ORGANIZATION OF TUBULIN IN NORMAL AND TRANSFORMED RAT KIDNEY CELLS

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ABSTRACT

We have carried out a quantitative biochemical and ultrastructural study of tubulin and microtubules in a normal rat kidney (NRK) cell line and its viral transformant (442) in culture. Under equivalent culture conditions, both cell lines contain the same amount of tubulin according to a colchicine-binding assay. The normal and transformed cells differ significantly, however, with respect to the state of organization of their tubulin. Counts of microtubules in sectioned cells indicate that NRK cells have almost twice as many microtubules per unit area of cytoplasm as the 442 cells. Centrifugation studies, on the other hand, show that 442 cells have almost twice as much pelletable tubulin as the NRK cells. We propose, therefore, that the transformed cells contain a large amount of tubulin which is in some alternative aggregate form that is not morphologically detectable as microtubules in the cytoplasm.

KEY WORDS microtubules · tubulin · polymerization · transformation

Malignant transformation is commonly associated with dramatic changes in cell shape. Since microtubules are involved in the maintenance of cell shape, there has been much interest in the role that these organelles play in cellular transformation. Fluorescent antibody methods have been most useful for the visualization of the microtubular network in normal cells (3, 5, 16, 28), but it has proven difficult to define quantitative changes in the microtubular apparatus with transformation using this technique. In particular, questions such as the following have been dealt with in only a limited way by previous studies: Are the numbers of microtubules in transformed cells altered by the transformation process? Does tubulin appear in aggregates other than microtubules in transformed cells, and does the total amount of tubulin in the cell change?

In the present investigation, we have sought to compare electron microscope and biochemical data on the organization of tubulin in normal rat kidney (NRK) cells and their Kirsten viral transformant, the 442 cell. These cells are highly stable model systems for the study of cellular malignancy. The NRK line flattens onto the substrate in an epithelial-like configuration and possesses numerous actin cables attached to the substrate. The transformed line (which unlike the NRK cells does not show contact inhibition) is fusiform in shape with much of the cytoplasm lifted off the substrate and it contains few actin cables (19). To quantitate microtubules in the cells, we have employed the glycerol stabilization method first suggested by Filner and Behnke (9) and later modified for use in determinations of the percentage polymerized tubulin by Rubin and Weiss (20). This method has been further modified, recently, to make it a more quantitative assay (18). We have employed this technique to measure the degree of tubulin organization in normal and transformed cells and have compared the results to a quanti-

tative EM analysis of microtubule number in these two cell types. In our EM analysis, we have taken special care to deal with the problems that may arise in attempting to compare microtubule numbers in cells of very different shape, and a method has been used whereby these problems can be circumvented.

MATERIALS AND METHODS

Cell Cultures

NRK and 442 cells were grown to confluence. It has been shown previously (19) that the NRK and 442 lines have different amounts of actin and protein per cell up to 5×10^6 cells per 75-cm^2 culture flask. Beyond these densities, however, both cell lines have the same amounts of actin and protein per cell. All our assays were carried out at culture densities greater than 5×10^6 cells per flask. The cells were renewed from pleuropneumonia-like organism-free frozen stocks and grown in Dulbecco's medium with 10% fetal calf serum. All experiments were performed on NRK and 442 cells which had identical plating histories.

Tritiated Colchicine-Binding Assay

For all assays, the contents of two culture flasks were pooled. One flask was treated first with 5 ml of microtubule stabilization buffer (MTS) (18) for 5 min. The cells were then scraped off with a rubber policeman, and the material was pipetted into the second flask. After the second flask was equilibrated and scraped, the pooled flask material (4.5-5 ml total) was sonicated for 5 s at 45 W on a Bronson sonifier model W851D (Heat Systems Ultrasonics, Inc., Plainview, N. Y.). This disrupted more than 95% of the cells. After more than 15 s of this treatment, there was a reduction in total bound colchicine. The resulting MTS-stabilized homogenate was then spun at 100,000 *g* for 45 min. The supernate was poured off and a 100-μl sample was treated with tritiated colchicine. The pellet was resuspended in 800 μl of tubulin depolymerizing buffer (TS) (18) at 0°C using a 1-ml Teflon homogenizer. The resulting suspension, after ½ h in the cold, was spun at 100,000 *g* for 30 min and 100 μl of supernate was used for the colchicine-binding assay of total pelletable tubulin.

The basic colchicine assay was patterned after the previous studies (18, 21, and P. Sherline, personal communication) and involved the addition of sufficient tritiated colchicine (0.25 Ci/0.0062 mg, New England Nuclear, Boston, Mass.) to produce 100,000 cpm. Enough cold colchicine and bovine serum albumin (BSA) was then added to 100 μl of sample to make the sample 50 μM in colchicine and 0.25% BSA. The MTS supernate was incubated for 2 h at 37°C and pelleted for 1 h at 37°C. This total colchicine concentration was chosen because it was 10 μM higher than saturation for the highest protein concentration used. The 1- and 2-h incubation times were chosen because beyond that period no increase in binding occurred. Thus, all colchicine-binding experiments were done under conditions of saturation. It was found that increasing the ratio of the amount of the tritiated to cold colchicine did not produce an increase in total cpm after the subtraction of the background. The background control for each experiment involved the use of an MTS or TS blank treated in the same manner as the experimental material. Only those results in which there was a sufficient total protein concentration to produce experimental values of twice the control were used. Total experimental counts minus controls ranged from 900,000 to 1.5 million per min per flask. The MTS and TS buffer systems were shown to stabilize colchicine-binding activity over a time period greater than the entire colchicine-binding assay (18).

To obtain more values for the total amount of colchicine-binding activity per cell, total colchicine-binding activity was assayed to replicate cultures. They were trypsinized, pelleted and washed, and a cell count was taken using a hemacytometer. The final cell pellet was then resuspended in 0.5 ml of TS buffer containing 0.5% Triton X-100. The cells were lysed by gentle homogenization with a Teflon homogenizer, and the total volume of the preparation was raised to 2 ml with TS. This preparation was then assayed for tritiated colchicine binding as described above. The addition of Triton X-100 did not affect the total amount of colchicine binding in any preparation. 0.5% was chosen because it was the lowest concentration that optimally lysed all of the cells. The use of Triton X-100 was necessary as sonication in TS tended to denature the tubulin as indicated by a reduction in the total amount of colchicine-binding activity after even short periods of sonication in TS. The results of these experiments compared favorably on a unit protein basis with those described above in the pelletable tubulin experiment when the pelletable and the supernatant fractions were added together to obtain an estimate of total binding activity per unit protein.

Electron Microscopy

NRK and 442 cells that were to be used for counts of microtubules were prepared using two different protocols. The first protocol involved fixation with a glutaraldehyde/paraformaldehyde fixative (13) which contained the following: 3% glutaraldehyde, 2% paraformaldehyde, and 0.1 M cacodylate buffer (pH 7.3). The second method of fixation used a buffer (microtubule-stabilizing buffer, MSB) designed for stabilizing the mitotic spindle (4) as a vehicle for the fixative. This fixative contained 3% glutaraldehyde in 0.1 M piperazine-*N*-N'-bis[2-ethane sulfonic acid] (pH 6.8), 3% polyethylene glycol, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 2.5 mM GTP, 0.1 mM dithiothreitol, and 1% NP-40 detergent. Both fixations were carried out at 37°C for 30 min. After fixation, the cells were washed in 0.1 M cacodylate buffer

containing 10% sucrose and postfixed in osmium tetroxide in 0.1 M cacodylate at pH 7.3 for 30 min. The cells were rinsed in 0.05 M maleate buffer (pH 5.2) and then stained with 0.5% uranyl acetate in the same buffer. After staining, the cells were dehydrated in alcohol and propylene oxide and embedded in Epon 812 resin. Thin sections of the embedded cells were stained with uranyl acetate and lead citrate.

The counts of microtubules in NRK and 442 cells were done with a method specifically designed to circumvent the problems of nonuniform cell shape and nonrandom microtubule distribution within the cells. To obtain representative, random samples of NRK and 442 cell layers on culture dishes, our data were gathered from micrographs taken at regular intervals along the cell layers. For each micrograph, microtubules were counted in the cytoplasm of all cells appearing in the micrograph, and the cytoplasmic area (in μm^2) for each cell was measured using an electronic planimeter.

A conventional method of determining the average density of microtubules per μm^2 of sectioned cytoplasm for a population of cells is to determine that ratio for each cell examined, and then to average those values for all the cells. This method, however, is satisfactory only in the case where the two cell populations have the same cell shape and the same general distribution of microtubules within the cells. The NRK and 442 cells, however, have very dissimilar shapes and microtubule distributions, and it can be shown that this method can lead to erroneous conclusions about which population of cells contains a greater microtubule density. Suppose, for example, that one population of cells has relatively few microtubules per cell overall, but that within this population there are a few cells or cell processes containing very large numbers of microtubules. It is apparent that the individual ratios of microtubules per μm^2 for these exceptional cells will tend to skew the distribution of values for all the cells so that the mean of all values will not be truly representative of that population.

It is possible to analyze the data so that cell shape and nonuniform microtubule distribution are not variables in the analysis. This is accomplished quite simply by treating each cell analyzed not as a single unit, having some defined shape and microtubule density, but rather adding together the individual area and microtubule measurements, respectively, for all cells. When this is done, one has single aggregate measures of microtubule number and cytoplasmic area for the entire cell population. The ratio of microtubules to cytoplasmic area obtained from these two numbers clearly provides a meaningful index of overall microtubule density in the cell population as long as a sufficiently large number of cells has been examined. The most important point is that this ratio is not unduly biased by the presence of a few cells displaying very high or low microtubule densities in a particular thin section.

A further requirement of our analysis is that we would like to compare statistically the microtubule densities between two different cell lines to see if any observed differences in microtubule density are significant. To use a parametric statistical assay to compare two cell populations, it is required that one have for each population a set of values which themselves approximate a normal distribution. To generate such values, we collected our data in the following manner: for each cell line, twelve different random samples of the entire cell population were taken, with each sample representing the pooled data for seven to nine different cells. Pooling means that the cytoplasmic areas and microtubule numbers for each cell were added together before a ratio was taken, as described in the preceding paragraph. Finally, the ratios of microtubules to cytoplasmic area obtained from 12 such samples for each cell population formed the normally distributed sets of values to be used for statistical comparison. A standard one-way analysis of variance was performed on the data.

RESULTS

Colchicine-Binding Assays

We initially examined the relationship of the pelletable to nonpelletable colchicine-binding activity in the normal and transformed cell lines. Using the procedures described in Materials and Methods, we obtained repeatable and statistically significant results indicating an almost twofold increase in the percent pelletable tubulin for the transformed 442 cell line (Table I). To establish whether this increase in the amount of the pelletable fraction is paralleled by an increase in the total tubulin concentration, the total amount of tubulin per cell and per unit protein was deter-

TABLE I
Ratio of Polymerized/Unpolymerized Tubulin

Exp No.	NRK	442
1	0.98	1.01
2	0.86	2.00
3	1.20	3.40
4	0.370	2.39
5	1.66	3.73
6	1.09	4.16
7	1.12	4.43
8	1.034	4.17
9	2.63	3.84
10	1.98	4.36
Mean	1.29*	3.35*

Although the absolute amounts of polymerized and unpolymerized tubulin vary considerably from one experiment to another for undetermined reasons, NRK and 442 cells, except for exp no. 1, always show the same relative difference. The means are highly significant.
* $P < 0.01$ (analysis of variance).

TABLE II
Total Colchicine-Binding Activity in NRK and 442 Cells

Exp No.	NRK	442
	cmp/μg Total protein	
1	452	512
2	469	538
3	428	514
4	719	530
5	569	445
6	440	409

mined (Table II). Clearly, the amounts of tubulin in the two cell lines are very similar.

To verify the validity of the percent polymerized tubulin determination procedure and to answer the question of whether the pelletable tubulin is in the form of microtubules or some other aggregated species, a quantitative electron microscope analysis of the normal and transformed cells at confluence was undertaken.

Electron Microscopy

In culture, the NRK cells are thinly spread on the culture dish (Fig. 1). The upper surfaces of the cells may bear a few short microvilli. The numerous microtubules in the NRK cells may be found in any region of the cytoplasm (Fig. 2). 442 cells are more nearly spherical or fusiform and adhere only minimally to the substrate (Fig. 3). These cells usually display elaborate surface membrane folding and may also form one or more cytoplasmic processes that are in the range of 1–2 μm in diameter. Transverse sections through a layer of 442 cells yield two general types of images: profiles of the large cell bodies interspersed with much smaller profiles of the slender processes. In general, only a few scattered microtubules are found in the cell bodies of the 442 cells (Fig. 4) while the cell processes often contain very large numbers of parallel, longitudinally oriented microtubules (Fig. 5 *a*), as well as significant numbers of 5- to 7-nm microfilaments and 10- to 12-nm intermediate filaments.

The two different fixation protocols yielded nearly equivalent images of the cells in section. In the cells fixed in the presence of detergent, the plasmalemma generally appeared to be intact although the background cytoplasm was often slightly clearer and the microtubules were thus more sharply defined. This may contribute to the somewhat higher microtubule counts with this fixation as reported below. In Figs. 2, 4, and 5, which display portions of cells actually included in our counts, all entities scored as microtubules are indicated on the figure (Figs. 2 and 4) or in an accompanying diagram (Fig. 5 *b*).

Our microtubule counts for NRK and 442 cells, using both fixation methods, are presented in Fig. 6. Every point in the histograms represents a ratio of microtubules per μm^2 of cytoplasm for several cell measurements pooled together as outlined in Materials and Methods. Thus, while only twelve points are used to generate the mean values obtained, each histogram actually represents measurements of 84–105 cells. For both fixation methods, the results indicate that NRK cells display almost twice as many microtubules per unit area of cytoplasm as do the 442 cells. The differences, moreover, are highly significant ($P < 0.01$). Comparison of the two different methods of fixation suggests that fixation in microtubule-stabilizing buffer is slightly more effective in preserving microtubules, but the differences are not statistically significant according to our data.

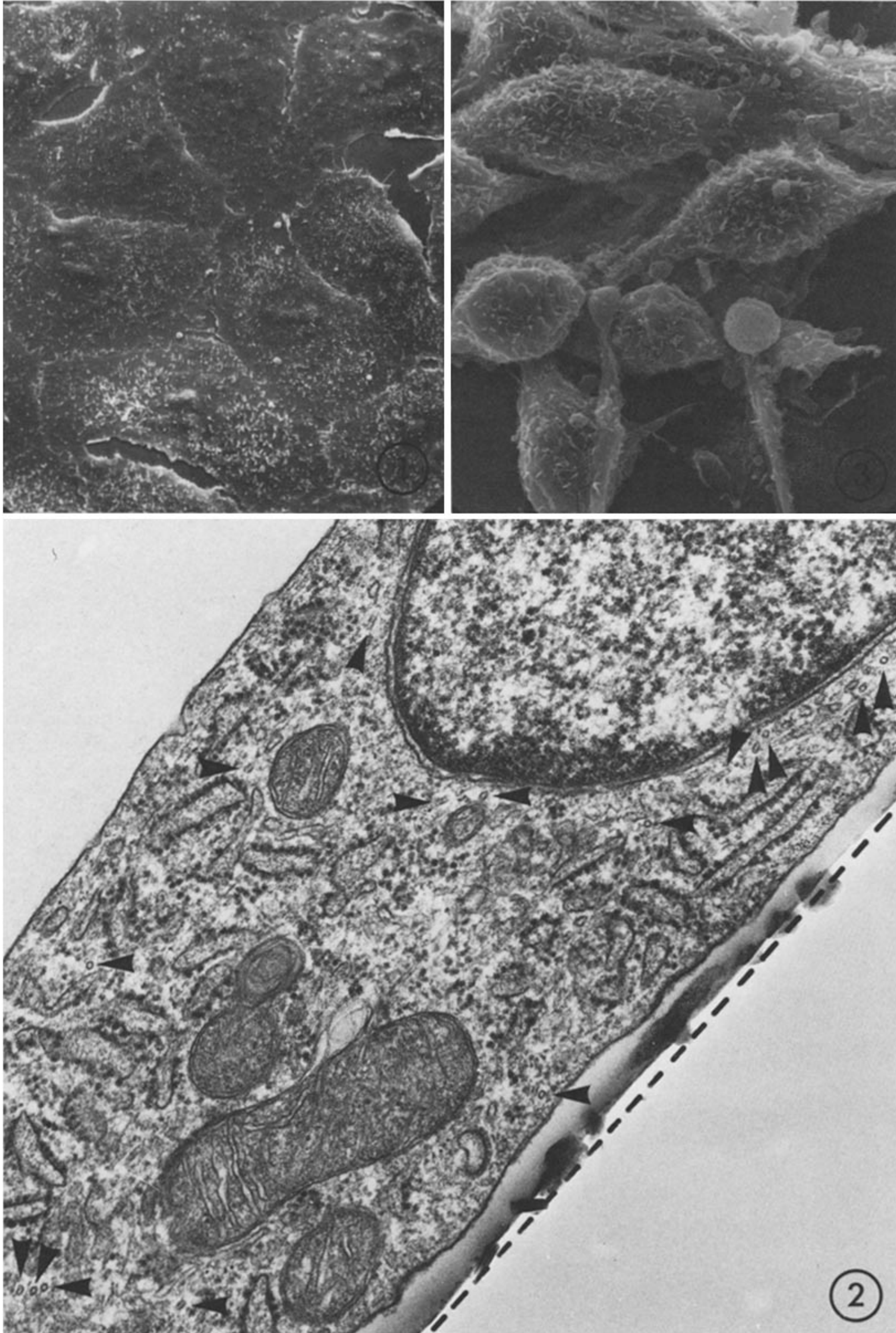
DISCUSSION

Our biochemical evidence, based on 10 replicate assays, indicates that there are equivalent amounts of tubulin in the NRK and 442 cells. There is some disparity among the results of other studies dealing with this question. In two of the earlier studies, it

FIGURE 1 Scanning image of confluent NRK cells, which flatten and adhere tightly to the substrate. × 800.

FIGURE 2 Thin section of NRK cell perpendicular to substrate (indicated by dash line). The numerous and widely distributed microtubules are indicated by the arrowheads. × 50,000.

FIGURE 3 Scanning image of high density 442 cells. The spherical to fusiform cells adhere poorly to the substrate and pile up on one another at high density. The cells display elaborate surface folding and may form 1- to 2-μm-wide processes (two of which are oriented vertically in lower portion of micrograph). × 800.



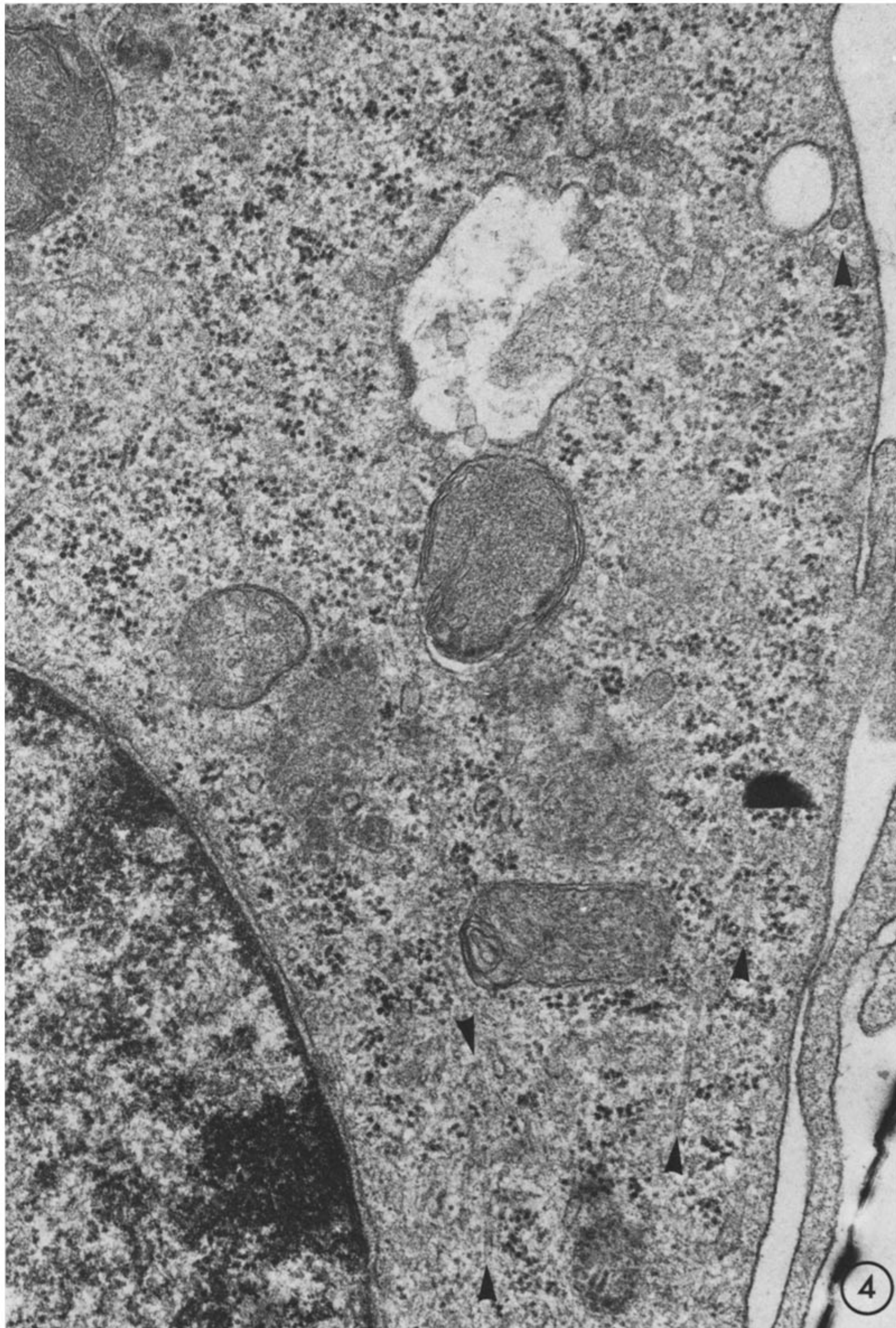
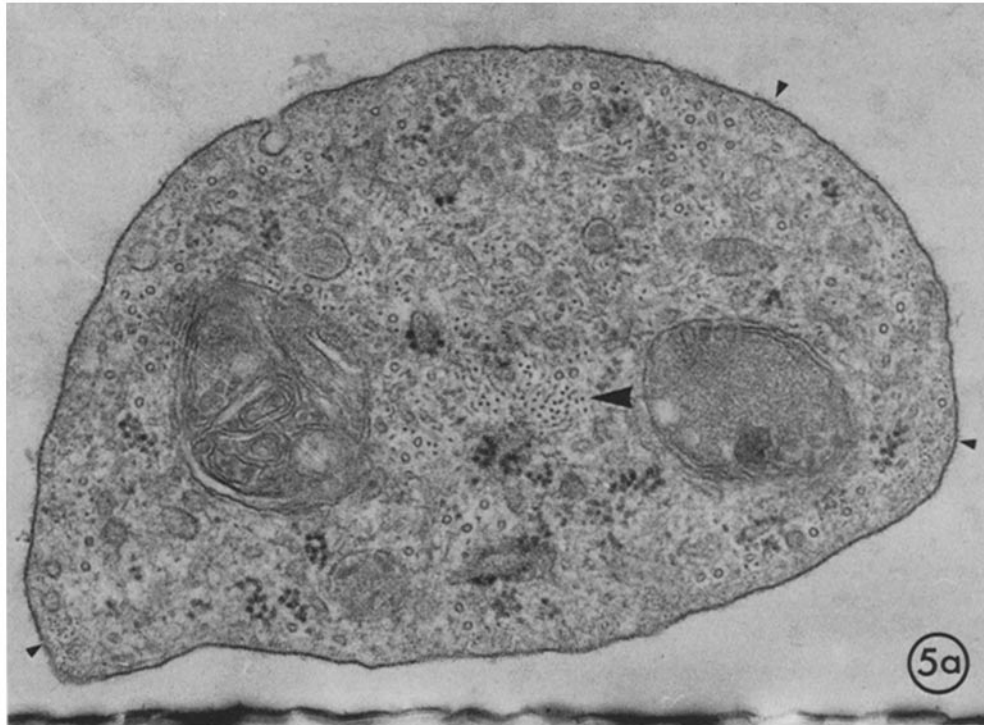


FIGURE 4 Thin section of a portion of a large 442 cell body (section perpendicular to substrate, located at bottom right corner). Microtubules (arrowheads) are infrequently encountered in sections of the cell body cytoplasm. × 50,000.



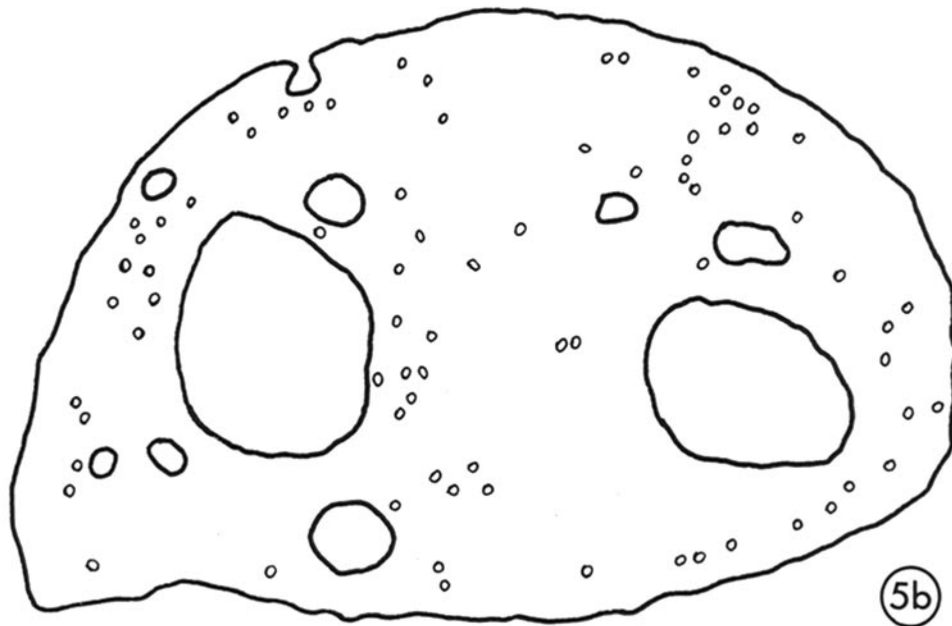


FIGURE 5 (*a*) Transverse sections of a larger process of a 442 cell displaying many microtubules. Intermediate filaments (large arrowhead) and cortical microfilaments (small arrowheads) also are present. × 50,000. (*b*) Tracing of the cell process in Fig. 5 *a*. The small profiles show location of microtubules.

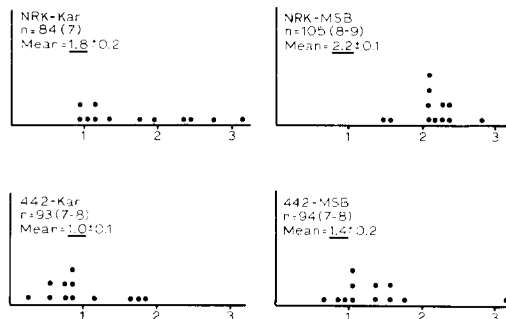


FIGURE 6 Histograms of microtubule counts for NRK and 442 cells fixed in Karnovsky (*Kar*) fixative and in microtubule-stabilizing buffer (*MSB*). For each of the four cell populations, n = total number of cells examined, and the number in parentheses indicates the number of cells pooled to yield one point on the histogram. Comparison of the means for both fixations shows that NRK cells display almost twice the numbers of microtubules per μm^2 of the cytoplasmic area as do the 442 cells.

was reported that both NRK cells (17) and mouse 3T3 cells (10) contained twice as much tubulin as their respective viral transformants. More recently, however, results similar to ours have been reported for 3T3 cells (12, 31) and for chick epidermal cells (12). Both of these studies, utilizing either colchicine binding (31) or a sensitive radioimmunoassay (12), report that equivalent amounts of tubulin are present in the normal cells and in their virally transformed counterparts. We cannot account for the disparities among the different studies at the present time, but variations in cell lines, culture conditions, and types of tubulin assay may contribute to the differences. It seems reasonable to conclude, however, that transformation does not necessarily lead to a reduction in cellular tubulin content.

Although total quantity of tubulin does not seem to differ in NRK and 442 cells, significantly more of the tubulin in 442 cells appears in the high-speed pellet of the cell homogenate. The form of the tubulin in this pellet is undefined. For this reason and because of the controversy over the state of organization of microtubules in normal and transformed cells, we turned to electron microscopy to obtain quantitative data about the extent of microtubule polymerization in both cell types.

Although it has been claimed in an earlier study (8) that 442 cells lack microtubules, it is clear from our micrographs that both cell types contain significant numbers of microtubules. The numbers and distributions, however, are quite different in the two cell types. The microtubules are spread throughout the cytoplasm in the NRK cells while in the 442 cells there are very few microtubules in the cell body and very many in the cytoplasmic processes. No counterpart for these cell processes exists in NRK cells, although similar processes, apparently containing microtubules (by criteria of antibody staining and polarized light birefringence), have been noted on other transformed cells in culture (27).

It is becoming evident that cell transformation per se does not entail loss or disorganization of the cytoplasmic microtubules (6, 16, 27), and many transformed cells may contain extensive microtubular arrays. It seems that the degree of cell contact with the substrate may influence the ability of microtubules to organize and, if there is an apparent reduction in microtubule number in a transformed cell, it may be secondary to the cell's ability to adhere and spread upon the growth substrate. Our 442 cells are clearly a type which adheres poorly to the substrate.

A factor which complicates quantitation of microtubule number with fluorescence when comparing cells of differing morphology is that microtubules may be more difficult to visualize in thick spherical cells due to overlapping or to masking by diffuse cytoplasmic fluorescence in the cell body. At the present time, direct assay of microtubule numbers in electron micrographs is the only quantitative method that is not troubled with these problems. Even with the EM method there may be artifacts, however. It has been suggested that fixation may affect the visualization of microtubules and there are indications that fixation in buffers designed for microtubule stabilization may give better results than conventional glutaraldehyde fixation (15). In terms of defining absolute numbers of microtubules in cells, this may be a very difficult problem, but for comparisons of relative numbers of microtubules between cell lines there is less difficulty as long as both cell types are treated identically. For the present work, we used two different fixation protocols. It appeared that the MSB fixation might be slightly superior (Fig. 4) although we cannot substantiate this statistically. It may be that the MSB fixation simply allows the existing microtubules to be visualized more readily. Since the relative differences in microtubule numbers in the NRK and 442 cells are the same with both fixation methods, we believe that fixation is not a factor in our comparisons between the two cell lines.

As noted in Materials and Methods and in the Appendix, irregular cell shapes and nonrandom distributions of microtubules within cells can complicate the comparison of microtubule numbers between two different populations of cells. If the data are highly skewed, it is quite possible that erroneous conclusions could be reached in comparing two such populations. We have avoided this problem by normalizing the data as described.

We have found that the 442 cells have about one-half the number of microtubules per unit of cytoplasmic area displayed by normal cells. Strictly speaking, we cannot draw the same conclusion about the numbers of microtubules per cell since we do not have direct measurements of cell volume. We do know, however, that NRK and 442 cells have essentially the same amounts of tubulin and total protein per cell, so it seems unlikely that numbers of microtubules per cell will differ significantly from the numbers per unit cytoplasmic area. We are therefore faced with the problem of accounting for the excess of sedimentable tubulin in the 442 compared to NRK cells.

One of the most likely explanations for the increased sedimentable tubulin in the 442 line is that this tubulin is aggregated in a form that is not morphologically detectable in the electron microscope, such as the "rings" that are observed in vitro (2). Microtubule-organizing centers in cells, such as the mitotic spindle organizers in the asters, usually are foci of amorphous, moderately dense material that may contribute to the fluorescence observed in the aster with antibody staining for tubulin (11, 29). It is especially interesting to note that in unfertilized sea urchin eggs (30) large amounts of tubulin are stored in the form of cytoplasmic particulates. These consist of large masses of amorphous, granular matrix containing only a few microtubules. It is thought that these particulates contain large amounts of unpolymerized tubulin which is later incorporated into the microtubules of the spindle during egg cleavage. Before ciliogenesis in many cells, granular bodies containing some amorphous material accumulate in the cytoplasm and are evidently transformed into basal bodies and cilia (1, 7). Such cytoplasmic particles have been isolated from cells undergoing ciliogenesis and have been shown to contain tubulin (23). In studies of the microtubule-rich arms in a Heliozoan (25, 26), it was shown that disruption of the microtubules by colchicine or cold was accompanied by an increase of amorphous matrix substance in the cytoplasm. There is evidence also that tubulin associates with the cell membrane (22, 24) in a form which should therefore be sedimentable but which is different in organization from the microtubule.

Regarding cells in culture, there is little evidence dealing directly with the question of nonmicrotubular tubulin aggregates. Antibody staining studies have indicated that there may be significant amounts of diffuse, nonlinear reaction product in cells, but the form of the tubulin giving rise to this reaction has been unclear due to the problems of superposition as noted above, and the often harsh fixation and staining methods used. With improvements in techniques, nonmicrotubular aggregates might be visualized. Such appears to be the case in a very recent study (14) in which small, patchy, foci of tubulin-antibody fluorescence have been noted in regions of very thinly spread and well-preserved cytoplasm of cultured cells.

Our present study suggests that such nonmicrotubule tubulin aggregates may represent a large portion of the total cell tubulin in a transformed cell. The amount of this material relative to the tubulin in microtubules and to that present as dimers may be important in the regulation of microtubule-dependent phenomena, such as morphologic alteration during malignant transformation.

APPENDIX

The example discussed here is provided to illustrate more graphically the type of problem we encountered in analyzing microtubule distributions in our cells and how it was dealt with. In Fig. 7, we have drawn highly idealized thin sections of cells as they might appear in a layer of 442 cells on a culture dish. The large profiles (cells 1 and 3) represent large, spherical cell bodies, each of which contains a relatively small number of microtubules per unit of cytoplasmic area (1 $MT/\mu m^2$, as indicated in Row A below the figure). The small profile (cell 2) is one of the slender cell processes occasionally encountered. These usually have a large number of microtubules (arbitrarily set at 10 $MT/\mu m^2$ in this instance). It can be seen that the presence of this one small process, when treated as a measurement of an individual cell, significantly skews the distribution of the measurements for all three cells. The mean (4 $MT/\mu m^2$) is obviously not truly representative of the population.

If the data are treated in a different manner (row B), this problem is circumvented. Instead of

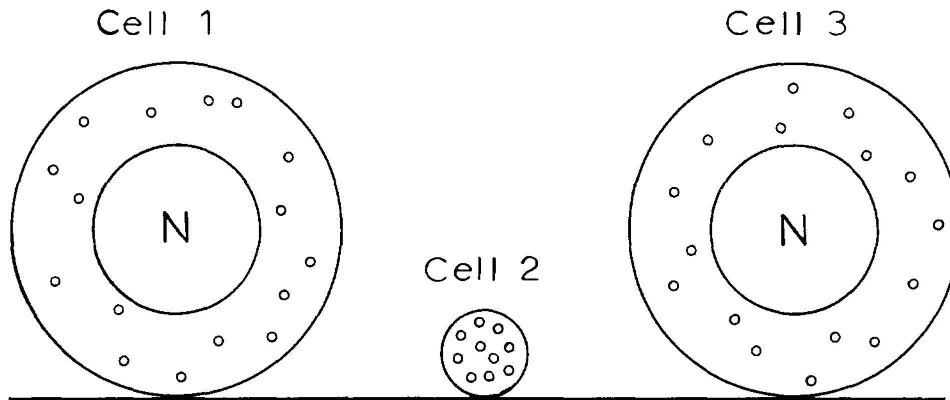


FIGURE 7

	Cell 1	Cell 2	Cell 3	
	No. MT = 16	No. MT = 10	No. MT = 16	
	Area = 16 μm^2	Area = 1 μm^2	Area = 16 μm^2	
A.	$Ratio_1$ = 1 MT/μm^2	$Ratio_2$ = 10 MT/μm^2	$Ratio_3$ = 1 MT/μm^2	Mean ratio = 4 MT/μm^2
B.	$MT_{\Sigma 1\text{-}3}$ = 42	$Area_{1\text{-}3}$ = 33 μm^2		Ratio = 1.3 MT/μm^2

a ratio of MT/μm^2 being taken for each cell, the microtubule counts and all areas are pooled separately and the sums are used to derive a ratio (1.3 MT/μm^2) that is more representative of the population. This is the method used in the accompanying paper. If this type of sampling process is carried out randomly a number of times (12 samples in our analysis), a set of values can be generated whose distribution approximates a normal curve, so that comparisons of the results for different cell population can be made using a parametric type of analysis.

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Note Added in Proof: Mean cell volumes of trypsinized and glutaraldehyde-fixed NRK and 442 cells have been determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.). As cultures become confluent, the volumes of 442 cells decrease from near equivalence with NRK cells to a mean volume that is 25% less than the mean volume of NRK cells. This result is consistent with our conclusions regarding the relative quantities of pelletable tubulin and microtubules per cell.

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