubulins from immune and preimmune sera (rabbit 2) were examined. Again, there was no effect on beat frequencies by either preparation and a selective amplitude inhibition by only the immune anti-tubulin; these results are shown in Fig. 5-3. These results were obtained with higher concentrations, approximately 15 $\mu\text{g/ml}$, of immune and preimmune anti-tubulins. Monovalent Fab fragments were made from the immune and pre-immune sera (rabbit 2) and purified by tubulin-affinity chromatography. Pre-immune Fab fragments had no effect on beat frequency or amplitude (Fig. 5-4a). Figure 5-4b shows the effects of immune Fab fragments on beat frequencies and bend angles of individual spermatozoa. Concentrations of the Fab fragments were adjusted to equivalent quantities of antigen binding sites as compared to amplitude inhibiting concentrations of the corresponding intact divalent antibody. These immune Fab fragments had no significant effect on either beat frequencies or amplitudes. Moreover, the addition of an excess of goat anti-rabbit Fab (Cappel Laboratories) after 3 min of preincubation with the immune Fab alone, Fig. 5-4c, had no effect on the frequencies or amplitudes of the flagella within the time period of this experiment.

In addition to the two pairs of affinity-purified anti-tubulins already discussed (from rabbits 2 and 4), the effects of three more anti-tubulins were also examined. Only two of these precipitated tubulin in an immunodiffusion assay (rabbits 3 and 4). Each of the five pairs was tested by adding affinity-purified antibody to a concentration of 10 $\mu\text{g/ml}$ in the reactivation solution. After incubation of demembranated spermatozoa in the reactivation solution + antibody

Figure 5-3. Effects of buffer, affinity-purified preimmune anti-tubulin, and affinity-purified immune anti-tubulin (from rabbit 2) on beat frequencies (open circles) and bend angles (solid circles) of individual reactivated spermatozoa as a function of time. Both anti-tubulins were added to reactivation solutions to a final concentration of 15 $\mu\text{g/ml}$ and an equivalent volume (90 μl) of buffer was added to 1 ml reactivation solution to obtain the first set of curves. Each curve represents one spermatozoon.

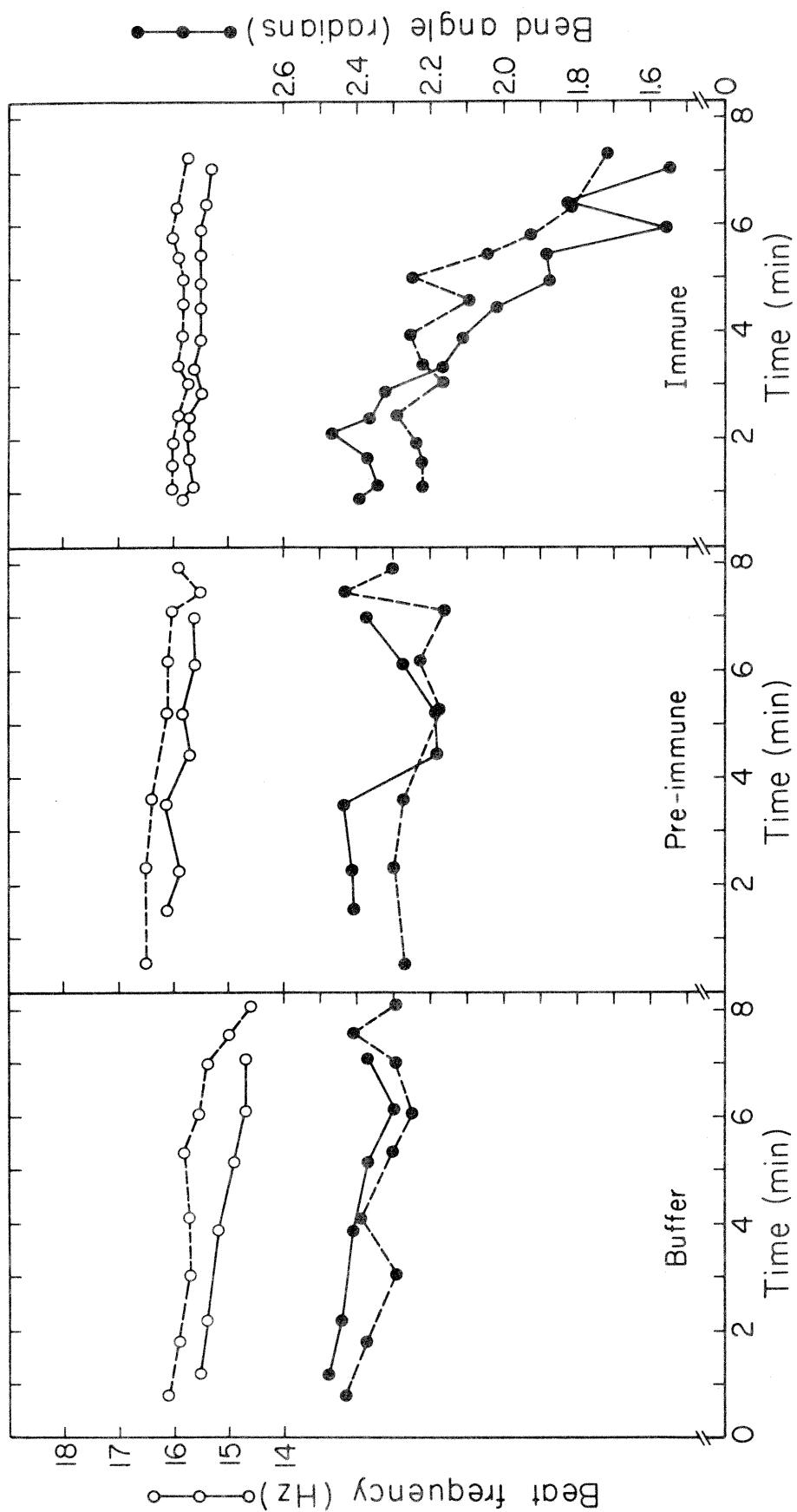


Figure 5-4. Effects of affinity-purified Fab fragments (from rabbit 2) on beat frequencies (open circles) and bend angles (solid circles) of individual reactivated spermatozoa as a function of time. Preimmune (a) and immune (b) Fab fragments were at a final concentration of approximately 10 $\mu\text{g/ml}$ which represented the same number of antigen binding sites per sample as that of the corresponding intact antibody (Fig. 5-3). In (c), immune Fab fragments (10 $\mu\text{g/ml}$) were first incubated with demembranated spermatozoa; after 3 min preincubation, excess goat anti-rabbit Fab (Cappel Laboratories) was added and individual spermatozoa observed. Each curve represents one spermatozoon.

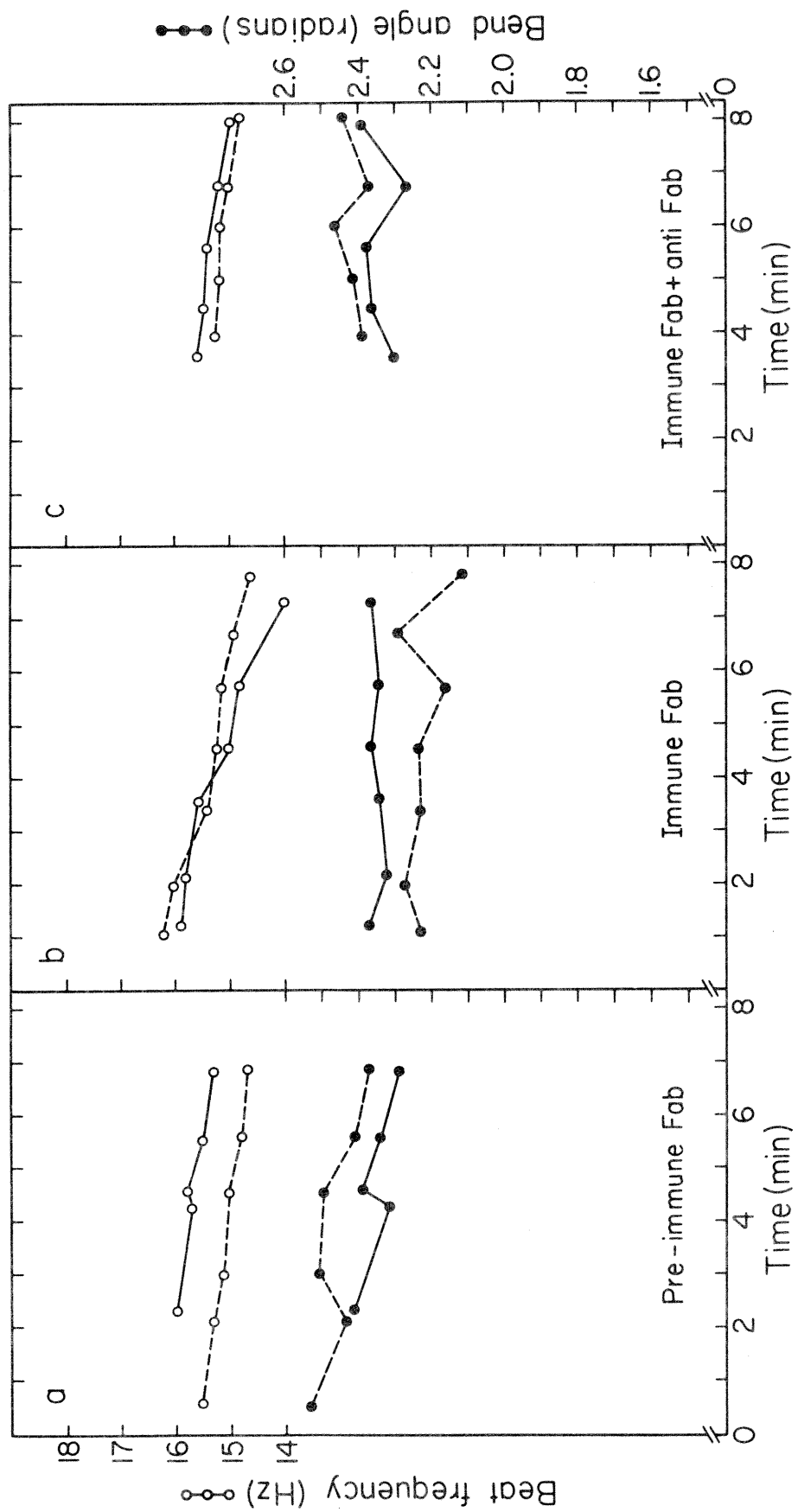


Figure 5-5. Comparison of affinity-purified anti-tubulins from preimmune and immune sera from five different rabbits (r1-5; see Table 4-1). Demembranated spermatozoa were incubated for 10 min with 10 µg/ml affinity-purified antibody and then several different spermatozoa were photographed. The open circles are averaged results for beat frequencies; the solid circles are averaged bend angles. Standard deviations are also shown for each averaged value. Sample sizes ranged from 6 to 13 spermatozoa. The first point of each pair of points joined by a line represents the value obtained from the preimmune antibody; the second point is the value from the immune antibody.

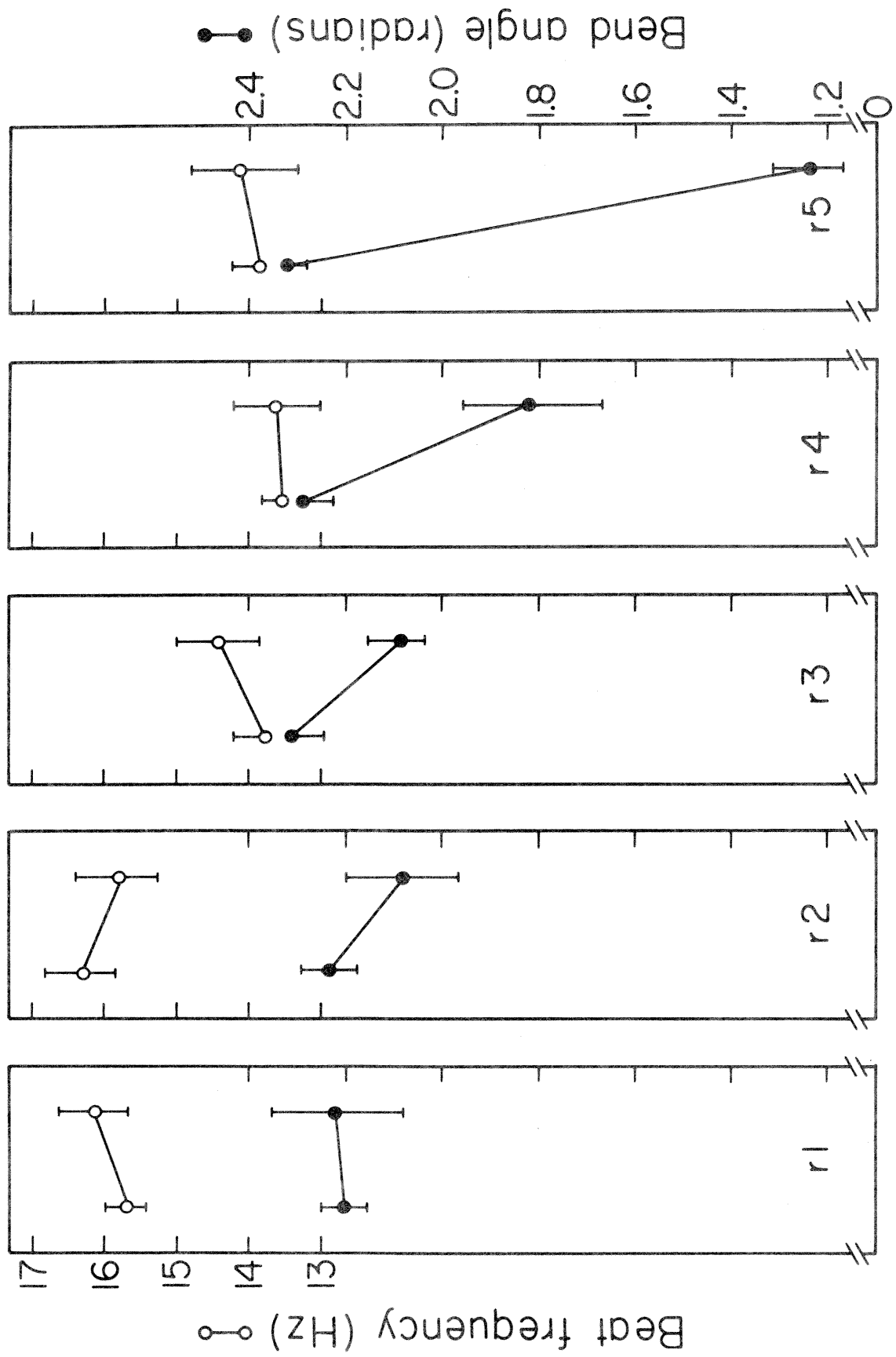
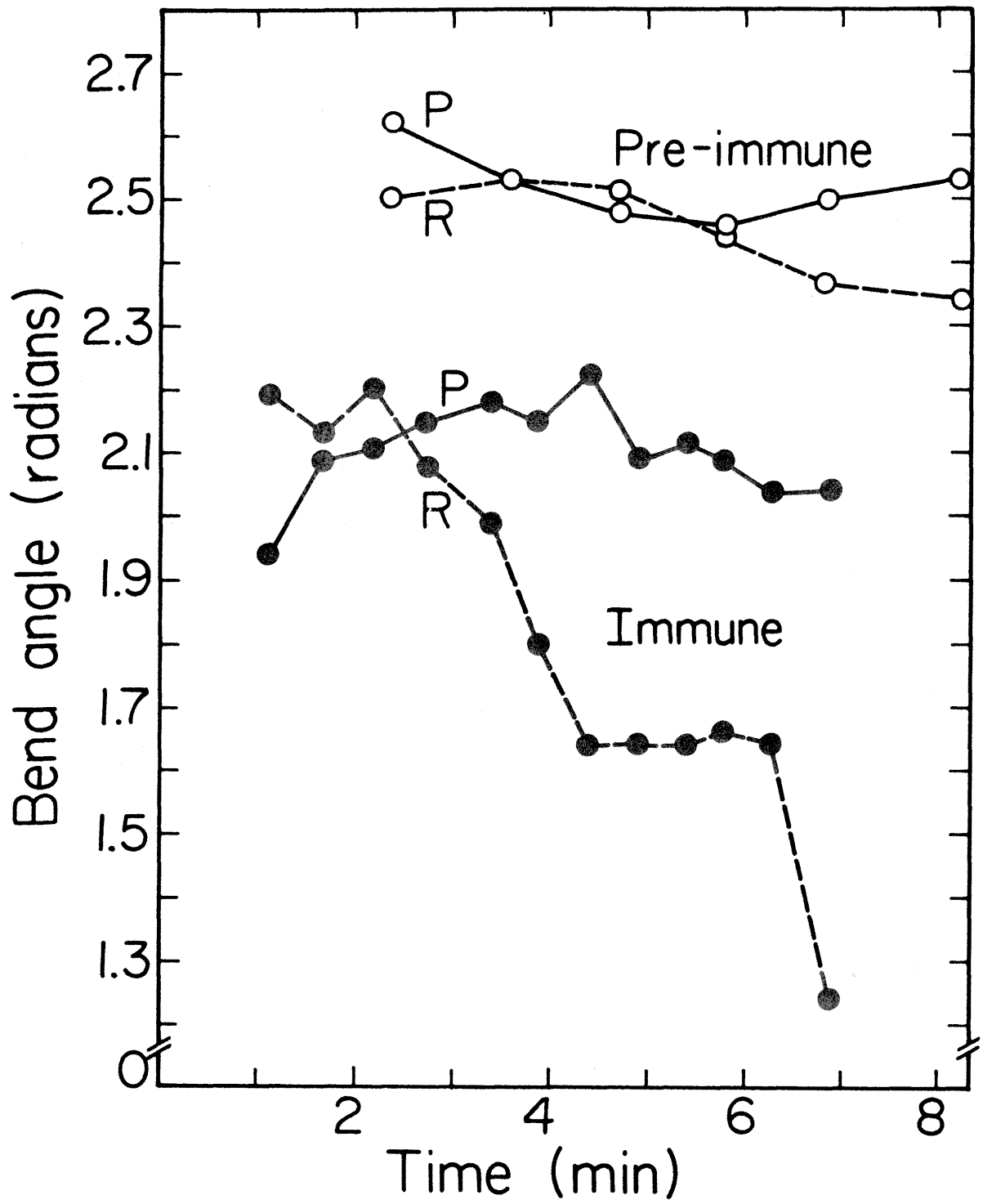


Figure 5-6. Measurements of principal (P) and reverse (R) bend angles of reactivated spermatozoa treated with affinity-purified preimmune or immune anti-tubulins. One example of each treatment is shown. Spermatozoa represent experiment shown in Fig. 5-1.



mixture for 10 min, samples of the spermatozoa were photographed by dark-field microscopy. Averaged results are shown in Fig. 5-5 which compares each pair of preimmune and immune anti-tubulins. None of the immune and preimmune anti-tubulins affected beat frequencies. As before, the preimmune anti-tubulins did not alter bend angles. All of the four anti-tubulins which gave positive immunodiffusion results significantly inhibited amplitudes, with each immune anti-tubulin having a different magnitude of amplitude effect under these conditions. Anti-tubulin from immune rabbit 5 at 20 $\mu\text{g/ml}$ was able to completely paralyze the flagella after 10 min exposure. With all of the immune antibodies which affected amplitude, the symmetry of the waveform was simultaneously affected. As an example of this, Fig. 5-6 shows the principal and reverse bend angles plotted for the immune and preimmune antibody preparations shown in Fig. 5-1 (from rabbit 4).

Summary to Chapter 5, Part 1.

Induced anti-tubulins from four different immunizations were all found to reduce the bend angle and the symmetry of the movement of demembranated spermatozoa without affecting the beat frequency. At identical concentrations, spontaneous anti-tubulins (five samples) and both of the Fab preparations had no effect on bend angle, symmetry, or frequency. At least one of the induced anti-tubulins (from rabbit 5), at high concentrations, was able to completely paralyze the reactivated spermatozoa.

Part 2. Effects of induced anti-tubulin on microtubule sliding.

The active sliding process which generates the bending movements of flagella and cilia can be observed in isolation from bending by utilizing demembrated flagellar axonemes which have been briefly digested with trypsin (11) or elastase (3). When MgATP^{2-} is added to these preparations, the axonemes disintegrate, and disintegration can be directly observed, using dark-field light microscopy, and can be seen to occur by sliding extrusion of doublet microtubules (11). These preparations have been used to demonstrate that the active sliding process is located between adjacent doublet microtubules and is unidirectional (10), to show that inhibitors of flagellar bending such as vanadate (9) and antibodies against fragment 1A of dynein (6) also inhibit tubule sliding, and to show that CO_2 inhibits flagellar bending but does not inhibit tubule sliding (4). In this part of Chapter 5, I examine the properties of induced anti-tubulin on the ATP-driven extrusion of elastase-treated axonemes in order to directly test their effect on active sliding. For comparison, I also tested the effects of anti-fragment 1A on extrusion.

MATERIALS AND METHODS

The preparation of a stock sperm suspension from concentrated *S. purpuratus* spermatozoa was the same as described in Part 1 of this chapter. Triton demembrating and reactivation solutions were identical to those previously used (2).

In order to prepare elastase-treated axonemes, the following protocol was adopted. Elastase (Sigma, #E0127) was added to ATP-free reactivation solution to a final concentration of 6 $\mu\text{g/ml}$. Stock sperm suspension was demembrated at 20 μl stock sperm per 1 ml triton solution for 60 sec, after which 400 μl of demembrated spermatozoa was added to 2 ml of the reactivation

solution containing elastase. Digestion was allowed to progress at 18°C for 2.5 min after which chicken ovomucoid (Sigma, #T 1886), a trypsin inhibitor which also inhibits elastase (1), was added to a final concentration of 12.8 µg/ml. The treated spermatozoa were then broken by passing them through a drawn-out Pasteur pipette. The resulting axonemes usually were nearly full length, but were uniformly detached from the sperm heads. These elastase-digested axonemes were stored at 0°C for subsequent experiments and were stable over an entire day.

Anti-tubulin antibodies were obtained from immune and preimmune sera from rabbit 5. The induced anti-tubulin from rabbit 5 was the most potent in inhibiting the movement of reactivated spermatozoa (Chapter 5, Part 1). The antigen for immunization was twice-extracted sea urchin axonemes (Table 4-1). Both immune and preimmune anti-tubulins were purified by tubulin-affinity chromatography of the immune and preimmune ammonium sulfate precipitated antibodies. The immunoabsorbent coupled to the Sepharose 4B affinity columns was sea urchin sperm flagella outer doublet tubulin which had been electrophoretically purified. Different but identical columns were used to process immune and pre-immune antibodies.

Anti-fragment 1A serum prepared against the ATPase-active tryptic fragment of dynein 1 from sperm flagella of the sea urchin A. crassispina was the gift of Dr. Kazuo Ogawa (8).

Observations were typically performed by mixing 100 µl of elastase-digested axonemes with 10 µl of freshly demembrated spermatozoa, in the absence of ATP. Antibody was added to this mixture, and after incubation for 30 min, a drop was transferred to a microscope slide and observed using dark-field illumination. Reactivation solution containing 1 mM MgATP²⁻ could be added to the edge of the cover glass and the effects of ATP directly observed as it diffused across

the field of vision. In order to quantitate the ATP concentration, known quantities of the antibody-incubated axonemes were mixed with small aliquots of ATP from a 1 mM MgATP^{2-} reactivation solution. These were then observed by dark-field microscopy.

RESULTS

Untreated control axoneme disintegration

In control experiments in the absence of antibody, as ATP diffused into the sperm suspension, its arrival was signalled by an initiation of beating of the undigested sperm flagella (which still had heads) and by the disintegration by tubule extrusion of the digested, headless, axonemes. In samples which had undergone disintegration, randomly-curved balls of extruded tubules were visible. From a series of experiments involving different concentrations of MgATP^{2-} , it was determined that, under these conditions, 2.0 μM ATP was not sufficient to drive extrusion but that the addition of MgATP^{2-} to 8.3 μM resulted in total disintegration. The photographs in Fig. 5-7 show axonemes and spermatozoa in the absence (Fig. 5-7a) of ATP and after the addition of ATP to 10 μM (Fig. 5-7b).

Effects of anti-fragment 1A

In the presence of 150 μM ATP, anti-fragment 1A (8), at a concentration of 10 $\mu\text{l/ml}$, was able to completely paralyze reactivated spermatozoa within 15 min. This concentration was approximately twice the minimal amount necessary to stop beating within 30 min. To study the effects of this antiserum on microtubule sliding, a mixture of elastase-treated axonemes and untreated demembranated spermatozoa was combined with anti-fragment 1A at 10 μl antibody per 1 ml in the absence of ATP. This mixture was incubated for 30 min at which time samples were removed and observed by dark-field microscopy. Even after the

Figure 5-7. Control axonemes before (a) and after (b) the addition of MgATP^{2-} to 10 μM . Axonemes were digested with elastase, incubated in the absence of antibody for 30 min and observed by dark-field light microscopy.

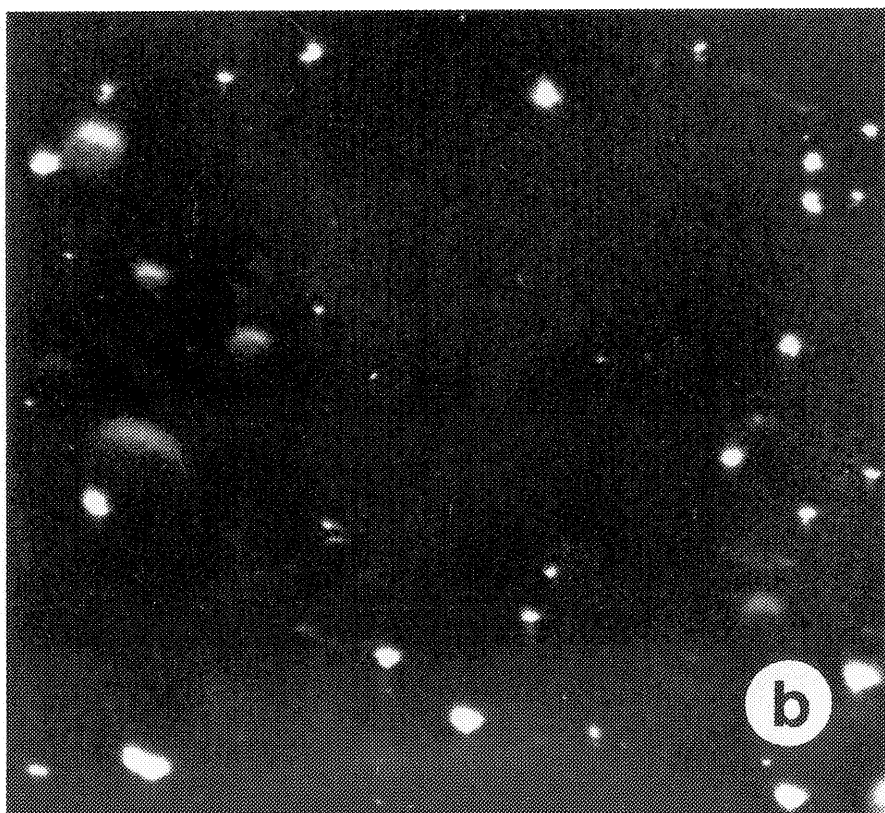
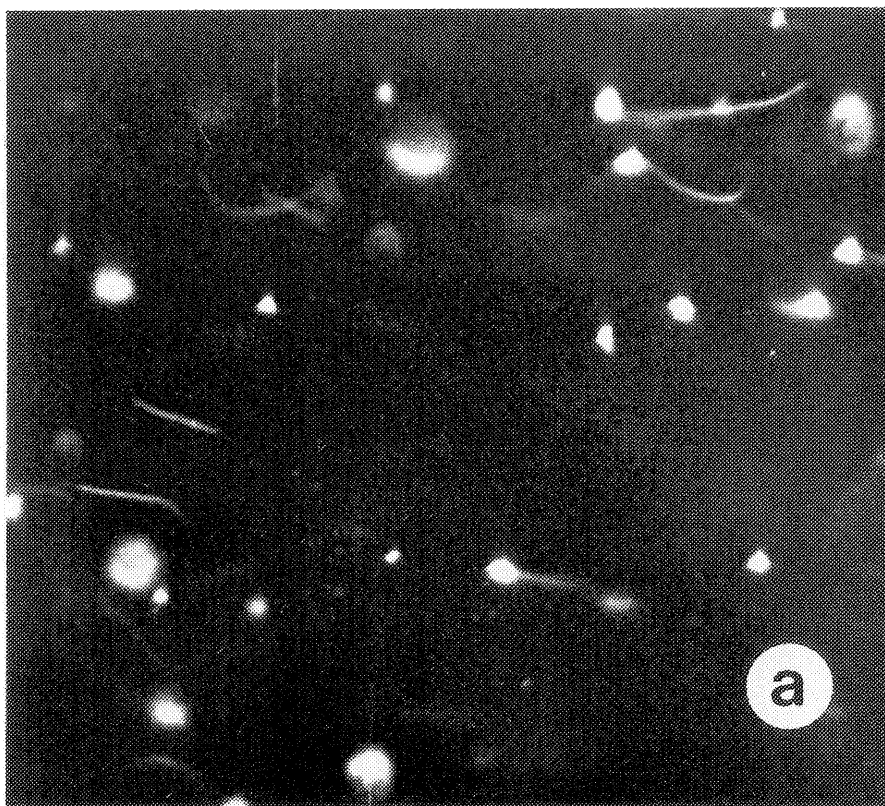
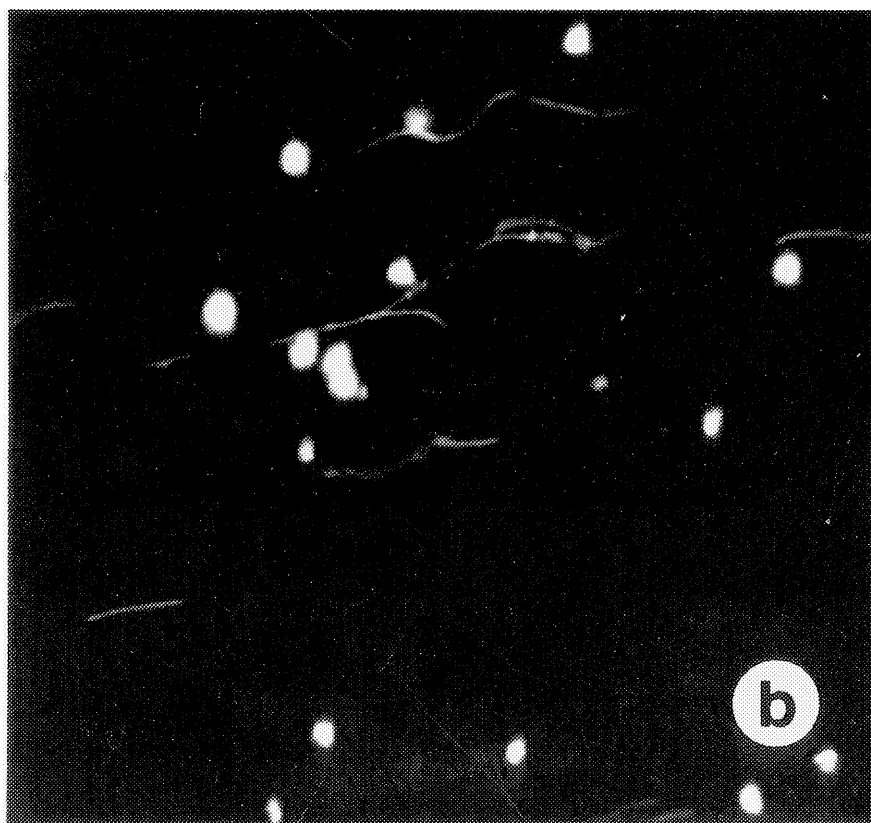
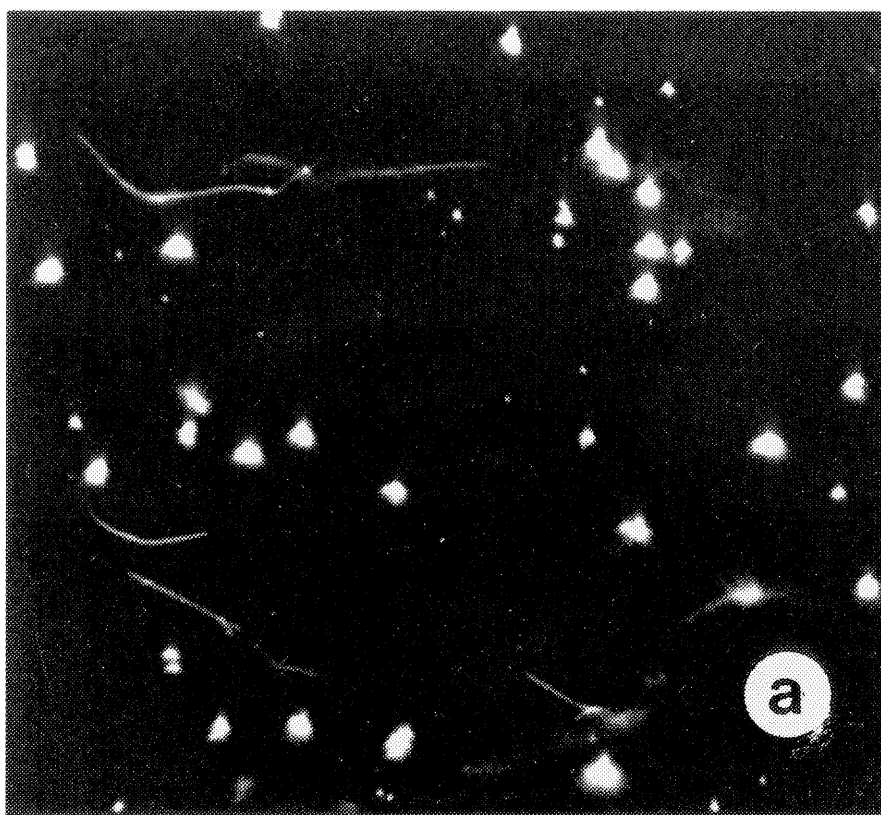


Figure 5-8. Anti-fragment 1A (10 $\mu\text{l/ml}$)-treated elastase-digested axonemes before (a) and after (b) the addition of MgATP^{2-} to 150 μM . Axonemes were digested with elastase and incubated with anti-fragment 1A for 30 min prior to observations.



addition of MgATP^{2-} to a final concentration of $150\ \mu\text{M}$, no disintegration was observed. These results are shown in the photographs in Fig. 5-8. Similar results were reported previously by Masuda *et al.* (6).

Effects of anti-tubulins

Using a protocol similar to that used to test the effects of anti-fragment 1A, affinity-purified spontaneous and induced anti-tubulins were tested. Spontaneous anti-tubulin antibody, at a concentration of $30\ \mu\text{g/ml}$, did not affect the disintegration of axonemes--the results were the same as with the control axonemes. This concentration ($30\ \mu\text{g/ml}$) of induced anti-tubulin was found to be approximately twice the minimal concentration required to paralyze beating after 15 min. Induced anti-tubulin at $30\ \mu\text{g/ml}$, after a 30 min incubation with elastase-treated axonemes and untreated demembranated spermatozoa in the absence of ATP, did not inhibit disintegration at $150\ \mu\text{M}$ ATP. However, it did raise the ATP concentration threshold necessary for disintegration to occur. No disintegration was observed at $0\ \mu\text{M}$ ATP, $8.3\ \mu\text{M}$ ATP, or $16.7\ \mu\text{M}$ ATP. Partial disintegration was noted at $25\ \mu\text{M}$ ATP and total disintegration occurred at $33.3\ \mu\text{M}$ ATP. These results are summarized in Table 5-1 and examples of these axonemes are shown in Fig. 5-9.

Figure 5-9. Induced anti-tubulin (30 $\mu\text{g/ml}$)-treated elastase-digested axonemes before (a) and after (b) the addition of MgATP^{2-} to 30 μM . Axonemes were incubated with induced anti-tubulin for 30 min prior to observation.

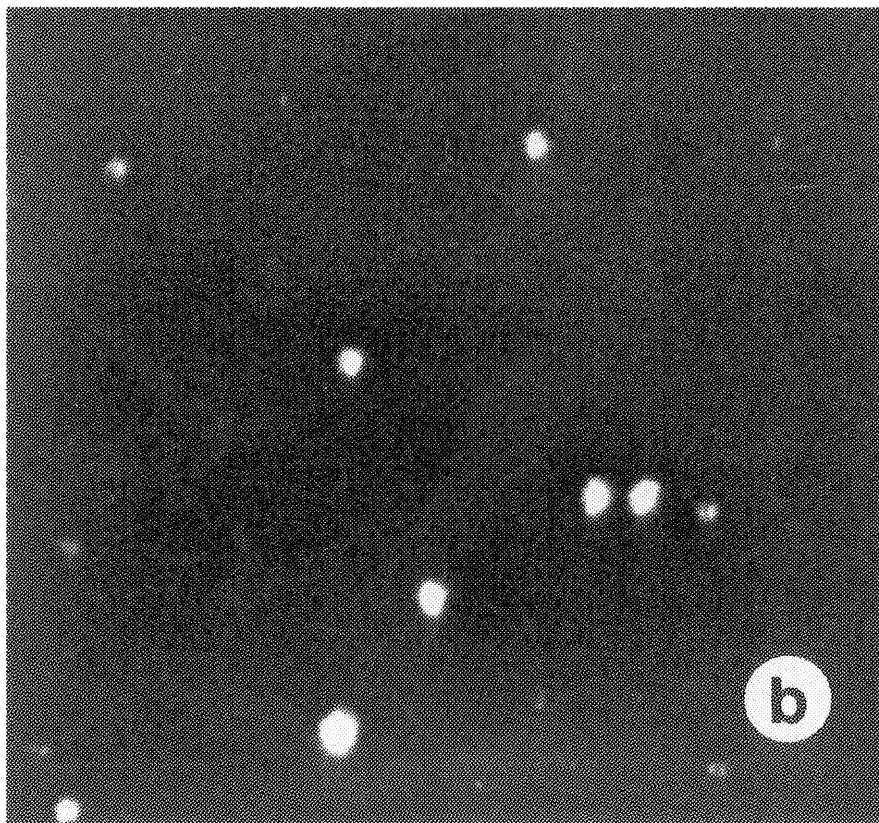
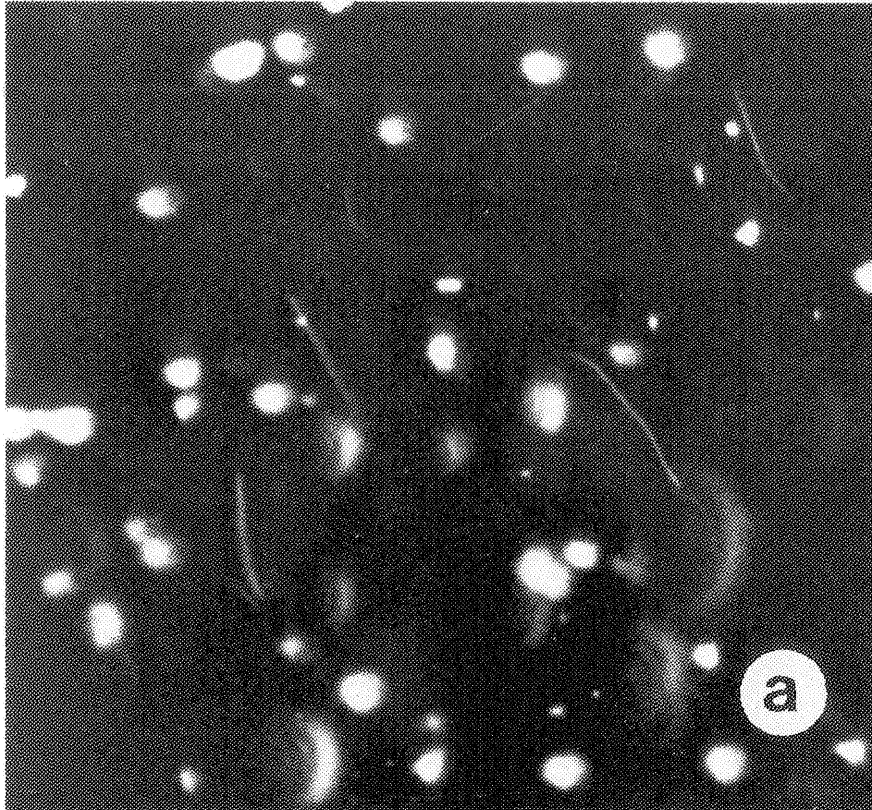


Table 5-1

Effects of Induced Anti-tubulin and Anti-fragment 1A Antibodies
on ATP-driven Disintegration of Elastase-digested Axonemes

Antibody	MgATP ²⁻ concentration (μ M)	Disintegration
None	2.0	-
None	8.3	+
anti-tubulin (30 μ g/ml)	0	-
	8.3	-
	16.7	-
	25.0	+
	33.3	+
anti-fragment 1A (10 μ l/ml)	150.0	-

Summary to Chapter 5, Part 2.

The ATP-mediated disintegration of axonemes that have been treated with elastase has been used to compare the effects of anti-fragment 1A and anti-tubulin antibodies. Axonemes unexposed to antibody disintegrate at ATP concentrations less than 9 μM . Anti-fragment 1A, at concentrations inhibitory of ATPase activity and flagellar movement, prevents the extrusion of tubules at ATP concentrations up to 150 μM . Spontaneous anti-tubulin antibody has no effect on the ATP sensitivity of treated axonemes to disintegration. Induced anti-tubulin antibody, in quantities exceeding those necessary to paralyze flagella reactivated in 150 μM ATP, does not inhibit the extrusion of microtubules, but does raise the ATP threshold concentration to approximately 25 μM .

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Chapter 6

DISCUSSION

Antibodies to tubulin

Induced anti-tubulins were produced in a total of five rabbits, of which four (r2, 3, 4, 5) are represented in this thesis. Different preparations of sea urchin sperm flagella tubulin were used to inject the rabbits--residue tubulin, electrophoretically purified outer doublet tubulin, and extracted axonemes. The immunization procedures using SDS denatured antigen were successful in producing antibodies which precipitated tubulin in immunodiffusion assays. However, as noted in Chapter 4, only 5 of the 17 rabbits injected with tubulins yielded precipitating antibodies. From all of my attempts at immunization, no pattern emerges for "good" antigen preparation nor for a successful injection protocol. It is possible that some of the serum samples that tested negative by immunodiffusion nevertheless contained induced anti-tubulins which could have been captured by tubulin-affinity chromatography. However, affinity-purified post-injection serum tested in Fig. 5-5 (rabbit 1) neither precipitated tubulin nor yielded antibodies that affected the flagellar waveform.

Immobilized, electrophoretically purified tubulin was used as an affinity absorbent to purify anti-tubulins. Special care was taken to use tubulin which was as pure as possible to couple to the Sepharose. In experiments such as that shown in Fig. 5-5, it is possible to compare the effects of the same quantity of anti-tubulin from different sera because they have all been purified over identical affinity columns.

In contrast to the low percentage of successful immunizations, all of the preimmune rabbit sera (eight) I have tested by tubulin-affinity chromatography contain spontaneous anti-tubulins. This finding of spontaneous antibodies to tubulin is confirmed by an earlier report by Karsenti and coworkers (13) in which they also used tubulin-affinity chromatography to isolate anti-tubulins from a variety

of nonimmune mammalian sera. Karsenti et al. also found that their spontaneous antibodies did not precipitate tubulin nor did they give efficient immunofluorescent staining of cytoplasmic microtubules. Similarly, I find that spontaneous anti-tubulin antibodies purified by affinity chromatography do not precipitate tubulin in immunodiffusion assays, in contrast to the precipitation obtained with equivalent amounts of induced anti-tubulin, and that the spontaneous antibodies do not strongly stain chick fibroblast microtubules by indirect immunofluorescence. I have further differentiated these two types of anti-tubulins on the basis of presence or absence of a specific inhibition of flagellar motility.

I have demonstrated that the anti-tubulins from both immune and preimmune sera are IgG type antibodies. As mentioned in Chapter 1, it is curious that spontaneous anti-tubulins are IgGs, and it remains to be determined how this apparent normal secondary response to tubulin is elicited.

Immunoglobulin-affinity chromatography demonstrates that these antibodies are specific for proteins which cannot be distinguished from tubulin by SDS gel electrophoresis when a mixture of axonemal proteins is chromatographed. Immunodiffusion assays showing only a single precipitin line between purified anti-tubulin immunoglobulin and crude axonemal extracts and the demonstration of fluorescent staining of chick fibroblast microtubules provide additional evidence that these antibodies are specifically directed against tubulin. I conclude that the inhibitory effects of the induced antibodies on the movement of reactivated spermatozoa are caused by antibodies binding specifically to tubulin.

Binding of ^{125}I -labeled anti-tubulins to axonemes demonstrates that both induced and spontaneous anti-tubulins bind to axonemes; it also appears that spontaneous anti-tubulin displays a 100-fold lower affinity for the axoneme.

It is useful to calculate the number of antibody molecules available to bind a tubulin dimer in the experiments presented in Chapters 4 and 5. The tubulin dimer is approximately 80 Å long (2) and a series of these dimers constitute a single protofilament. If the flagellum is 40 μ in length, each protofilament is a chain of about 5×10^3 dimers. Each outer doublet is a total of 24 protofilaments, or 1.2×10^5 dimers; and nine doublets, or one axoneme, contain approximately 1×10^6 tubulin subunits. Brokaw and Simonick (9) counted the number of spermatozoa in a stock sperm suspension which had the same concentration as the stock suspension used in my work. They found an average of 2×10^7 spermatozoa per 1 μl suspension. A given reactivated preparation in these studies uses the equivalent of 0.1 μl stock of 2×10^6 spermatozoa, which represent a total of approximately 2×10^{12} tubulin dimers. In the experiments studying the effects of anti-tubulin antibodies on reactivated spermatozoa, antibody was added to a final concentration of 2.5 μg/ml (for r4, Fig. 5-1) and 15 μg/ml (for r2, Fig. 5-3). Based on a molecular weight of 150,000 for IgG, this means that induced anti-tubulin from r4 inhibited motility at 5 antibody molecules per 1 tubulin dimer, and induced anti-tubulin from r2 inhibited motility at a concentration of 30 antibody molecules per tubulin dimer.

The radiobinding assays cannot give a total number of available binding sites per axoneme because it was not possible to raise the antibody concentrations to saturating conditions. However, since the axoneme concentration (100 μl used per assay point) in the binding assay was approximately one-eighth that of the stock sperm suspension used in the reactivation experiments, the highest amount of antibody (0.042 μg) used in the binding assay represents an antibody/tubulin ratio of 50,000 times less than in the reactivation experiments with anti-tubulin from r2. In the binding assays, at axoneme concentrations equivalent to those

used in the experiments with reactivated sperm flagella, about 25% of the antibody was bound, and most of the binding occurred within 30 minutes (results not shown). In the motility experiments, after 5-10 minutes incubation, at least 5% of the antibody might therefore be expected to bind to the axonemes. In the motility experiments with induced anti-tubulin from r2, where there were 30 antibody molecules per tubulin dimer, this would represent antibody binding to a large fraction of the exposed tubulin dimers.

These figures indicate that the radiobinding assay used in these experiments is extremely sensitive and that a substantial quantity of anti-tubulin, with respect to the total tubulin content of the axonemes, was added to reactivated spermatozoa.

Since monovalent Fab fragments were absorbed to the tubulin-affinity columns and also showed strong binding to the axonemes in the radiobinding assay, I infer that the binding of both induced and spontaneous anti-tubulins is a true antibody binding and not a recognition of tubulin by some other portion of the antibody (e.g., Fc portion).

Indirect immunofluorescence also demonstrates that both anti-tubulins are able to bind demembranated sperm tails but that only induced anti-tubulin decorates chick fibroblast cytoplasmic microtubules. I interpret these immunofluorescence data to reflect the immunization and anti-tubulin purification procedures. Sea urchin tubulin-affinity chromatography purifies those antibodies from both immune and preimmune sera which bind to sea urchin flagella. The sea urchin tubulin determinants to which the spontaneous anti-tubulins bind may not be available in sufficient quantity on chick microtubules. In addition, the SDS denaturation of sea urchin tubulin during immunization apparently increases the antigenicity of determinants to which only the induced anti-tubulins are able to respond. These additional sites may be present on both species' tubulin, therefore allowing the induced anti-tubulin to react with chick microtubules.

From the characterization of these anti-tubulins, I conclude that both the induced and the spontaneous antibodies and their monovalent Fab fragments purified by affinity chromatography are specific for tubulin and are capable of binding to sea urchin sperm axonemes.

Motility effects

The effects of the different anti-tubulins on the motility of reactivated spermatozoa were qualitatively similar. All four of the induced anti-tubulins specifically inhibited, to different degrees, the flagellar bend angle without affecting the beat frequency. This amplitude effect was accompanied by a loss of wave symmetry. None of the five spontaneous anti-tubulins tested, at the same concentration as their induced counterparts, affected bend angle, beat frequency, or symmetry. This may be due to binding of the spontaneous anti-tubulins only to "harmless" determinants or to a lower affinity for axonemes, which prevents them from affecting the waveform at the antibody concentrations which were obtainable in my experiments.

Anti-fragment 1A is able to completely inhibit microtubule sliding in axonemes digested by trypsin (14) or elastase. This is interpreted to show that if the dynein ATPase is suppressed, the cross-bridges cannot force the displacement of microtubules. In contrast, induced anti-tubulin, at concentrations sufficient to completely paralyze reactivated flagella, does not inhibit microtubule sliding. This suggests that anti-tubulin is not affecting cross-bridge activity, either by disabling the dynein or by binding to microtubules in such a way as to interfere with cross-bridge activity. Beat frequency is sensitive to parameters controlling the active sliding process including changes in the ATP concentration (3, 12) and the number of functional dynein arms (11). The lack of inhibition of beat frequency

by induced anti-tubulin suggests that this antibody does not interfere with the active sliding process.

Induced anti-tubulins belong to a class of probes which have the property of affecting flagellar bending with minimal effect on sliding. These probes include CO_2 (8), N-ethylmaleimide (10), and Soft Pellet Extract.¹ Also, flagellar mutants of Chlamydomonas have been reported which have impaired bending but which still can extrude tubules normally after trypsin digestion (1, 17). The chemical mechanisms of the probes which specifically affect amplitude are probably very different but it will be interesting to ascertain if this shared property of specifically affecting amplitude is due to their action on a common target structure, such as the outer doublet microtubules.

One possible explanation for the inhibition of motility by induced anti-tubulin might be that the antibodies adhere to the outer doublets and alter movement by increasing flagellar bulk and viscous drag. This explanation does not seem to be sufficient since spontaneous anti-tubulins, which also bind to axonemes, have no effect on the movement, and because of the lack of effect of adding anti-Fab serum to spermatozoa incubated with immune and preimmune Fab fragments. Also, changes in external viscosity do not give a specific amplitude reduction (6).

It is also possible that binding by anti-tubulin molecules might greatly increase the bending resistance of the microtubules, by preventing cooperative configurational changes required when a microtubule bends. This possibility could be tested by direct measurements of the bending resistance of axonemes exposed to anti-tubulin, using the method of Okuno and Hiramoto (16). However, flagella have a low bending resistance in the presence of CO_2 (16).

¹Asai, D. J., R. D. Owen and C. J. Brokaw, unpublished observations.

A third possibility is that the antibodies may be able to penetrate and bind to the central pair microtubules and interfere with participation of the radial spokes-central pair structures in the control of bending wave propagation. This possibility must take into account the fact that the antibodies were raised against and later purified with outer doublet tubulins. It seems quite probable that the antibodies are prone to bind preferentially to the externally-exposed outer doublet tubules.

Another possibility is that the anti-tubulins are binding to outer doublets in such a way as to prevent cross-bridge attachment to sites on the outer doublets. The absence of any effect by equivalent concentrations of monovalent Fab fragments, which presumably bind to the same sites as the inhibitory, intact, anti-tubulin, argues against such an intercalation of antibody preventing dynein-tubule interaction. It is still possible that the anti-tubulin acts as a blocking agent which binds close enough to dynein attachment sites to sterically impede cross-bridge action--the Fab fragments might be too small to produce adequate blocking. This hypothesis would contrast with the more potent inhibition by anti-fragment 1A which could be preventing cross-bridge detachment by inhibiting ATPase enzyme activity, and in this way inhibiting both sliding and bending. However, since induced anti-tubulin, at greater than paralyzing concentrations, does not prevent microtubule sliding, it is difficult to imagine a simple blocking of cross-bridge attachment by the antibody.

The only anti-tubulins shown to affect reactivated motility are intact, divalent induced antibodies. The success of these preparations in altering waveform may reflect some cooperative effect of the two antigen-binding sites on each molecule. The amplitude effect could be explained if the anti-tubulin was spanning the inter-doublet spaces, or preventing cross-bridge detachment, to create enough

unyielding "cross-bridges" to eventually paralyze the flagellum. Anti-dyneins may do this, but the experiment showing microtubule sliding in the presence of paralyzing quantities of anti-tubulin rules out this possibility for anti-tubulins.

Theoretical work (4, 5, 7) has hypothesized a feedback loop which utilizes the amount of bending to control cross-bridge action on one half of the flagellum. The feedback signal could involve microtubule configuration and it is possible to envision how an antibody, or other probe, which interfered with microtubule conformational changes could affect this feedback signal.

Since the amplitude effect of induced anti-tubulins occurs only with divalent antibodies, it may be due to an alteration of the relative positions of adjacent tubulin subunits, or by preventing new conformations of microtubules. If these conformational effects are sufficient, they might prevent cooperative microtubule conformational changes which are required for normal modulation of cross-bridge activity. This effect would be evident in the waveform of reactivated intact flagella which requires some sort of modulation of active cross-bridges. Alteration in tubule conformation would not be expected to interfere with the action of cross-bridges in elastase-digested axonemes because, under these conditions, cross-bridges appear to stay "on" and unidirectionally displace microtubules.

An antibody against native dynein 1 and an antibody against a tryptic fragment of dynein 1 were examined in previous work (15). Both of these anti-dyneins showed inhibitory effects on both the amplitude and beat frequency of reactivated sperm flagella, and the patterns of inhibition were different from the pattern I have obtained with induced anti-tubulins. With the antibody against a tryptic fragment of dynein, there was a steady decrease in both beat frequency and bend angle; with the antibody against native dynein 1, the inhibition of beat

frequency was relatively less, and there was a more rapid decrease during the first 2 minutes, followed by a gradual decrease. So far, each of the three types of antibody preparations tested for inhibitory effects on the movement of reactivated S. purpuratus sperm flagella has given a distinct pattern of inhibition, and only the induced anti-tubulin prepared from immune serum has given a specific inhibition of a single parameter of movement. Furthermore, this specific amplitude inhibition is shared by all four of the induced anti-tubulins tested; this is so far the only case where it has been demonstrated that the effects on motility of a particular type of antibody are common to antibody preparations from several different immune animals. Anti-tubulins may be a particularly useful probe for further studies of the function of microtubules in flagellar movements.

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