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Review of Cytology

EDITED BY

G. H. BOURNE J. F. DANIELLI

ASSISTANT EDITOR
K. W. JEON

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EDITED BY

G. H. BOURNE
Yerkes Regional Primate Research Center
Emory University
Atlanta, Georgia

J. F. DANIELLI
Worcester Polytechnic Institute
Worcester, Massachusetts

ASSISTANT EDITOR
K. W. JEON
Department of Zoology
University of Tennessee
Knoxville, Tennessee

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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

LILY C. A. CONRAD (245), *The Rockefeller University, New York, New York 10021*

J. DOERR-SCHOTT (193), *Laboratoire de Cytologie Animale et E.R.A., Université Louis Pasteur, 67000 Strasbourg, France*

M. P. DUBOIS (193), *Institüt National de la Recherche Agronomique, Station de Physiologie de la Reproduction, 37380 Monnaie, France*

E. FOLLÉNIUS (193), *Laboratoire de Cytologie Animale et E.R.A., Université Louis Pasteur, 67000 Strasbourg, France*

MARC W. KIRSCHNER (1), *Department of Biochemical Sciences, Princeton University, Princeton, New Jersey 08540*

JAMES K. KOEHLER (73), *Department of Biological Structure SM-20, University of Washington, School of Medicine, Seattle, Washington 98195*

EDWARD M. KOSOWER* (109), *Department of Chemistry, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel, and Department of Chemistry, State University of New York, Stony Brook, New York 11794*

NECHAMA S. KOSOWER (109), *Department of Human Genetics, Sackler School of Medicine, Tel Aviv University, Ramat Aviv, Tel Aviv, Israel*

LYNN MARGULIS (267), *Department of Biology, Boston University, Boston, Massachusetts 02215*

DONALD W. PFAFF (245), *The Rockefeller University, New York, New York 10021*

ROBERT ROSEN (161), *Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7*

SRINIVAS K. SAIDAPUR (225), *Departments of Obstetrics and Gynecology, University of Kansas Medical Center, Kansas City, Kansas 66103*

LELENG P. TO (267), *Department of Biology, Boston University, Boston, Massachusetts 02215*

I. B. ZBARSKY (295), *Biochemistry Laboratory, N. K. Koltzov Institute of Developmental Biology, Academy of Sciences of the U.S.S.R., Moscow V-334, U.S.S.R.*

**Present address:* Departments of Medicine and Chemistry, University of California, San Diego, La Jolla, California 92093.

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Department of Biochemical Sciences, Princeton University, Princeton, New Jersey

I. Introduction

The study of cell structure has undergone two periods of intense progress. Histological observations in the late nineteenth and early twentieth century described many features of cellular differentiation, as well as common cellular structures such as the mitotic spindle. The conclusions reached from these studies were often far-reaching, accurate, and uncannily prophetic. A second period of inquiry employed the electron microscope and ultrastructural histochemical methods to give more molecular detail to the structures described earlier by histologists, as well as to reveal structures unseen before. There is a feeling now that we are at the beginning of a third wave of inquiry, employing biochemical methods to study cellular structure. We can expect that earlier observations will be reexamined in greater detail and that much will be explained, but in the early stages of these investigations we cannot always tell what experiments have extended our insight into biological processes.

It is curious perhaps that the biochemical study of cellular structure has been so slow in developing when at the same time so much progress has been made in

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complex biochemical investigations of gene expression, enzyme regulation, and virus assembly. Despite the fact that actin is in general the most common cellular protein and that so much was known about it from studies of muscle, not until recently has its role in nonmuscle cells been appreciated. Tubulin, perhaps the second most common intracellular protein, was not described until the 1960s. Functional studies of tubulin assembly were not performed until 1972. There are many reasons for this. In some cases, new techniques had to be developed and some of the pitfalls in the older methods had to be rediscovered by later investigators. During this period, it became clear that assays for structural proteins are different from enzymic assays. They are more ambiguous and often more awkward. Studying the biochemistry of these proteins is difficult, since despite their high concentrations they have often proven difficult to purify to homogeneity. A few pioneers, however, have defined the biochemical questions in a cellular context, thus opening the trail to problems and solutions which we all could follow.

The biochemical study of structural proteins such as tubulin and actin is therefore a rather new area. It has nevertheless received a considerable amount of attention recently which, it is hoped, reflects progress. In the last 3 years there have been three major conferences on microtubules, which have resulted in published compilations of papers. There have been a few recent review articles which have summarized this rapidly changing field, as well as numerous journal articles. However, so much progress has been made recently that in many areas old reviews are of little use, and an attempt to codify the experimental facts would be worthwhile. Some aspects of the problem such as the structure of microtubules and the biochemical properties of tubulin are, it seems, now rather well understood. For questions such as those involving the pathway of assembly and the role of nucleotides, there appears to be a clearer consensus emerging. Even for some questions, such as those dealing with the function of accessory proteins, there may be hope that some general principles are becoming apparent. For others, however, such as those involving the mechanism of microtubule nucleation at the centriole and centrosome and *in vivo* regulation of microtubule assembly, the best one can do is try to sort the clearer experiments from the more obscure ones. The probability is not negligible of course that the best experiments are wrong and that the more poorly documented ones will in fact be proven to be correct.

What we have tried to do in this article is to view the field of microtubule biochemistry from the perspective of a cell biologist wishing to know the facts potentially useful for extending his or her biological insight. Establishing the facts in some cases is difficult. There is an awkward period in the development of a new field of biology when it leaves the familiar confines of formal description for the confusing terrain of chemical investigation. The results come quickly and are often contradictory. Things that should not be important or are intrinsically uninteresting become crucial, such as how exactly a substance was purified. Yet

it is usually worthwhile to persevere in trying to extract the important conclusions. The purpose of this article is to establish from the recent studies on *in vitro* assembly, nucleation, and growth of microtubules the properties which shed light on their biological role, suggest means of regulation, and demonstrate interesting principles of self-assembly. This article is concerned with the experiments which emanated from the discovery of conditions for assembly of microtubules from brain extracts (Weisenberg, 1972), which relate to the biochemical properties of tubulin, and which utilize *in vitro* systems for studying nucleation and growth of cytoplasmic microtubules. We do not summarize the biological role of microtubules in this article. We have chosen certain topics in microtubule assembly which seem to have been well studied in the past few years and have not discussed or mentioned many other findings. The choices and particularly the omissions do not reflect the value of the particular experiments but merely our view of the appropriateness of discussing them in this context at this time, as well as the limitations of space. Since we believe reviewers should make judgments in their reviews, we have also tried to make judgments. Whether the judgments ultimately prove correct or incorrect, we hope they will at least help to clarify the issues.

II. Structure of Microtubules

Microtubules are found in all eukaryotic cells. They are part of a large number of structures such as mitotic spindles, eukaryotic flagella, cilia, nerve cell processes, cytoplasmic cytoskeletal elements, and so on (for recent reviews, see Olmsted and Borisy, 1973a; Snyder and McIntosh, 1976; Stephens and Edds, 1976). With only a few exceptions (Burton and Hinkley, 1974; Nagano and Suzuki, 1975), the general morphology of cytoplasmic microtubules appears to be identical in all cell types and in all species studied. It is likely that varying dimensions reported in various ultrastructural studies reflect differences due to problems with fixation or staining rather than differences in structure. The morphology of typical brain microtubules as revealed by negative staining is shown in Figure 1. In transverse section they appear as hollow cylinders 24 nm in diameter made up of 13 discrete subunits (Tilney *et al.*, 1973). These subunits are stacked in parallel fashion, giving rise to 13 longitudinal protofilaments. In flagella, centrioles, and basal bodies, modified microtubules are found which are composed of one complete microtubule of 13 protofilaments and one (flagella) or two (basal bodies and centrioles) incomplete microtubules which share a common wall made up of 3 protofilaments (Ledbetter and Porter, 1964; Porter, 1966; Phillips, 1969; Ringo, 1967; Tilney *et al.*, 1973; Warner and Satir, 1974).

A description of the detailed subunit arrangement in microtubules is important to an understanding of the mechanism of microtubule assembly and possible structural interactions with other materials. The surface lattice of subunits in the

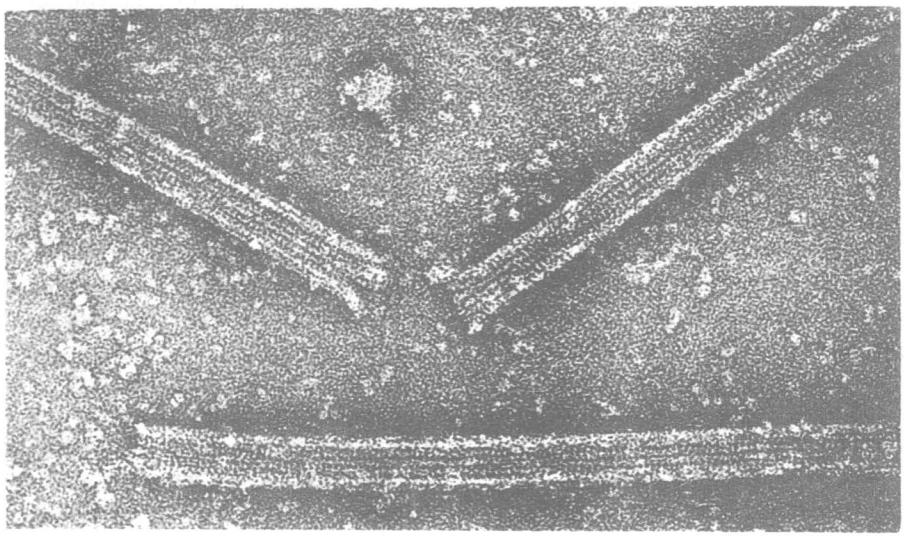


FIG. 1. Electron micrograph of pig brain microtubules negatively stained with uranyl acetate. ×205,000. (Courtesy of Dr. Robley Williams, University of California, Berkeley.)

microtubule has been studied by optical diffraction of negatively stained electron microscope images and by x-ray diffraction of oriented flagellar microtubules or repolymerized brain microtubules. Optical diffraction of electron micrographs clearly shows a 4-nm spacing (see Fig. 2a) which is easily observed as longitudinal repeats along the protofilament in good electron micrographs of tubules (Grimstone and Klug, 1966; Chasey, 1972; Erickson, 1974b; Amos and Klug, 1974; Linck and Amos, 1974). The 4-nm spacing is thought to represent the repeat of a single 55,000-molecular-weight polypeptide chain of tubulin. From the position of the peaks and the 4-nm layer line, Amos and Klug (1974) and Erickson (1974b) computed a model for the surface lattice that involves two helical families, a 10-start right-handed and a 3-start left-handed helix with 4-nm spacing. Since the 10^5-molecular-weight dimer is almost certainly the unit of assembly in the microtubule, the lattice can be subdivided, in dimers with linear dimensions of 8 nm, into either a right-handed 5-start helix or a left-handed 8-start helix. Figure 2c is a diagram of the surface lattice of a microtubule as reported by Amos (1975), showing the 3- and 10-start as well as the 5- and 8-start helical families. At a higher resolution Erickson (1974b) saw a splitting of the individual proteins in the reconstituted microtubule, and Amos reported that flagella microtubules seem to be "dumbell dimers joined end to end to form longitudinal protofilaments" (Amos, 1975). The subunits within a protofilament show the greatest structural cohesion. Each protofilament is staggered in order to produce the 5- and 8-start helical families, although the 8-nm reflection is sometimes not observed.

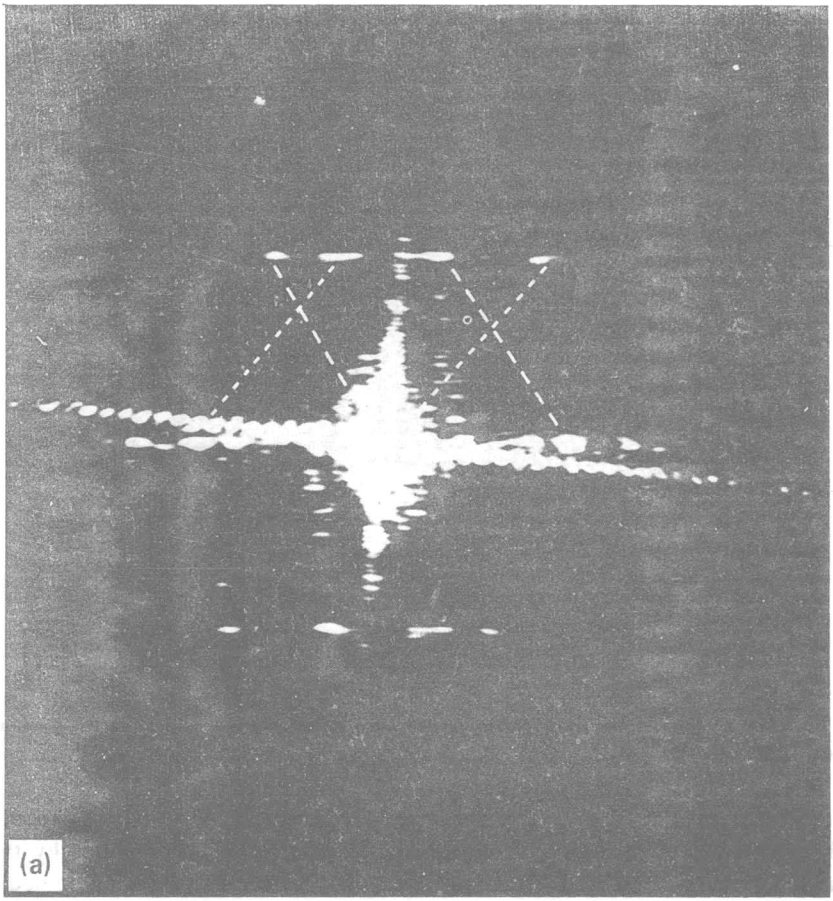


FIG. 2a. Optical diffraction pattern from an electron micrograph of microtubules stained with uranyl acetate. The mask is at an angle to the microtubule axis which is horizontal in this photograph. Courtesy of Dr. Linda Amos, MRC, Cambridge. Fig. 2b and c on following pages.

Initial interpretations of the x-ray diffraction patterns of hydrated microtubules from sperm tails were similar to those of optical diffraction patterns of electron microscope images. Figure 2a is an actual optical diffraction pattern from an electron micrograph of microtubules stained with uranyl acetate (Amos, 1975). The two spots on the 40-Å-layer line closest to the meridian are due to the 3-start helical families, and the two spots on the same 40-Å-layer line farthest from the meridian are due to the 10-start helical families. Figure 2b is an x-ray diffraction pattern of brain microtubules (C. Cohen, personal communication). The same 40-Å repeat is clearly visible. However, the intensity of the spots is quite dif-

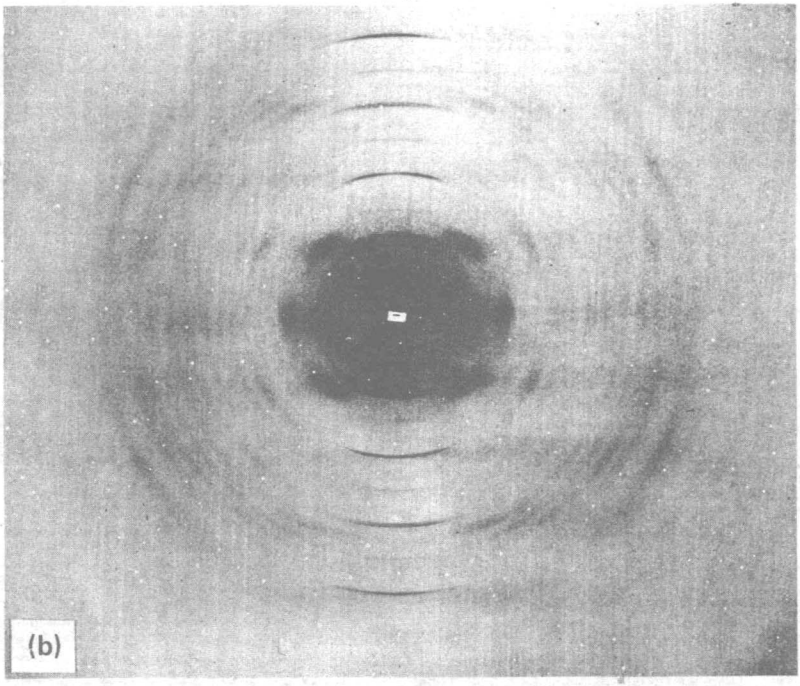


FIG. 2b. X-ray fiber diffraction pattern of calf brain microtubules. (Courtesy of Dr. Carolyn Cohen, Brandeis University.)

ferent (Cohen *et al.*, 1971). This apparent discrepancy can be attributed either to the artifactual production and relative obliteration of some structures by the negative stain, or to actual rearrangement of the subunits in the microtubule during preparation of the samples for electron microscopy. In addition, one of the important meridianal reflections was lacking in the early x-ray patterns, and the diameter of the microtubule was not known accurately. This led to an interpretation of the x-ray patterns different from that of the optical diffraction patterns. However, recent x-ray patterns of repolymerized brain microtubules show reflections completely consistent with the surface lattice proposed by Amos and Klug (Cohen *et al.*, 1975). The intensities, however, are still quite different from those of the optical diffraction pattern of the negatively stained preparations. The x-ray pattern shows a deeply grooved 10-start helix, and there is little intensity from the 3-start helix (C. Cohen, personal communication). This may mean that the actual shape of the subunit as revealed by electron microscopy, which reflects stain distribution and penetration, is different from that revealed by x-ray diffraction, which reflects mass distribution. The arrangement of the subunits, however, appears to be that shown in Fig. 2c.

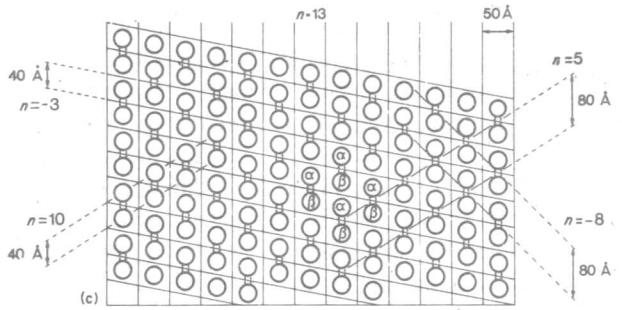


FIG. 2c. Surface lattice of a microtubule from Amos and Klug (1974), showing left-handed 3- and 8-start helices and right-handed 5- and 10-start helices. By permission of Company of Biologists Limited.

III. Purification and Identification of Tubulin

Tubulin was originally identified as a soluble protein found in most eukaryotic cells, which bound the antimitotic drug colchicine (Wilson and Friedkin, 1966; Borisy and Taylor, 1967), or as a major component of ciliary microtubules (Renaud *et al.*, 1968; Shelanski and Taylor, 1967, 1968). The tight binding of radioactive colchicine by some component in the extract made possible a simple and sensitive assay for the presence of this protein (Borisy and Taylor, 1967). Numerous experiments by Taylor and his students correlated the presence of colchicine binding with the presence of microtubules in tissues as well as with the presence of microtubules in structures such as the mitotic apparatus, cilia, and flagella. The colchicine-binding factor or tubulin had remarkably similar properties in many types of cells. The colchicine-binding reaction was slow, and the activity was labile and sedimented at 6S (Weisenberg *et al.*, 1968).

Tubulin assayed on the basis of its colchicine-binding activity has been purified from neuronal tissues by the method or modifications of the method of Weisenberg *et al.* (1968). This procedure involves separating the acidic tubulin molecules from other cell proteins by DEAE-Sephadex chromatography. With the use of this method tubulin has been purified from various sources.

Tubulin has also been obtained by precipitation with vinblastine. Vinblastine is an alkaloid which binds tightly to tubulin (Owellen *et al.*, 1972; Bryan, 1972; Lee *et al.*, 1975; Wilson *et al.*, 1975) and induces the formation of tubulin complexes and paracrystals both *in vivo* and *in vitro* (Krishan and Hsu, 1969; Schochet *et al.*, 1968; Tyson and Bulger, 1973; Bensch and Malawista, 1968; Marantz and Shelanski, 1970). Although vinblastine precipitation when used as a general precipitating reaction has been criticized as being nonselective for acidic proteins (Wilson *et al.*, 1970), it is likely that paracrystal formation is a specific reaction for tubulin. Precipitation with vinblastine has also been used to prepare

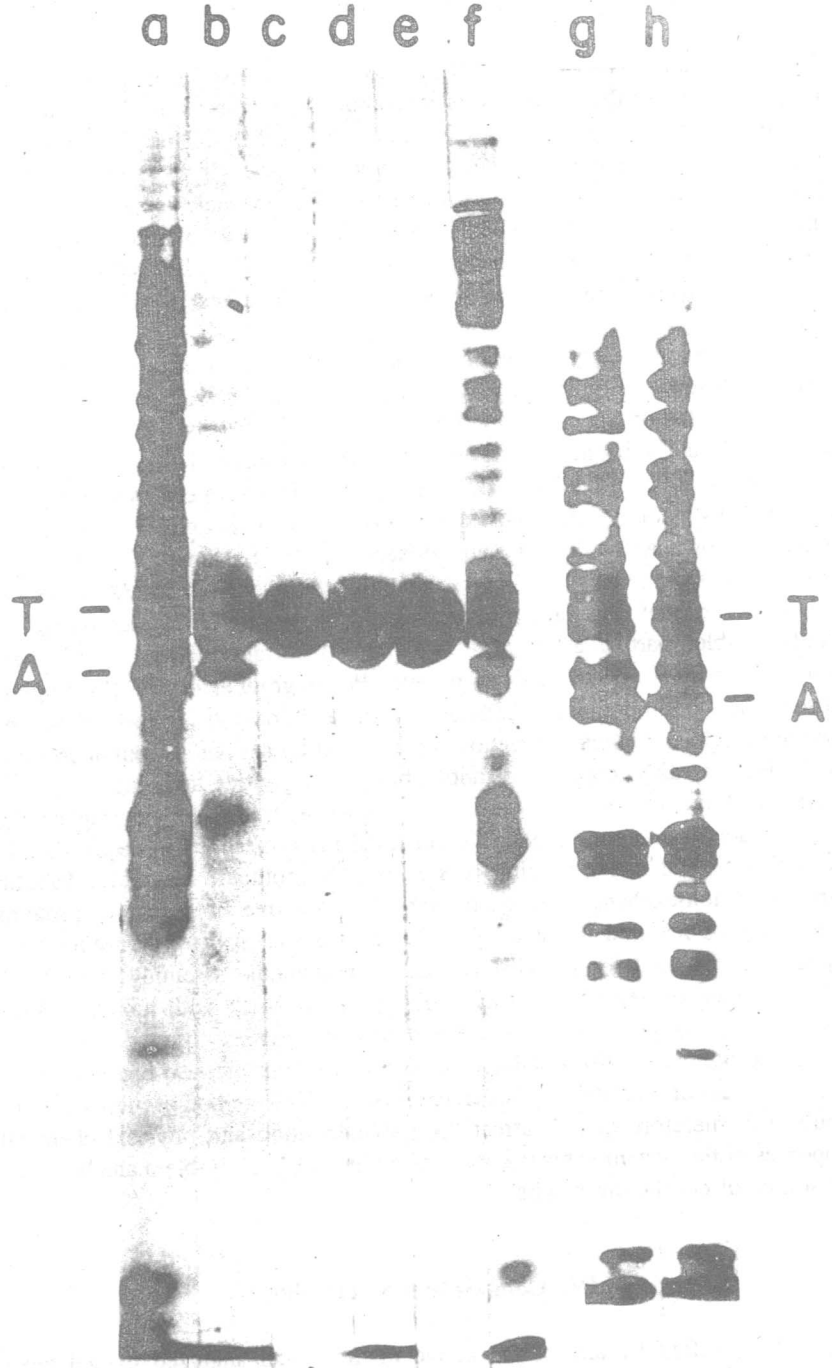
tubulin from various nonneuronal sources such as *Chlamydomonas* and mouse L cells (Olmsted *et al.*, 1970; Nagayama and Dales, 1970).

Recently, the major method for tubulin preparation has been the use of microtubule purification through reversible polymerization and depolymerization (Shelanski *et al.*, 1973; Borisy *et al.*, 1974). Extracts are warmed to polymerize the microtubules, and the latter are removed by sedimentation. The microtubules are then depolymerized, and nonspecific aggregates are removed. This technique has been used to prepare fractions enriched in tubulin from both neuronal and nonneuronal sources. It is not uniformly applicable, since it requires a sufficiently high concentration of tubulin to overcome the initially difficult nucleation steps in assembly, and therefore it may not succeed in the presence of inhibitors or if there is an insufficient concentration of tubulin or accessory proteins. For example, Wiche and Cole (1976) polymerized microtubules reversibly from a rat glial cell line, but using the same procedure were unable to obtain tubulin from a mouse neuroblastoma line. This procedure, however, has been used to obtain microtubules, hence tubulin, from *Drosophila* embryos (Green *et al.*, 1975), renal medulla (Barnes *et al.*, 1975), and blood platelets (Crawford *et al.*, 1975), as well as neuronal tissue (Shelanski *et al.*, 1973; Borisy *et al.*, 1974).

When it is difficult to achieve polymerization from extracts, small quantities of radioactively labeled tubulin can be prepared by copolymerization with carrier brain microtubules. This has been useful with *Chlamydomonas* gametes (Weeks and Collis, 1976), cultured Chinese hamster ovary (CHO) cells (Spiegelman *et al.*, 1977), *Aspergillus* (Sheir-Neiss *et al.*, 1976), and yeast (Water and Kleinsmith, 1976). Figure 3 shows autoradiographs of electrophorograms of methionine-^{35}S-labeled tubulin from cultured CHO cells purified by copolymerization with porcine brain microtubules. Slot a is the soluble extract of CHO cells in which the α and β peptides of tubulin together comprise about 14% of the total soluble protein. After the first polymerization and depolymerization step tubulin represents 80% of the protein (slot b). After the second cycle of assembly and disassembly, 90% of the radioactive protein labeled with methionine-^{35}S is tubulin (slot d). Slots g and h show that purification is dependent on added carrier brain microtubules.

The use of a method of purification designed to yield tubulin is not in itself adequate for identifying tubulin, as graphically demonstrated by Kane (1975). He obtained actin from sea urchin eggs by a polymerization procedure designed

FIG. 3. Gel electrophoretic analysis of the copolymerization of methionine-^{35}S-labeled tubulin from CHO cells with pig brain microtubulins. Pig brain microtubules are mixed with a labeled CHO extract and carried through cycles of thermally induced polymerization and depolymerization. (a) CHO cell extract; (b) one cycle of assembly; (c) two cycles of assembly; (d) three cycles of assembly; (e) phosphocellulose-purified CHO tubulin; (f) associated protein fraction from phosphocellulose; (g) CHO crude extract; (h) one cycle of assembly without carrier microtubules. T, Brain tubulin position; A, muscle actin position.(Spiegelman *et al.*, 1977. By permission of the MIT Press. Copyright © MIT.)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to purify tubulin. A colchicine affinity column has also been used in which deacylated colchicine is linked by esterification to the column matrix (Hinman *et al.*, 1973; Sandoval and Cuatrecasas, 1976a,b). Although this column has an affinity for tubulin, it seems to operate at low ionic strength by a general hydrophobic interaction and not a specific interaction at the colchicine-binding site, since tubulin pretreated with colchicine still binds to the column (Morgan and Seeds, 1975a). While this column purifies brain tubulin, it may not be as effective for nonneuronal tissues, and one cannot assume that it will yield pure tubulin.

The criterion of purity for tubulin which has been most often employed has been the presence of only two polypeptides of molecular weight 55,000 which can be separated on urea–sodium dodecyl sulfate (SDS) gels and stacking SDS gels (Bryan and Wilson, 1971; Feit *et al.*, 1971; Eipper, 1972; Luduena and Woodward, 1973). All tubulin examined so far has had these two proteins in roughly equal amounts, and reported deviations are now thought to be artifacts of preparation or analysis (Bibring and Baxandall, 1974). Nonstacking SDS systems (Weisenberg *et al.*, 1968) reveal a single tubulin band at 55,000 molecular weight. Additional criteria, however, should be employed in identifying the tubulin doublet, particularly because two other structural proteins, neurofilament protein (Yen *et al.*, 1976) and tau protein (Penningroth *et al.*, 1976), migrate close to the tubulin doublet in SDS gels. Some additional criteria which may be used are colchicine binding, reaction with antitubulin antibodies, polymerizability or (more weakly) copolymerization ability, and peptide mapping.

All the above methods of purification, coupled with SDS gel electrophoresis, are adequate for preparing tubulin for chemical analysis but are not equivalent in the preparation of native tubulin for studies of microtubule assembly. Tubulin purified by ion-exchange chromatography is often free of accessory proteins which may be required for assembly. No one has shown that vinblastine paracrystals can be used for physical studies of microtubule assembly. Reversible polymerization or depolymerization yields proteins along with tubulin, which may or may not be functionally related to microtubules. Immunoadsorption (Ikeda and Steiner, 1976) or colchicine affinity columns are also likely to select their own class of microtubule-associated proteins. The method of preparation of tubulin can therefore greatly affect the polymerization and physical chemical properties of the system. This is unfortunately not a trivial problem and has been the source of conflicting results.

IV. Composition of Tubulin

Tubulin purified by any of the above methods and analyzed by gel electrophoresis was seen to fractionate into two closely spaced bands. The split bands