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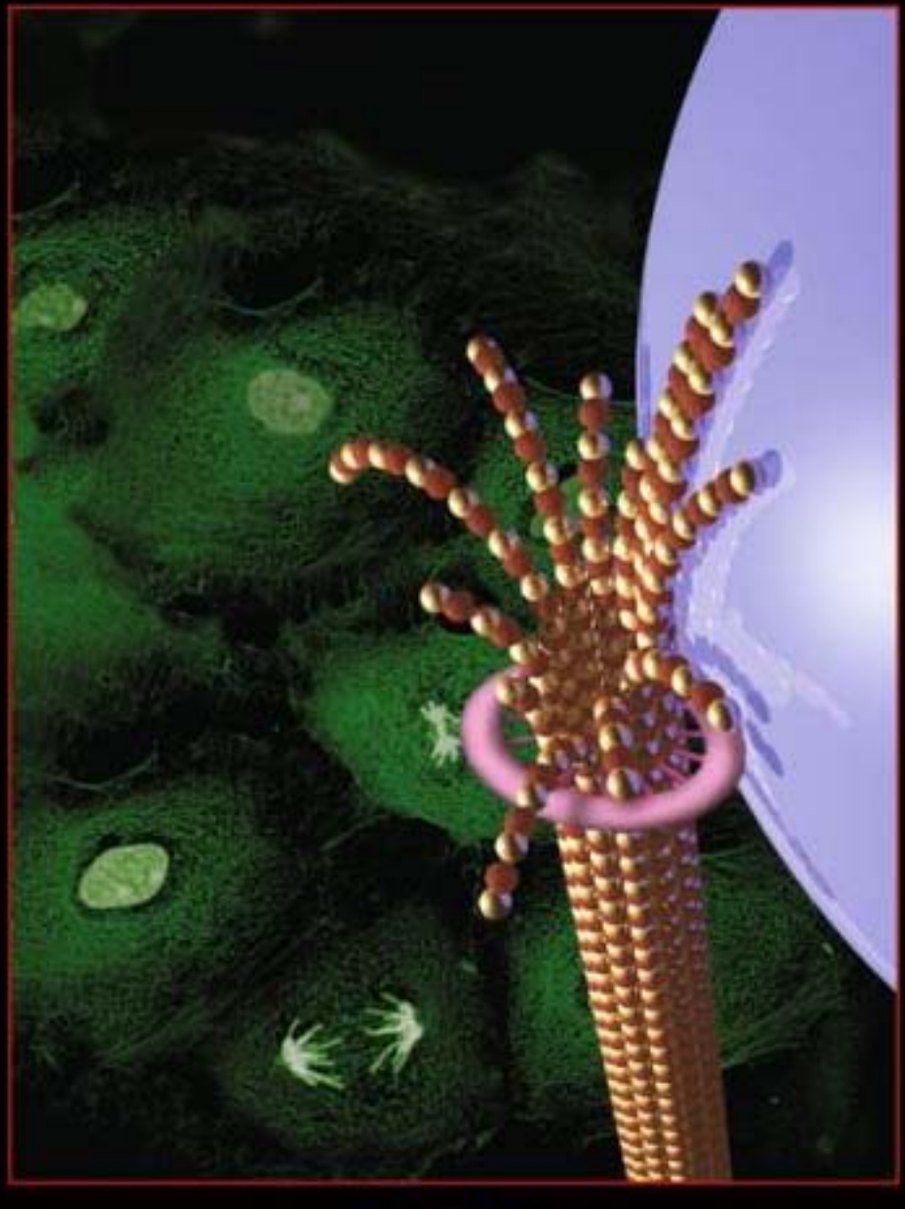
MICROTUBULES, *IN VITRO*



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PREFACE

It has been almost 30 years since the first Methods in Cell Biology volumes that contained methods for analysis of tubulin and microtubules were published. These were two volumes on The Cytoskeleton, edited by Les Wilson, covering the entire cytoskeleton field, which at the time was a relatively new emerging area of cell biology. Volume 24, Part A of the two-part series, focused on the isolation and characterization of cytoskeletal proteins. Of the 24 chapters, 18 were on microtubules; 5 on purification methods, 6 more on length distributions, polarity, assembly kinetics, and tubulin flux measurements, 3 on tyrosination, tubulin tyrosine ligase reactions, and only 2 on dynein. The nontubulin methods chapters covered actin, myosin, and intermediate filament purification and characterization, with 2 chapters on actin assembly measurements. The second volume, Part B, was Volume 25 and focused on biological systems and in vitro models. Of the 18 chapters, 1 discussed axonal transport, 1 discussed microtubule mediated motility of sperm flagella, 2 were on the role of microtubules in secretion and intracellular transport, 3 discussed mitotic spindles and mitosis in either PtK1 cells, sea urchin eggs, or Aspergillus nidulans, and 1 described electron microscopic methods for visualizing microtubules in spindles. The remaining methods chapters focused on actin-based systems with only one chapter devoted to intermediate filaments in muscle. While most of the chapters in the earlier volumes are classics and remain extremely useful today, so much has happened since then. At the time these volumes were assembled and published, dynamic instability had not yet been described, kinesin had not been isolated, video-enhanced differential interference contrast microscopy had not yet been developed, laser trap methods were just being discovered, and the three-dimensional (3D) structure of tubulin and microtubules was a distant dream. As advanced as the state of knowledge on microtubules was at that time, a continuous wave of new discoveries, dominated by the advancement of single-molecule methods and high-resolution techniques and their application to new families of remarkable motor proteins and microtubule-associated proteins (MAPs), has emerged that has sent the microtubule field into unimagined directions.

Here, with a focus on analysis of purified (*in vitro*) systems, 33 groups of experts have written state-of-the-art methods chapters that reflect many of the advances that have taken place in the analysis of tubulin and microtubules and their functions that have occurred in the subsequent 28 years. Volume 95, Microtubules, *In Vitro*, is organized into five sections. Section 1, on Isolation, Biochemistry, and Characterization of Antibodies and Isotypes, consists of seven chapters on tubulin isolation, β-tubulin antibody production and characterization methods, PCR methods for tubulin isotypes and MAPs, and native electrophoresis methods together with two chapters on mass spectroscopy, C-terminal posttranslational modifications, and tubulin proteomics. Section 2, Microtubule Structure and Dynamics, consists of seven chapters on cyro-electron microscopic- and atomic

force microscopic imaging of microtubules, analysis of microtubule dynamic instability at steady state, and at nanometer resolution, single-molecule imaging of microtubule dynamics and kinesin motors, and mathematical modeling of microtubule dynamics. Section 3 (Drugs) includes seven chapters on methods for measuring drug binding to tubulin and to microtubules. Section 4 (Interactions with Motors and MAPs) includes seven methods chapters useful for studying MAP tubulin interactions. These include analysis by nuclear magnetic resonance spectroscopy, isothermal titration calorimetry (ITC), fluorescence, total internal reflection fluorescence (TIRF), fluorescence polarization microscopy (FPM), and the methods for functionalizing the surfaces for these measurements. Finally, Section 5 (Functional Extracts and Force Measurements) includes five chapters describing methods for performing dynamics measurements in functional extracts, for extracting mechanical properties from thermal fluctuations of microtubules, for measuring force at dynamic microtubule ends, and for reconstructing kinetochore complexes and measuring the associated forces.

This volume in part reflects the continuing importance of tubulin and microtubule biochemistry and the emergence of modern physical methods for studying molecular interactions including immunological and PCR techniques. It also reflects the critical importance of antimitotic drugs as drugs for treatment of cancer and as tools in cell and molecular biology, and the development of assays to study them, especially at high resolution. While the antimitotic field is currently dominated by the drug discovery biotechnology and questions about biomarkers and clinical outcomes (see the recent volume edited by Tito Fojo, *The Role of Microtubules in Cell Biology, Neurobiology and Oncology*), the assays described here play a major role in drug development and in our understanding of the microtubule cytoskeleton. Recent advances in cryo-electron microscopy, nuclear magnetic resonance spectroscopy, atomic force microscopy, and proteomic methodology are driving detailed studies into the structure and function of microtubule-based systems. The single-molecule imaging and the techniques that allow nanometer and 3D-nanometer resolution, microtubule plus end tip tracking, and detailed structural pictures of kinesin motility clearly play a major role in the new experimental approaches described in this volume. The merging of single-molecule nanotechnology with high-resolution imaging has significantly changed the questions we can ask and the way microtubule structure, function, and regulation can be analyzed. While this volume is not meant to be all inclusive, it does bring together the breath of biochemical, physical, structural, dynamic, microscopic, and nanomolecular techniques that are now available for study of the microtubule cytoskeleton with purified microtubule systems. An accompanying *Methods in Cell Biology* volume that will be published later this year, *Microtubules*, *In Vivo*, edited by Lynne Cassimeris and Phong Tran, will focus on analysis of microtubule function in living cells.

We wish to thank all the authors for their contributions and the immense effort they put into making this volume an over whelming success. Their willingness to contribute to this project has been essential. (To those authors who were unable to meet the deadline for this volume, we anticipate assembling a follow-up volume on microtubules *in vitro* to this volume). We especially want to thank Tara Hoey, Zoe Kruze, and Narmada Thangavelu at Elsevier for their support and organizational skills

throughout this long and seemingly endless process. Finally, we thank our colleagues, collaborators, and families, who only politely harassed us when we seemed to be distracted from our other more noble pursuits.

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SECTION I

Isolation and Biochemistry of Tubulin and Characterization of Antibodies and Isotypes

CHAPTER 1

Preparation of Microtubule Protein and Purified Tubulin from Bovine Brain by Cycles of Assembly and Disassembly and Phosphocellulose Chromatography

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I. Introduction

Tubulin can be purified from a number of nonmammalian and mammalian nonneural sources (e.g., Farrell, 1982; Detrich and Wilson, 1983; Newton *et al.*, 2002; Bellocq *et al.*, 2005), but for most applications mammalian brain is the most commonly used starting material (Borisy *et al.*, 1975; Lee and Timasheff, 1975; Asnes and Wilson, 1979; Murphy, 1982; Williams and Lee, 1982; Sloboda and Belfi, 1998; Andreu, 2007). There are several important reasons that mammalian brain is so highly favored. First, the tubulin concentration in brain is very high, especially in

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brains from young animals (Bamburg *et al.*, 1973). Second, microtubules from mammalian brain readily assemble at 30–37°C and quickly depolymerize at cold temperature (0–4°C), facilitating purification by straight forward alternating cycles of warm temperature polymerization and cold temperature depolymerization. Third, mammalian brain contains a high content of assembly-promoting/stabilizing microtubule-associated proteins (MAPs) such as MAP2 and tau. These MAPs, which are mainly found in large amounts in the axonal and dentritic processes of neurons, strongly promote microtubule nucleation and elongation and reduce the critical tubulin concentration required for self-assembly. Beginning with fresh brains from cows, pigs, chickens, goats, mice, rats (or other mammals), and especially from young animals, one can obtain tens of milligrams of assembly-competent microtubule protein (MTP) and purified tubulin in 6–10 h.

Solvents such as glycerol or dimethylsulfoxide (DMSO), and drugs like taxol, strongly promote microtubule polymerization, and a number of purification protocols based upon alternating cycles of warm assembly and cold disassembly have been developed over the years in which such solvents or taxol are added to promote polymerization. While the use of these agents increases the yields of tubulin, because of the high MAP content, purification of tubulin from brain tissue by cycles of assembly and disassembly is very efficient in the absence of such agents as long as the concentration of tubulin present during the assembly reaction is well above the critical tubulin concentration required for polymerization in the presence of the MAPs (~0.3–0.7 mg/ml). This is easy to accomplish simply by careful adjustment of the buffer volume when resuspending microtubules assembled during the preceding cycle into cold depolymerizing buffer (see below).

Also depending upon the application, the use of assembly-promoting solvents or assembly-promoting agents could present important disadvantages. The most important is that they modify the polymerization, treadmilling, and dynamic instability behaviors of microtubules and can mask the activities of drug molecules or specific MAPs that one would like to study (e.g., Schlistra *et al.*, 1991; Panda *et al.*, 1999). Thus, if protocols that involve assembly in assembly-promoting agents are used, it is critical to ensure that all of the agents are completely removed in order to study the effects of modulating proteins or drugs—a procedure that takes additional time and could result in some degradation of the tubulin. Here we present an efficient high yield and relatively easy protocol for purification of MTP (tubulin plus stabilizing MAPs, consisting of ~70–75% tubulin and 25–30% MAPs) and, subsequently, for purifying tubulin from the MTP, in the absence of assembly-promoting solvents.

II. Protocols

A. Optimal Starting Material and Initial Processing

1. Freshness of Brains: Age and Sex of the Animals

The yield of polymerization-competent tubulin is highly dependent upon the freshness of the brain tissue, which is difficult to control. We obtain bovine brains at a commercial slaughter house from Mr. Ramero Carlos of Manning Beef LLC, in Pico Rivera, CA, which is about a 3-h drive from our laboratory. There is a complex and somewhat time-consuming process defined by law that slaughter houses must

follow, which affects brain freshness. As a rule, when cattle arrive at the slaughter house they are first inspected to ensure good health. Animals are then filed onto a killing floor where they are rendered unconscious with a captive bolt pistol fired against the forehead centered just above the eyes. The jugular vein and carotid artery are then cut to drain the blood. Although head removal from the carcass is one of the initial steps in the slaughtering process, the brain cannot be removed from the head until the rest of the carcass has been inspected. First the internal organs are excised and inspected for parasites or disease. Then the tongue is taken and the glands around the head are examined. Only after the carcass passes inspection can the brain be removed from the head. We must arrange to obtain the brains in advance. Murphy (1982) has reported the half-time for loss of tubulin polymerization activity from death of the animal until brain removal to be approximately 19 min. So this process should be carried out quickly. We always request that removal of the brain from the skull be carried out as rapidly as possible—a process that under good conditions takes 20–30 min from the time of kill. The brain is immediately separated into two approximately 125 g hemispheres that are individually placed in plastic sealable bags and buried in ice. This reduces the time to chill the brain to ~0°C and greatly increases the half-time for loss of assembly competence. We have obtained good yields of assembly-competent tubulin from brains that were quickly buried in ice and kept on ice for as long as 5 h after slaughter.

We have found that cow brains yield only one-third the amount of assembly-competent MTP as steer brains. Thus we try to obtain steer brains. Perhaps most important is the age of the animals. Dairy cows at the slaughter house we use are usually 5–7 years old. On the other hand steers, which are raised primarily for beef, are usually no more than 2 years old when slaughtered. Since we have found that young heifers yield similar amounts of assembly-competent protein as steers, it seems that the age of the animal is much more important than the gender, with younger being better. Bulls, like cows, are usually older animals when sent to slaughter and also yield less MTP per gram of fresh brain tissue than steers. Thus, it is best to obtain brains from young steers.

2. Condition of the Brains

An important factor that influences the yield of MTP is the condition of the brains. Because the animals are initially stunned with a pistol fired against the forehead, the skull is usually shattered at the point of impact, sending fragments of bone deep into portions of the brain. This results in localized hemorrhage and damage to the surrounding brain tissue (perhaps due to protease activity), which in our experience reduces yields. Thus, we remove all damaged brain tissue as soon as possible.

3. Removal of the Meninges

We also have found that the yield of assembly-competent tubulin is greatly increased by removing the meninges and free-flowing and coagulated blood. We do this as quickly as possible at the slaughter house. The presence of blood and blood clots seems to reduce yields. Removal of the meninges also facilitates homogenization of the brain tissue. We remove the meninges with blunt tipped forceps. It is important to handle the brain tissue as gingerly as possible during the process to

minimize tissue damage, to minimize the amount of blood forced into the interstitial spaces of the tissue, and to minimize the presence of free-flowing and coagulated blood prior to homogenization.

4. Processing Speed and Optimal Cycling Temperatures

Once the brain is homogenized (see below) it is important to isolate the tubulin and associated proteins as quickly as possible. The tubulin degrades rapidly once the brain is homogenized even at 0°C and each successive polymerization cycle removes proteases and other degradative activities. It is important to keep in mind that all steps (except for warm temperature polymerization) should be carried out at 0°C with 1 mM GTP and 1 mM Mg^{++} present. It is also important that soluble free calcium is removed with EGTA and that the pH is maintained in a narrow range. We use 30°C to polymerize MTP rather than 37°C. Polymerization is robust at 30°C and experience has shown that recovery of assembly-competent tubulin drops off dramatically as the temperature rises above 37°C. There can be as much as a 5°C variation in temperature above the set point on centrifuges that use a friction-based heating system. In general superspeed centrifuges use a refrigeration unit to cool the chamber and thus the rotor, but rely on friction from the movement of air to heat the chamber and rotor. Temperature is maintained by balancing refrigerated cooling and friction-generated heating. By contrast, most ultracentrifuges are equipped with both heating and cooling units. It is important to confirm the temperature of samples and adjust the centrifuge controls accordingly.

B. Protocol for Purification of Microtubule Protein (Tubulin Plus Microtubule-Associated Proteins) in the Absence of Glycerol

While the protocol has been considerably fine-tuned and standardized, the basic protocol for isolating microtubules by temperature-dependent cycles of assembly and disassembly in the absence of glycerol remains similar to the protocols that were first introduced (Borisy *et al.*, 1975; Asnes and Wilson, 1979; Murphy, 1982). The procedure we describe here is a modification of the Asnes and Wilson procedure (1979). It should work well with some modifications for purification of MTP and tubulin from any brain source. Purification of tubulin from cultured cells or other sources that contain much less tubulin than brain presents special problems that will not be described here (see Farrell, 1982; Detrich and Wilson, 1983; Newton *et al.*, 2002; Bellocq *et al.*, 2005). A flowchart documenting the purification protocol is shown in Fig. 1.

1. Preparation of the Crude Brain Extract (CBE)

We usually begin with two steer brains, weighing a total of between 400 and 600 g. Processing this quantity of tissue requires the equivalent of six Sorvall refrigerated superspeed centrifuges. For example Sorvall RC5B Plus centrifuges equipped with SS-34 rotors work well for the initial purification steps. It is convenient the day before carrying out the purification to prepare two buffers. The first is one l of homogenizing buffer, L-GNPEM buffer, consisting of 100 mM sodium glutamate, 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 6.85,

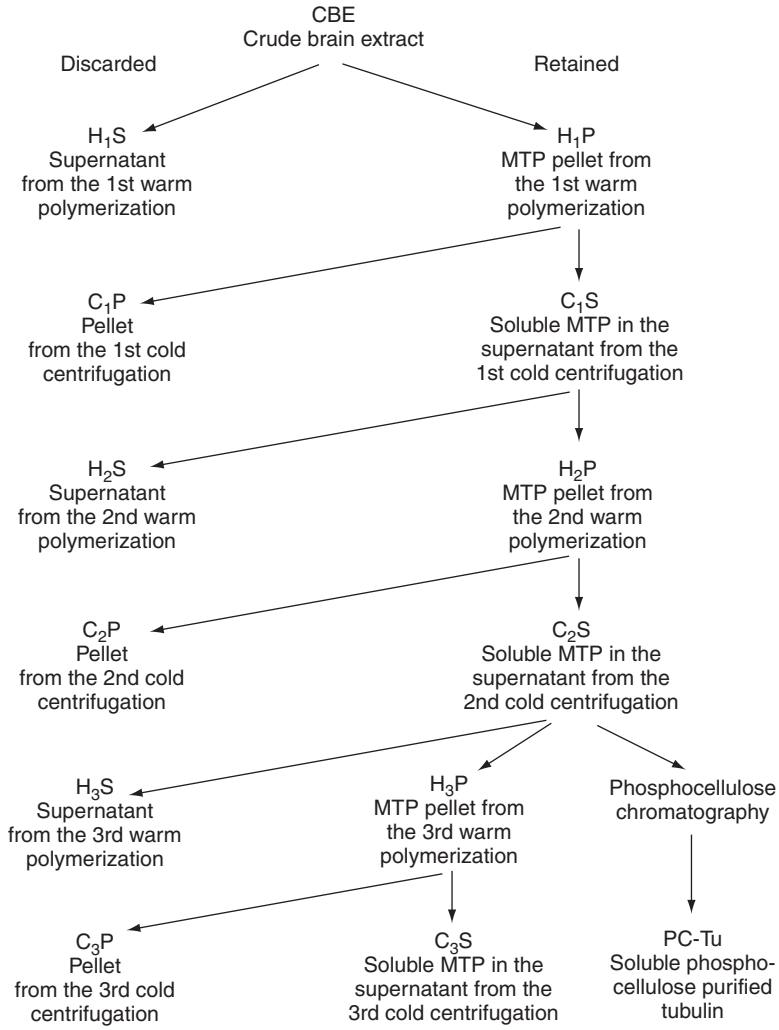

Fig. 1 Flowchart illustrating the steps in the purification of microtubule protein and phosphocellulose-purified tubulin.

and the second is 250 ml of the same buffer at pH 6.75. Both buffers should be refrigerated. Beginning at the slaughter house, the meninges, superficial blood vessels, and blood clots are removed and each brain hemisphere is placed in a separate plastic bag and buried in ice for transport back to the laboratory (see above). Once back at the laboratory, add 1 mM dithiothreitol (DTT) to one l of L-GNPEM buffer, pH 6.85. This buffer will be used to blend the brains. Brains are initially blended in a Waring Commercial Blender at a ratio of 1.5 ml of buffer per gram of wet brain weight at low speed for 30 s. Next, the blended brains are homogenized by using one pass in a motor-driven Teflon pestle/glass homogenizer (Kimble-Chase, Vinland, NJ) operated at the maximum speed (we use a Tri R Stir R motor). Next, the brain homogenate is centrifuged at 32,500 $\times$ *g* (average RCF) for 40 min at 4°C

(we use 50-ml centrifuge tubes). The supernatant, now called the crude brain extract or CBE (Fig. 1), is collected in a liter graduated cylinder to measure the volume and serve as the polymerization vessel.

2. First Polymerization and Depolymerization Cycle

The appropriate amount of GTP is first added in dry form to the CBE at a final concentration of 2.5 mM. Next, the CBE is warmed to 30°C and incubated in a water bath for 30 min to polymerize the MTP into microtubules. We use a 20-gal aquarium with a Fisher Isotemp immersion circulator for all warm incubations. This is followed by centrifugation at 45,000 × *g* for 30 min at 30°C again using 50-ml centrifuge tubes to obtain microtubule pellets (the H_1P). It is important to note the total volume of the warm supernatant, now called the H_1S, which is then discarded.

Next the H_1P microtubule pellets are resuspended in 10% of the volume of the H_1S into cold L-GNPEM buffer, pH 6.75. The suspended microtubules are then homogenized with three passes in a 15-ml glass pestle "A"/glass tissue homogenizer (we prefer the Kontes K885300–0015) and incubated on ice for 40 min to fully depolymerize the microtubules. The solubilized H_1P fraction is then centrifuged at 45,000 × *g* for 45 min at 4°C to clarify it and remove any particulate debris. Next the clarified supernatant, the C_1S, is collected and retained and the C_1P pellet is discarded (Fig. 1).

3. Second Polymerization Cycle

First, 2.5 mM GTP from a stock solution of 100 mM GTP is added to the C_1S, which is then incubated for 30 min at 30°C. The C_1S, now a suspension of microtubules, is centrifuged at 45,000 × *g* for 60 min at 30°C. The H_2S is decanted and the volume is noted before it is discarded. At this point, the H_2P can be quick-frozen (see below) in liquid nitrogen and stored as pellets in a −70°C ultracold freezer until further purification or processing is convenient. As a rule, this will be ∼8–9 h after beginning to prepare the CBE. The H_2P can either be taken through another cycle of assembly/disassembly to obtain a clean MAP-rich tubulin preparation or be subjected to phosphocellulose chromatography (see below) to prepare MAP-free purified tubulin, often called PC-Tubulin (Pc-Tu), for use in experiments.

4. Third Purification Cycle

We often use MTP that has been processed through three cycles of warm assembly and cold disassembly. This third cycle involves centrifuging through 50% sucrose cushions to remove any proteins that do not adhere to the microtubules. The third cycling process is similar to the two previous cycles with the following modifications. The frozen H_2P pellets are quickly thawed by initially placing the tubes containing the pellets in a beaker of room temperature water, followed by cooling the tubes on ice. The thawed H_2P pellets are then suspended in L-GNPEM buffer, pH 6.75, using one-third the volume of the former H_2S, homogenized with three passes in a 15-ml glass pestle "A"/glass tissue homogenizer, and incubated on ice for 20 min to fully depolymerize the microtubules. The solubilized H_2P solution is then centrifuged to clarify it at 105,000 × *g* (average RCF) for 1 h at