DIRECT BIOCHEMICAL MEASUREMENTS OF MICROTUBULE ASSEMBLY AND DISASSEMBLY IN CHINESE HAMSTER OVARY CELLS

The Effect of Intercellular Contact, Cold, D_2O, and $N^6,O^{2'}$-Dibutyryl Cyclic Adenosine Monophosphate

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ABSTRACT

A study was undertaken to develop a means of quantitating the amount of tubulin present as a soluble pool and as intact microtubules in cultured Chinese hamster ovary cells. A procedure was developed in which these cells grown on monolayer culture in Petri dishes were placed in a "microtubule stabilizing medium" (MTM) consisting of 50% glycerol, 10% dimethylsulfoxide and sodium phosphate magnesium buffer, as described previously by Filner and Behnke. These cells then were homogenized and the homogenate was spun in the ultracentrifuge. Colchicine binding activity was then determined in the supernates and the pellets. The values, when compared with total colchicine binding activity present in replicate homogenates, were used to determine the percentage of tubulin present as intact microtubules. A statistical analysis of thin sections of cells treated with MTM revealed no statistically significant difference between MTM-treated cells and untreated controls. It was further discovered that the relative amount of colchicine binding activity recovered in the high speed pellet varied dramatically, depending upon the cell number of the culture being studied. Preconfluent cultures showed very low colchicine binding activity averaging less than 5%, while confluent and postconfluent cultures often possessed as high as 25% of their total colchicine binding activity in pelletable material. Although cold and D_2O treatment had little or no effect on these values, $N^6,O^{2'}$-dibutyryl cyclic adenosine monophosphate increased them. It is hoped that this study will serve as the basis for a reliable quantitative procedure for measuring microtubule polymerization and depolymerization in vivo.

Microtubule polymerization in living cells and the regulatory processes involved in microtubule assembly are current topics stimulating great interest. Cellular morphogenesis and motility are mediated, at least in part, by cytoplasmic microtubules (e.g. Gibbons and Grimstone, 1960; Byers and Porter, 1964; and Satir, 1968), and the regulation of such activity is thought to be facilitated by the

control of tubulin polymerization (e.g. Inoué, 1964; Inoué and Sato, 1967; Borisy and Taylor, 1967; and Rosenbaum and Child, 1967 *a*, *b*). However, the factors or conditions influencing assembly and disassembly of microtubules in living cells are not well understood. In the few cases studied (e.g. Piatigorsky et al., 1972; and Wilt et al., 1967), protein synthesis has not been shown to be a primary regulatory factor. Recently, dibutyryl cyclic AMP has been implicated in the process of regulating tubulin polymerization (e.g. Rubin and Filner, 1973; Goodman et al., 1970; Soifer, 1972; and Kirkland and Burton, 1972), but the exact nature of its role remains obscure. A major difficulty that faces an investigator studying the assembly of microtubules is the lack of a reliable means to measure quantitatively the amount of tubulin present as intact microtubules. This paper describes a procedure that can be used to quantify the amount of polymerized tubulin present in cultured cells, as well as to measure soluble tubulin pools in the same system. The reliability of the procedure is verified here by statistical analysis, and the technique is then used to study the effects of various treatments, such as dibutyryl cyclic AMP, on microtubule assembly and disassembly in Chinese hamster ovary cells.

MATERIALS AND METHODS

Culturing Procedure

Chinese hamster ovary cells were obtained initially from David M. Prescott (University of Colorado, Boulder, Colorado) and maintained in large Blake bottles at 37°C in a high humidity (5% CO_2-95% air) incubator. The cells were plated at variable densities in a total volume of 4 ml of media in 60-mm Falcon plastic petri dishes (Falcon Plastics, Div. BioQuest, Oxnard, Calif.) and allowed to stabilize for at least 24 h before use. The growth medium employed was Ham's F-12 supplemented with 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml Fungizone (Grand Island Biological Co., Grand Island, N. Y.).

Electron Microscopy

Cells prepared for scanning electron microscopy (SEM) were grown on glass cover slips in 3.0-cm diameter plastic petri dishes in which the cells were cultured. The cultures were fixed for 20 min with 3% glutaraldehyde in an aqueous solution of 0.05 M cacodylate buffer and 50% by volume Puck's Saline G (pH 7.4). After a brief wash in Puck's Saline G, the cells were postfixed for 15 min in 1% OsO_4 buffered with 0.2 M cacodylate (pH 7.2) and then dehydrated in an acetone series. A Sorvall critical point drying apparatus (Ivan Sorvall, Inc., Newtown, Conn.) was employed to dry the cells via the critical point technique described by Porter et al. (1972). A thin layer of carbon and gold was evaporated onto the specimen surfaces, and observations of the preparation were made with a Cambridge Stereoscan S-4 electron microscope operating at 20 kV.

Cells prepared for transmission electron microscopy were grown on glass cover slips coated with a layer of carbon. The fixation procedure was identical to that for SEM, except for an increased wash interval in Puck's Saline G between the glutaraldehyde fixation and osmification. The cells were then washed in 10% acetone to remove the residual buffer and stained en bloc with 0.5% uranyl acetate in 0.1 M collidine (pH 4.55) overnight at 4°C. These preparations were dehydrated in an acetone series and placed in a solution of 50% acetone and 50% Epon-Araldite in a desiccator at room temperature for 18 h. At the end of this period, the specimens were transferred to 100% resin for 8 h at room temperature and allowed to polymerize in a 60°C oven for 24 h. The embedded cells were sectioned on a Porter-Blum MT-2 ultramicrotome (Ivan Sorvall, Inc.), and the sections obtained were poststained in lead citrate (Reynolds, 1963). The preparations were viewed in a Philips 300 transmission electron microscope.

Sonication

Cells suspended in either the Weisenberg buffer (Weisenberg et al., 1968) or microtubule stabilizing medium (MTM) (Filner and Behnke, 1973) were lysed with ultrasonic waves produced by a Branson sonifying apparatus (Branson Instruments Co., Stamford, Conn.). The probe was placed just beneath the surface meniscus of the cell suspensions held in 15-mm test tubes. The apparatus was operated at a frequency of 200,000 Hz and at the third intensity setting for continuous intervals of 30 s in a room maintained at 20°C.

DEAE Assay

The [^{3}H]colchicine-DEAE technique outlined by Weisenberg et al. (1968) was used to assay tubulin quantities. We elected to use only one DEAE filter paper per aliquot for the majority of our experiments, rather than follow the protocol outlined by Borisy (1972), because large numbers of counts are retained when [^{3}H]colchicine in culture media is applied to multiple DEAE filters after extensive washing. This was also the case for background values. ("Background" is defined as counts per minute/disk from homogenates of cultures pretreated for 1 h with 10^{-3} M "cold" colchicine before the [^{3}H]colchicine incubation.) These background values were always equal to those obtained by applying [^{3}H]colchicine in cell-free media to the filters and then washing. We assume that the high concentration of cold colchicine has effectively out-competed the [^{3}H]colchicine since no detectable tritiated colchicine binding was observed.

Statistical Analysis

To investigate whether the MTM had an effect on microtubule length or number within the cells, we employed transmission electron microscopy. MTM-treated and control cultures were fixed, dehydrated, embedded, and stained identically, as outlined above. Random electron micrographs were made from sections of individual cells, and no cell was photographed more than once. All micrographs were printed at final magnifications of × 44,000. The areas bounded by the cells were then excised from the photographic paper and counts of individual microtubules (including microtubules in all planes of section) were made (method suggested by K. R. Porter). We assumed the density of the photographic paper to be constant within the experimental error of our measurements. The trimmed areas of cells were then weighed; the data (microtubules counted within a cross-sectional area of cytoplasm) obtained were expressed as units of microtubule counts per unit weight of paper, in grams. The results from the controls and MTM treatments are plotted as histograms (Figs. 6 *a* and 6 *b*). Simply upon inspection, the two sample distributions appear remarkably similar.

Because these distributions of data were skewed and the sample sizes were not overly large, we elected to use a nonparametric statistical method, the Mann-Whitney U[1] test, to examine the two sample groups. The null hypothesis is that the two distributions are identical. We found a calculated z value of 0.722 ($p < 0.20$) which failed to provide sufficient evidence to reject the null hypothesis. The MTM treatment appeared to have no effect on length or number of microtubules.

However, aware that distribution-free statistical methods are not as powerful as parametric methods, we conducted a t test (two-tailed), recognizing the deviation from a normal distribution. Again, we failed to find significance with a calculated t value of 1.35 ($p < 0.20$) and accepted the null hypothesis of no MTM treatment effects on preexisting microtubules in glutaraldehyde-fixed cells.

RESULTS

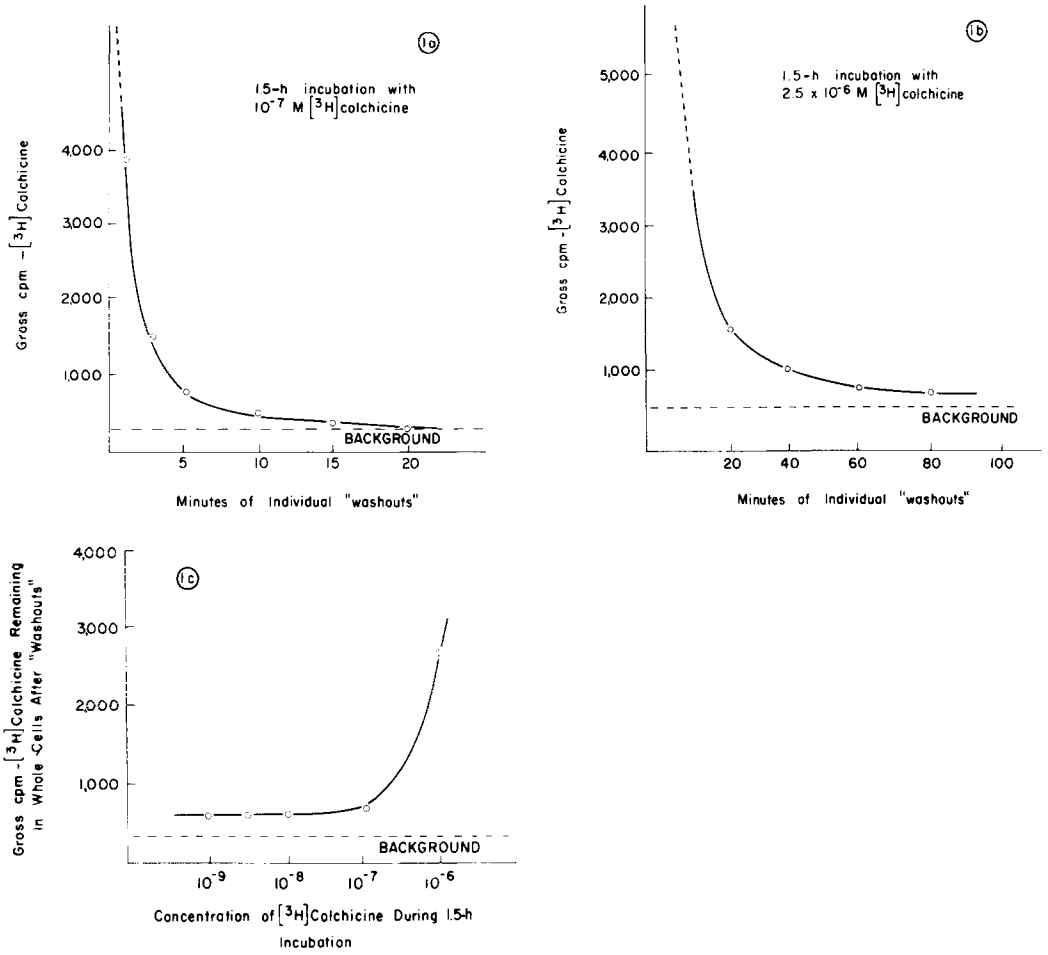
The material reported in this section may be categorized as follows. First, the preliminary experiments outlined below deal with efforts to bind [^{3}H]colchicine to living cells for the purpose of evaluating the soluble tubulin pool. Second, a procedure is described for determining the amount of tubulin present in vitro as polymerized microtubules. This procedure involves stabilizing preexisting microtubules, separating the soluble fraction from the stabilized tubules via centrifugation, and assaying the two fractions for microtubule protein. Finally, the effects of several treatments known to influence microtubule stability in Chinese hamster ovary (CHO) cells are examined by the stabilization procedure outlined above.

Initially, we attempted to measure the tubulin pool by the following in vivo [^{3}H]colchicine binding assay. Replicate confluent cultures of CHO cells were grown on coverslips in 2.5 cm petri dishes. The procedure utilized for the production of replicate cultures involves the use of a re-pipetting, according to the procedure of Everhart et al. (1973). This procedure provided excellent reproducibility and allowed the plating of many cultures which, after 48 h in culture, varied in their cell number by less than 5%. Various concentrations of tritiated colchicine (of constant specific activity) were added, and the cultures were incubated for 1.5 h. After incubation, the cover slips were washed with seven exchanges of warm conditioned media; the duration of each "wash" was 5 min. At low colchicine concentrations, the number of counts retained in the wash media remained constant after four to five washes (Figs. 1 *a* and 1 *b*). When the amount of [^{3}H]colchicine bound per cover slip after six washes was plotted against [^{3}H]colchicine concentration, a steep increase was observed at 10^{-6} M (Fig. 1 *c*). The shape of this curve was found to be very similar to that plotted from data obtained from high speed supernates of sonicated cells which were preincubated for 1 h before sonication with triated colchicine and were then assayed by the DEAE filter paper method of Weisenberg et al. (1968).

When the effect of variable [^{3}H]colchicine incubation time was investigated, it was found that only a slight increase (5–10%/h) of DEAE-bound counts per minute occurred after 2 h. Because the colchicine-tubulin complex was known to possess a short half-life (approximately 4–7 h), we routinely used incubation intervals of 1.5 h. We also found that increasing the number of DEAE filter paper disks used in the [^{3}H]colchicine assay increased the background radioactivity to the point where some experimental groups yielded quantities of DEAE-bound tritium nearly equal to background values. Consequently, only one disk per culture was used in these experiments.

The results of these preliminary experiments

[1] For information concerning this nonparametric test, the reader is referred to Hay, William L., and Robert L. Winkler. 1971. Statistics: Probability, Inference, and Decision. Holt, Rinehart, and Winston, Inc. 827–830.



FIGURES 1 *a* and 1 *b* Counts per minute released from "washed" cells into successive 5-min exchanges of conditioned medium. Note the continuing gradual loss of cpm at the higher [³H]colchicine concentration, even when the time intervals between medium exchanges are increased.

FIGURE 1 *c* Counts per minute remaining bound to whole cells after seven exchanges of medium versus [³H]colchicine concentration.

suggested that CHO cell microtubules are stable until the colchicine concentration reaches 10^{-6} M, at which time they rapidly disassemble and release dimer protein, which becomes available for colchicine binding. Alternatively, we may simply be measuring tubulin's affinity for colchicine, and the microtubules may be breaking down at the lower concentrations. To test this hypothesis, we fixed and sectioned cells pretreated with colchicine at differing concentrations and examined them with the electron microscope. For all cells examined, no microtubules were observed in cells treated with colchicine concentrations of 2×10^{-6} M or greater. Cells treated with 10^{-6} M or less appeared qualitatively to possess the same general number of microtubules per unit area sectioned. This does not rule out the alternative explanations for this phenomenon discussed below.

We then attempted to devise a method to stabilize existing microtubules. Investigation of various hexylene glycol-based buffers revealed that hexylene glycol irreversibly inhibited colchicine binding activity. This precluded the possibility of quantitative [³H]colchicine assays. During this

period, Dr. Philip Filner made available to us a preprint of a paper (see Filner and Behnke, 1973) describing a "microtubule stabilizing medium" (MTM) containing 50% glycerol and 10% dimethylsulfoxide. We treated cells with MTM for 1 h at room temperature, fixed them in 3% glutaraldehyde (in MTM), and prepared them for examination by scanning and transmission electron microscopy (Figs. 2 and 3 show treated and control cultures prepared for scanning electron microscopy).

Except for the increased numbers of blebs present on the surface of the MTM-treated cells, the control and experimental cell groups were essentially identical with respect to gross external morphology. Cellular extensions and general cellular asymmetries of the treated cells appeared normal when compared to those of the control cells. When MTM-treated cultures were exposed to very mild sonication before fixation, many individual cells came off the substrate. When viewed with phase optics, these cells appeared to

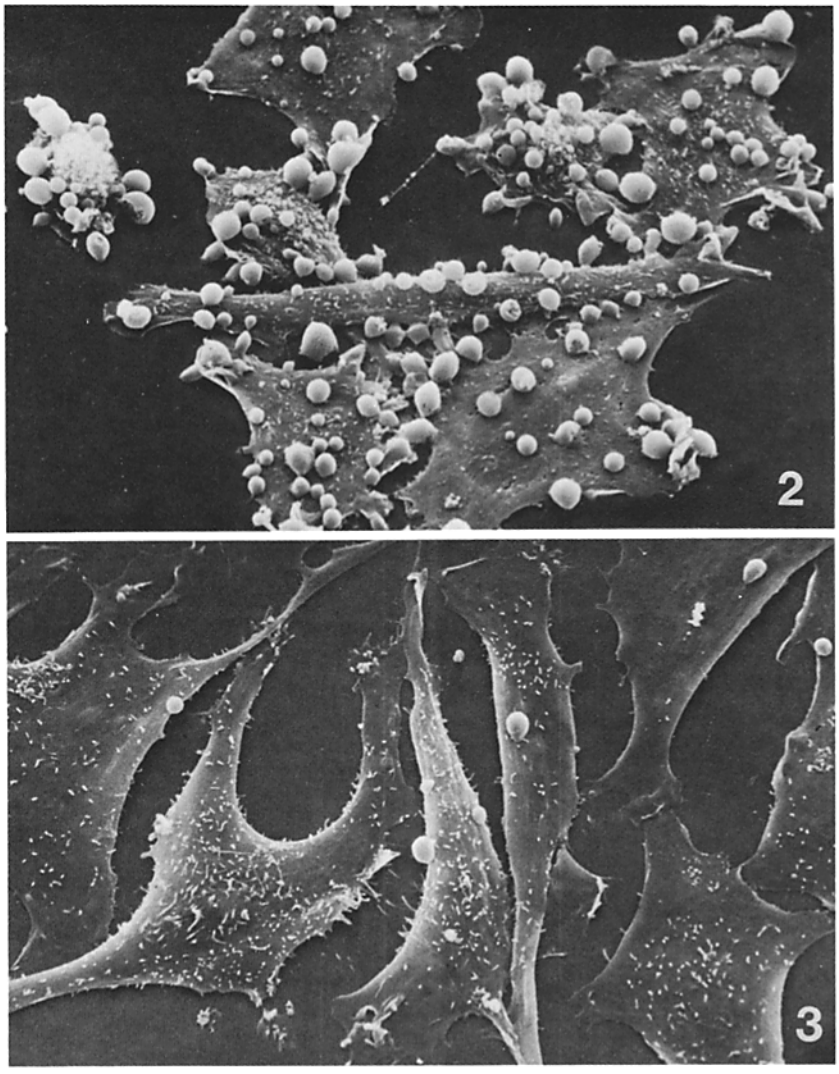


FIGURE 2 CHO cells treated for 1 h with MTM. The maintenance of cellular asymmetries and the presence of numerous surface blebs are prominent features of these treated cells. × 1,300.

FIGURE 3 Control CHO cells fixed at the same cell density as those in Fig. 2. × 1,300.

maintain the morphology that they had originally possessed while attached. Transmission electron microscopy revealed the presence of numerous microtubules, although considerable disruption of most other organelles was observed. The "ground substance" appeared to be entirely leached from the cells (Figs. 4 and 5).

To determine whether MTM actually "froze" existing microtubules without subsequent tubule assembly or breakdown, we made extensive microtubule counts from random thin sections. Both parametric and nonparametric statistical analyses were undertaken (see Materials and Methods). Although there was a slight increase in the mean microtubule number of the MTM-treated cells, there was no statistically significant difference in

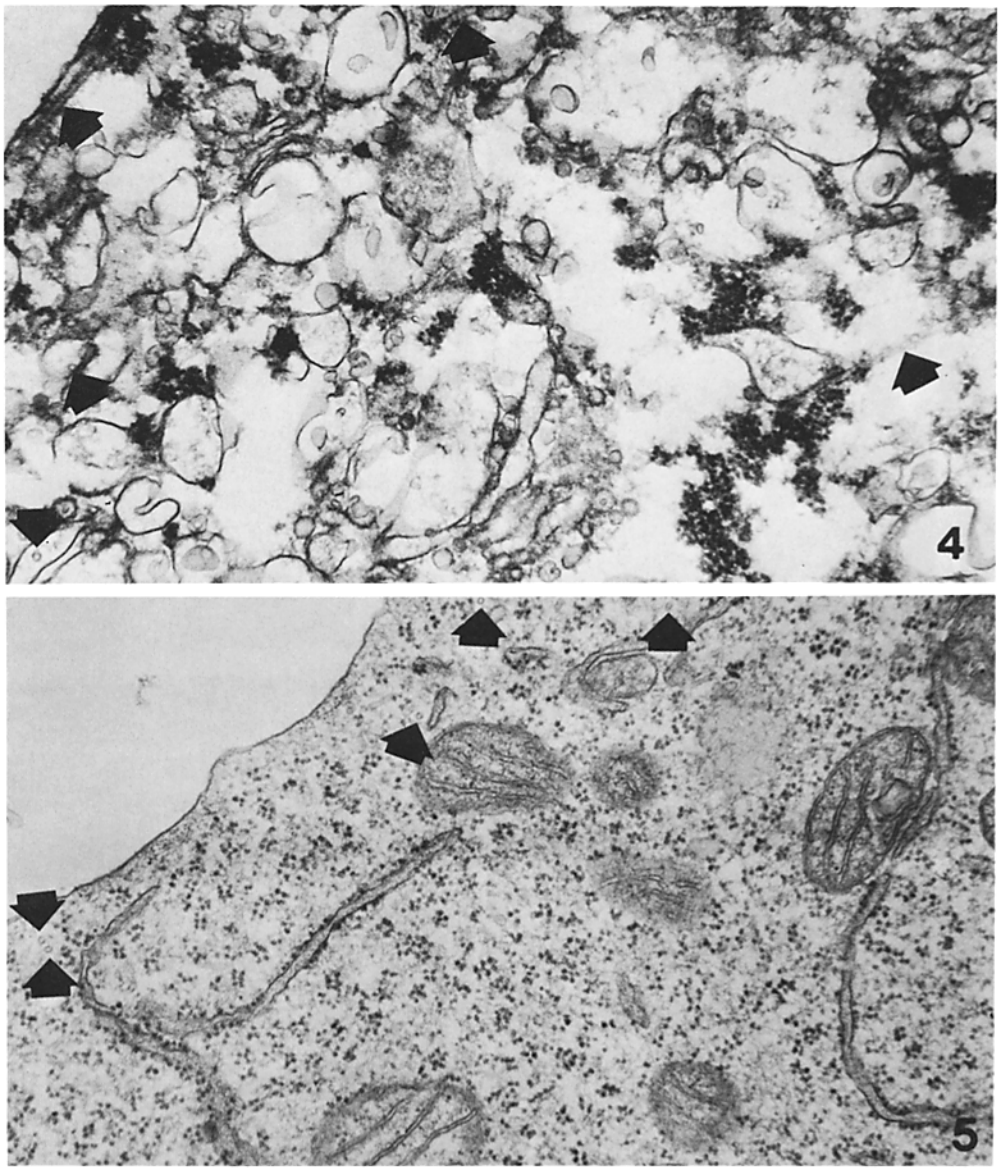


FIGURE 4 Thin section of a CHO cell treated with MTM for 1 h. Note the presence of numerous microtubules (arrows). × 44,500.

FIGURE 5 Thin section of control CHO cell. × 44,500.

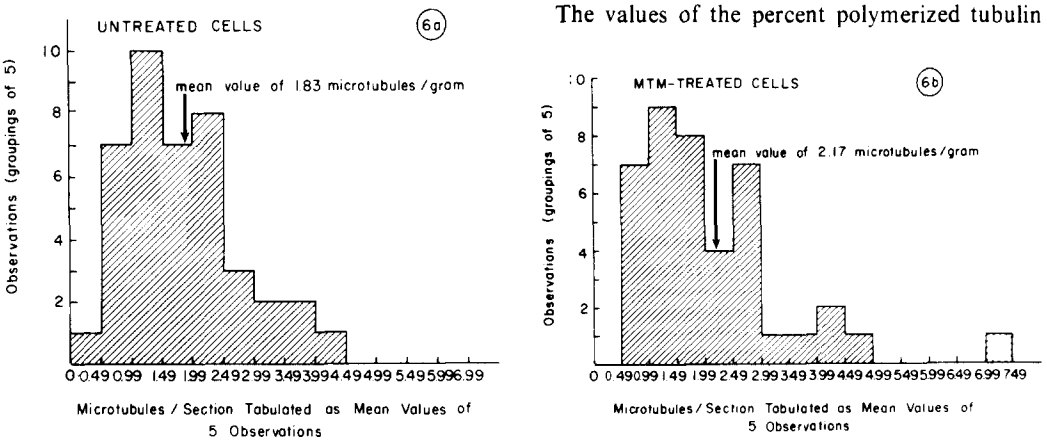
the numbers of microtubules present in MTM-treated and control cell thin sections (Fig. 6 and Table I).

Assured that MTM did stabilize microtubules, we devised a procedure to quantitatively measure the percentage of the total cellular tubulin present as intact microtubules.

Because it had been our experience that MTM partially and reversibly inhibited colchicine binding activity, we designed the following indirect approach toward a quantitative tubulin assay. Replicate cultures grown on 5.5-cm plastic petri dishes were removed by trypsinization with 0.005% trypsin for 5 min and were then placed in 15-ml centrifuge tubes containing 1% trypsin inhibitor. These control cell suspensions were centrifuged at 1,000 *g* for 3 min, and were resuspended in 0.7 ml of cold 0.01 M sodium phosphate buffer supplemented with 0.01 M $MgCl_2$ and 10^{-3} M GTP, pH 6.8. The cells were then lysed with a sonic probe, incubated for 1.5 h in 2×10^{-6} M [^{3}H]colchicine and assayed for bound counts using the Weisenberg DEAE filter paper assay. Replicate cultures were treated with MTM at 37°C for 5 min. These cells were removed from the petri dishes with a rubber policeman, lysed by sonication, and centrifuged on a SS-34 Sorvall head at 50,000 *g* for 60 min or on a 50.1 Spinco head (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 150,000 *g* for 60 min. No difference in the final results could be attributed to the different centrifuge heads when gravitational forces above 50,000 *g* were used; however, the pellets were more easily drained when they were compacted at higher speeds of centrifugation. The pellets were carefully dried, cut out of the 5-ml cellulose nitrate centrifuge tubes, and resuspended in 0.7 ml cold sodium phosphate buffer. Electron microscopy revealed that these pellets contained microtubules and that when the cells were resuspended in the cold sodium phosphate buffer and again spun down, the resulting pellet contained no microtubules.

The cell suspension was then assayed by the DEAE procedure. The resulting experimental value (counts per minute obtained from the DEAE assay), when compared with the value for the total bound colchicine from control cell culture homogenates, was taken as the percentage tubulin present as intact microtubules. The magnitude of the differences between experimental and control cultures described below did not vary when different times for the DEAE assay were used. Some of the times were varied from as little as 0.5-h treatment, followed by 1 h of tritiated colchicine binding at 37°C, to as long as a total time of 4–5 h. In each case the percentage differences were the same. We concluded that the differences in the half-life of the tubulin-colchicine complex in control and in experimentally treated cultures were negligible and could be ignored. While it would have been preferable to utilize the zero time extrapolation procedure of Wilson (1970), the low values of counts per minute per dish we obtained, combined with the relatively large number of replicate cultures required to do this assay at each point, made it impossible to utilize this procedure.

The values of the percent polymerized tubulin



FIGURES 6 *a* and 6 *b* Mean values of microtubule numbers per gram of photographic paper from five randomly grouped thin sections plotted as histograms. Note the general similarity of the distributions of MTM data and control data.

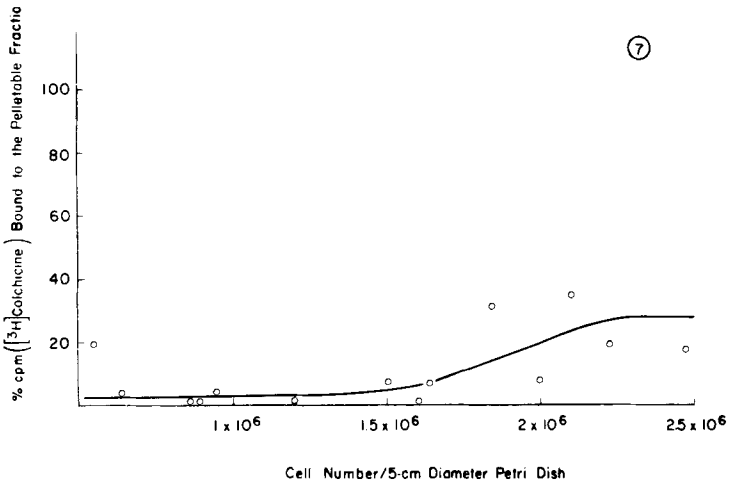


FIGURE 7 The percent of polymerized tubulin plotted against cell numbers.

TABLE I
Statistical Analysis

Parametrical statistical results	
Test employed: *t* test	
Mean values:	
Control 1.83 microtubules/g	
Experimental 2:17 microtubules/g	
Standard deviations:	
Control 1.28 microtubules/g	
Experimental 0.961 microtubules/g	
$n = 40$	
Significance level: $p \leq 0.05$ where $t_c = 2.00$	
Calculated *t*: 1.35	
Nonparametric statistical results	
Test employed: Mann-Whitney U	
Significance level: $p \leq 0.05$ where $F(z) = 1.65$	
Calculated *a*: 0.722	
Calculated $F(z)$: 0.764	

varied greatly, depending on number of cells in the cultures (Fig. 7). A mean value of 21% polymerized tubulin was obtained for confluent cultures (>180,000 cells per dish or >37,000 cells per cm^2). At densities below 18,000 cells per dish, the value was always much lower and varied from less than 1% to 4% polymerized tubulin. One possibly spurious point (20% polymerized tubulin) was obtained at the lowest density.

To further demonstrate that MTM treatment stabilized existing tubules, we exposed CHO cells to various treatments known to affect microtubule assembly or disassembly. The first treatment was exposure to low temperature. When cells were subjected to a temperature of 2°C 1 h or more before MTM treatment, no quantitative alteration in the free tubulin versus polymerized tubulin ratio was observed (i.e., the amounts of [^{3}H]colchicine bound to 2°C and 37°C MTM-treated cellular lysate pellets were identical). The same was true for cells treated with Ham's F-12 medium made with 50% D_2O and 50% H_2O. However, cells treated with cold or with D_2O displayed no morphological alteration from the control cells (using scanning electron microscopy and phase-contrast light microscopy). Sectioned cells previously treated with cold temperatures for 2 h showed no discernible differences in microtubule number when compared to control cells.

Since it has recently been shown that N^6,O^2-dibutryl-3',5'-cyclic adenosine monophosphate (dibutyryl cAMP) induces a twofold increase in microtubule number in CHO cells (Porter et al., 1973),[2] we investigated this phenomenon using the [^{3}H]colchicine-MTM assay. In Table II it can be seen that in all experiments dibutyryl cAMP induced a pronounced increase in the percentage of tubulin present as microtubules. The magnitudes of these values varied considerably with differing cell numbers.

In an attempt to determine the possible mecha-

[2] Porter, K. R., T. T. Puck, A. W. Hsie, and D. Kelley. 1973. An electron microscope study of the effects of cyclic AMP on Chinese hamster ovary cells. Manuscript to be submitted to *The Journal of Cell Biology*.

TABLE II

Comparison of Percent Polymerized Tubulin in Dibutyryl cAMP and Control Cultures

Exp. no.	% Pelletable counts [^{3}H]colchicine			Cell no.		
	Dibutyryl cAMP	Range	Untreated	Range	Dibutyryl cAMP	Untreated
	%	%	%	%		
1	9.1	8.1–10.3	7.0	6.8–7.9	738,560	956,000
2	3.1	2.0–3.5	1.2	1.5–2.9	847,300	1,115,000
3	10.1	7.5–11.2	7.1	6.0–7.5	1,555,000	1,640,000
4	36	30–41	27	20.2–39.1	2,110,000	2,110,000

Note: each value was calculated from a mean of three values.

nism of action of dibutyryl cAMP on microtubule polymerization, we grew cells in phosphate-free medium in the presence of [^{32}P]phosphate (2 μc/ml) and 0.3 mM dibutyryl cAMP plus 0.5 mM aminophylline. After 6 h the cells had become elongated. They were rapidly removed by treatment with 0.04% EDTA and immediately frozen. The total soluble fraction (100,000 *g* for 1 h) was run on slab gels and examined by autoradiography for the presence of labeled peptides. It was found that the bands which coelectrophoresed with mammalian brain tubulin were never labeled. This was the case for both dibutyryl cAMP-treated cultures and untreated controls, even when the incubation period was increased to 13 h. This failure to demonstrate tubulin phosphorylation occurred in a system in which well over 25 individual bands on the x-ray film could be resolved from a preparation made from a high speed supernate of cells pretreated with [^{32}P]phosphate. A number of these bands did not even correspond to a stained portion of the gel, using Coomassie blue stain. This indicated that extensive protein phosphorylation was occurring in this system, that the ^{32}P was taken up by the cells, and that even submicrogram amounts of phospho-protein could be detected by this system.

DISCUSSION

Of the previous attempts to quantitatively measure the relative amount of tubulin present as microtubules and as free dimer in the living cell, the most notable are those of Weisenberg (1972) and Gillespie (1972). In the former case, a hexylene glycol-based stabilization buffer was used; in the latter case, a colchicine-binding assay alone was employed. Neither investigator verified directly and quantitatively the assumption that the treatments failed to alter the ratio of free tubulin in the pool versus tubulin polymerized as microtubules. Kirkpatrick (1969) found that microtubules displayed a very short half-life when stabilized in a hexylene glycol-based buffer, while we found that hexylene glycol irreversibly inhibited colchicine binding activity. We thought that the use of unstabilized tissue slices and homogenates to study relative tubulin pool sizes was a questionable procedure, since these manipulations of the tissue tend to induce microtubule disassembly (see Filner and Behnke, 1973).

Our initial attempt to develop a quantitative tubulin assay system led to somewhat ambiguous results. The curve showing the amount of DEAE-bound tritium versus colchicine concentration (Fig. 1 *c*) implied that the depolymerization reaction was a threshold phenomenon with little or no tubule disassembly occurring until a critical colchicine concentration was reached. It may be possible to quantitate and compare the stability of the microtubule systems of various cell types by determining the lowest colchicine concentration which initiates tubule breakdown. It is perhaps reasonable to assume that it is at this concentration that the assembly has been inhibited enough to shift the equilibrium toward depolymerization, and that an abrupt increase in DEAE-bound radioactivity should be observed at such a concentration. Studying grasshopper embryos, Wilson and Friedkin (1967) reported a similar relationship between colchicine concentration and the amount of [^{3}H]colchicine bound in vivo, with abrupt shifts occurring at 10^{-6} M and 4×10^{-4} M colchicine. Borisy and Taylor (1967) described large increases in [^{3}H]colchicine bound to *Lytechinus pictus* eggs at a concentration of 5×10^{-7} M. These results are consistent with ours.

There are a number of possible explanations for this phenomenon of sudden increases in the amounts of [^{3}H]colchicine binding as one increases the concentration of the added colchicine. It is entirely possible, for example, that these results are a result of a colchicine uptake phenomenon such that little or no colchicine enters the cell until a critical concentration is reached. Alternatively, it is possible that these results indicate some unusual aspects of the colchicine tubulin affinity, as has been described in in vitro systems. It may be that no appreciable amount of colchicine is bound to tubulin until a threshold of concentration is reached, at which point a large amount of binding occurs.

Initially, we had thought that in vivo colchicine-binding studies could provide a quantitative means of determining the amount of free dimer which, when eliminated from the polymerizable pool, brought about a sufficient alteration in the equilibrium (tubules $\leftrightarrows$ dimer) to cause the tubules to begin to break down. A study of the amount of colchicine binding activity before and after this breakdown process had been initiated might provide a means for obtaining meaningful values for the percentage of tubulin present as pool and as intact microtubules. However, this type of study must assume that phenomena such as those discussed above were not operating. Furthermore, if our initial contention was correct, then the results would indicate that there is almost no free tubulin pool existing in these cells, since there is almost no colchicine bound at the lower concentrations. This is contrary to current evidence which suggests the presence of considerable tubulin pools in many cell types (Weisenberg, 1972; Piatigorsky et al., 1972; and Inoué and Sato, 1967). Therefore, our interpretation of these results runs counter to the work of others and, since a number of untested assumptions were involved in accepting our hypothesis, we abandoned the use of in vivo colchicine-binding studies as a means of determining what percentage of the total microtubule protein exists as intact tubules and as a free pool of soluble protein.

These initial experiments did help to confirm that the DEAE filter paper assay used in later experiments (those in which [^{3}H]colchicine was bound to MTM-treated pelletable material and whole lysed cells) quantitatively measured the amount of free dimer, because the amount of [^{3}H]colchicine (at concentrations greater than 10^{-6} M) bound to living cells always equalled that bound to DEAE filters from replicate culture homogenates pretreated in an identical manner.

The use of MTM buffer first described by Filner and Behnke (1973) provided more reliable results. Electron and light microscopy clearly indicated that this buffer "freezes" existing microtubules without measurably inducing tubulin polymerization or depolymerization. This conclusion was based on three observations. First, cells pretreated with MTM and freed from their substrate retain their morphological asymmetries. Second, except for an increase in surface blebbing, MTM-treated cells were indistinguishable from control cells when observed in the scanning electron microscope (Figs. 2 and 3). Third, a thorough statistical analysis of actual microtubule counts revealed no statistically significant difference between MTM-treated and control cells (Table I). The results of the statistical analyses are particularly compelling. Both parametric and nonparametric tests were employed and the assumptions of the parametric analysis were tested. Upon inspection of Figs. 6 *a* and 6 *b*, it is apparent that the single outlying point at 7.14 microtubules/g strongly contributes to the upward shift of the TMM mean value (2.17 microtubules/g) away from that of the control mean value (1.83 microtubules/g). This particular point was obtained in a random manner from a section of a distinctly flattened region of a cell and is regarded as a rare occurrence; it could have occurred as easily in the control sample. If this outlying point is removed from the MTM sample, the resultant mean value falls to 2.04 microtubules/g, which agrees with that of the control very well. However, without removing the outlying point, the difference in mean values is not statistically significant. On the basis of the statistical data provided, this is strong evidence to suggest that the MTM treatment does not interfere with the number or length of microtubules present in the cell. Since each individual value used in the statistical analysis was taken from microtubule counts of random thin sections of single cells, and since no two values come from the same cell, we should have been able to determine statistically whether a decrease or an increase in microtubule number or, in fact, in the net length of existing microtubules had occurred. This conclusion assumes that the number of microtubules present in glutaraldehyde-fixed cells is equal to that present in living cells. It is also assumed that there is no differential microtubule fixation that occurs in MTM-treated as

compared to untreated control cells. These assumptions could be tested in part by comparing the birefringences of the mitotic apparatus in fixed and unfixed control and MTM-treated cells. However, microtubule quantitation by measurement of birefringence cannot be made on these interphase cells.

Gross changes in the morphology of a cell are a less sensitive indicator of microtubule assembly or disassembly. However, Puck et al. (1972) have shown that CHO cells are altered morphologically in a very radical way by treatment such as colchicine application, known to affect tubulin polymerization in vivo. Hence, any sudden, drastic loss of cellular asymmetry may be a valid measure of microtubule disassembly.

The use of the MTM centrifugation method for determining the percentage of tubulin present as intact microtubules provided highly successful, although our failure to measure a change in the values of the percent polymerized tubulin after pretreatment with cold and D_2O was initially disconcerting. Since cold- and D_2O-treated cells showed no morphological alterations and since the cells exposed to cold temperatures appeared to possess qualitatively the same number of microtubules relative to controls, we assumed that CHO cells were resistant to these treatments. This implied that their microtubule systems are more stable than those of other cell types. Rubin (1973)[3], for example, has shown that 4337 rat sarcoma cells are extremely sensitive to cold; they rounded up within minutes after being placed in a 2°C cold room. Further, it is commonly accepted that practically all tissue culture cell types when placed in a 2°C environment will round up, often very rapidly. One possible complicating factor might be cell density. Rubin and Everhart (1973) have shown that cell contact causes cells in the S and G_2 phase of the cell cycle to flatten out extensively onto the substrate. It may be that confluent or nearly confluent cultures possess a more stable microtubule system; direct comparisons from cell type to cell type must take into consideration the degree of contact present.

When we first used the MTM centrifugation method, we obtained widely varying percentages of polymerized tubulin from the CHO cultures. We discovered that the cause of this inconsistency was related to the growth cycle of the culture. Cultures containing less than 1.5 million cells per dish showed an extremely low percentage of tubulin present as centrifugable microtubules. In cultures of greater than 1.5 million cells per dish, as high as 20–30% of the total colchicine binding activity was in centrifugable form (Fig. 7). It seemed to us not coincidental that the density point of 1.5 million cells per dish was the stage in the growth cycle where intercellular contact occurred. A monolayer confluent culture was reached at 2 million cells per dish. Otten et al. (1971) have shown that as 3T3 cells approach confluency, an increase in cyclic AMP levels occurs. If the same is true of CHO cells, the sudden increase in the percentage of tubulin polymerized as microtubules during the approach toward confluency might have been due to increased intracellular cyclic AMP levels. Our results indicated that dibutyryl cAMP increased this percentage (Table II). Since a study employing direct microtubule counts (see Porter et al., 1974) has independently confirmed the preliminary results of our dibutyryl cAMP experiments, it would appear that the MTM centrifugation procedure is a valid technique for the measurement of changes in the process of microtubule assembly and disassembly.

The mechanism of action of dibutyryl cAMP in inducing microtubule assembly remains obscure. Our failure to find phosphorylated tubulin implies that dibutyryl cAMP does not act by stimulating the covalent phosphorylation of the 100,000-dalton tubulin dimer. This conclusion has been confirmed for *Chlamydomonas* flagellar tubulin (Rubin and Filner, 1973), although Eipper (1972) reported ^{32}P-labeled brain tubulin on sodium dodecyl sulfate gels.

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