Kinetics and Intermediates of Marginal Band Reformation: Evidence for Peripheral Determinants of Microtubule Organization

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ABSTRACT The microtubules of the mature erythrocyte of the chicken are confined to a band at the periphery. Whole-mount electron microscopy after extraction reveals that the number of microtubules in each cell is almost the same. All the microtubules can be depolymerized by incubation in the cold, and the marginal band can be quantitatively and qualitatively reformed by return to 39°C. These properties allow the reformation of the marginal band to be treated as an in vivo microtubule assembly reaction. The kinetics of this reaction and the intermediates detected during reformation suggest a mechanism of microtubule organization that is distinct from that observed in other cell types. Apparently only one or two growing microtubule ends are available for assembly—assembly is only detected at the cell periphery, even at early times—and there is no evidence of the participation of a microtubule-organizing center.

The participation of microtubules in the assumption and maintenance of cell polarity and morphology has been deduced largely from the results of drug-interference experiments. These functions can also be inferred by comparing microtubule configurations with the morphologies of the disparate structures of which they are part—for example, the mitotic spindle, the polygonal cytoplasm of the interphase fibroblast, and the processes extended by neuronal cells. We are interested in understanding how microtubules of essentially identical ultrastructure and sharing a major component, tubulin, which is among the most highly conserved of all proteins, can end up in such different configurations. One explanation proffered is that the determinant of microtubule organization, and of cellular asymmetry itself, lies in a microtubule-organizing center. In this view, such centers would have the capacity not only to nucleate microtubule assembly but also to specify microtubule shape, number, length, and trajectory. Another possibility is that lateral interactions with other microtubules and other cell structures are responsible for microtubule organization. In this view, the determinants of microtubule shape are separable from sites of nucleating activity (1).

We have studied the origins of the detailed morphology, and, therefore, of microtubule arrangement, in individual cells. The cells in a neuroblastoma culture exhibit a very broad range of neurite morphologies, but we found that these morphologies are not arrived at randomly. Cells that are mitotically related are also strikingly morphologically related (2, 3). Even after the cytoskeleton is disrupted by microtubule-depolymerizing drugs, causing the neurites to retract, the original morphology of the cell can be restored when the drug is removed (4). These results suggest the existence of determinants of the shape of individual cells. In the work presented in this paper, we have turned to a normal cell type that contains an extraordinary microtubule organelle. The marginal band of the chicken red blood cell apparently contains all the cell's microtubules in a ribbon at the periphery and in one plane (5). There is morphological evidence that this band interacts with the very periphery of the cell (6). The band is quantitatively indistinguishable in the individual cells in a population from mature chickens (7), making quantitative study of its formation after cold-induced depolymerization possible. Our experiments reveal a novel in vivo microtubule assembly reaction that does not rely upon a central microtubule-organizing center.

MATERIALS AND METHODS

Extraction of Chicken Erythrocytes: Blood from one white Cornish chicken (*Gallus domesticus*), weighing 2 kg, was collected into 200 ml of

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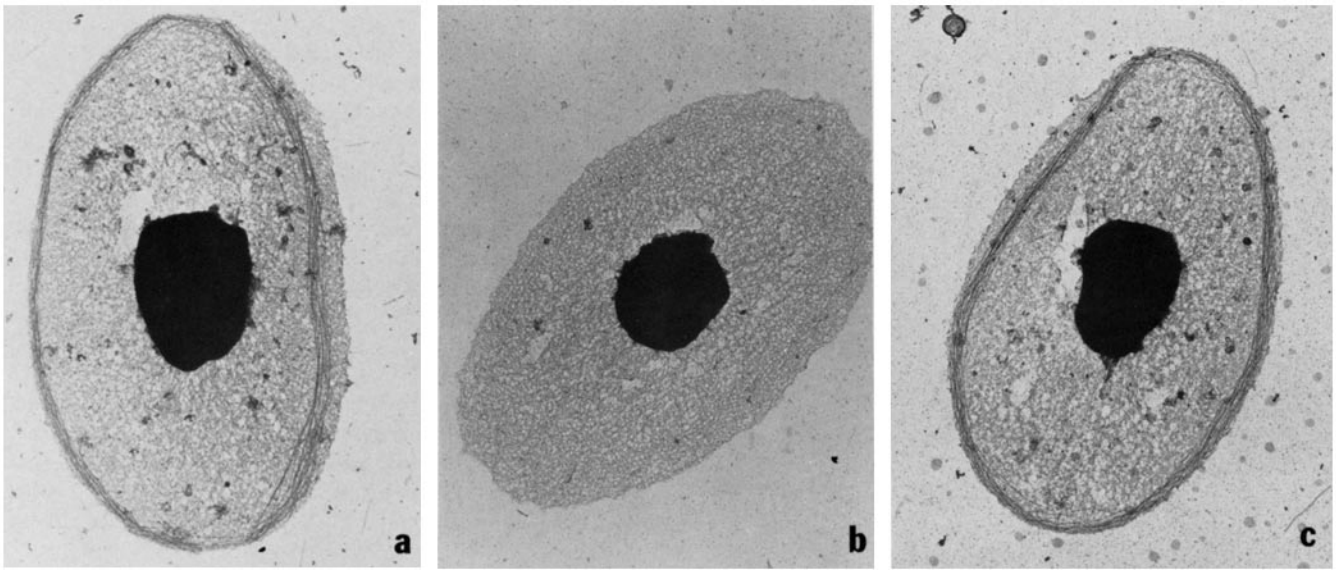

FIGURE 1 Whole mount views of extracted cells. Cells were extracted as described after incubation (*a*) at 39°C for 120 min, (*b*) at 0°C for 60 min, and (*c*) at 0°C for 60 min, then at 39°C for 60 min.

phosphate-buffered saline (PBS)[1] containing 0.1 mg of heparin per milliliter (Na salt, Grade I, Sigma Chemical Co., St. Louis, MO). The cells were centrifuged at 2,000 rpm (5 min, 37°C). The supernatant and buffy coat were removed by aspiration, and the cells were resuspended and centrifuged two more times in PBS containing 0.025 M glucose. The cells in the final resuspension were incubated at 0° or 39°C and then plated onto grid-coverslip assemblies (see below).

After the cell suspension had been in contact with the grids 30–45 s, the excess fluid was removed by blotting. The coverslip was dipped gently at a 45° angle into a beaker of PBS at the incubation temperature, then blotted on edge. This process was repeated until no red color was visible. The coverslip was then rinsed in PM2G extraction buffer (0.1 M piperazine-*N,N'*-bisethanesulfonic acid; 10^{-3} M magnesium sulfate; 2×10^{-3} M ethylene glycol bis-(β-aminoethyl ether)-*N,N'*-tetracetic acid, 2 M glycerol, pH 6.9), placed in a petri dish, and immediately covered with PM2G plus 0.1% NP-40. Taxol (5 μg/ml) was included to enhance microtubule preservation (8). Present only in the extraction buffer, taxol does not recruit tubulin into *de novo* microtubule assembly. In addition, the extraction buffer included the protease inhibitors aprotinin and PMSF. After 12 min, the coverslips were rinsed with PM2G and fixed in PM2G containing 3.7% formaldehyde and 0.5% glutaraldehyde at room temperature for 30 min.

Grid-Coverslip Assemblies: Four or five 300-mesh Athene copper grids (Polysciences, Inc., Warrington, PA) were sealed onto glass coverslips (No. 1, 18 mm) with a film cast from an anhydrous 0.7% (wt/vol) Formvar-dichloroethane solution. The Formvar coat was sealed on all four sides by attaching a second coverslip to the back of the first with cyanoacrylate adhesive.

Preparation of Samples for Electron Microscopy: The coverslips were rinsed free of their first fixation medium with PBS, and the cells were fixed in 2.5% electron microscopy grade glutaraldehyde and 0.2% tannic acid in PBS for 20–60 min at room temperature, rinsed four times, and incubated in 1% OsO_4 for 10 min in the dark. They were rinsed free of osmium, dehydrated through a series of ethanol washes (35, 50, 70, and 95%, and 100% three times, all at 4°C). The grids were then removed from the coverslips and dried by the critical-point method. A final carbon coat was applied to reduce beam damage, and samples were examined at 100 kV with an 18-μm gold foil objective aperture.

Scoring Samples: Whole mounts of the extracted erythrocytes were photographed, usually at × 7,000. The negatives were printed at a further 2.5-fold enlargement. The thickness of the microtubules made them easily distinguishable from other elements in these cells. Each print was inspected for microtubule profiles by each of the authors independently using a magnifying lens. The number of microtubules was determined at three or more randomly selected sites at the periphery, and the rest of the cell was carefully scanned. The viewers' counts usually agreed with one another, and never differed by more than one profile.

[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; MTOC, microtubule-organizing center.

RESULTS

Reversible Depolymerization of Marginal Band Microtubules

Gentle extraction of chicken erythrocytes with nonionic detergents in the presence of microtubule stabilization buffer, as described in Materials and Methods, permits visualization of each of the individual microtubules in the marginal band (Fig. 1*a*). Their shape is not noticeably distorted compared with that of unextracted cells. Throughout most of the perimeter, individual microtubules can be distinguished either by using a magnifying lens or at high magnification, so that the number of microtubules can be counted easily, even in complete bands (Fig. 2). Erythrocytes extracted in solution do not adhere well to grids and are typically torn and bent by the time they can be observed. Incubation of the cells at 0°C for 60 min before extraction causes apparent complete depolymerization of the microtubules in the marginal band; every cell observed after this treatment looks like the one shown in Fig. 1*b*. The microtubules returned upon incubation at 39°C for 30–60 min (Fig. 1*c*). The marginal bands in the control cells and in the chilled and rewarmed cells were essentially the same. The average number of microtubule profiles per cell was 10.3 in the marginal bands of the control and 10.6 in those of the rewarmed sample. That number agrees well with values obtained by other methods (7). The distribution of microtubule number per cell in the two populations was virtually identical (Fig. 3*a*). In both cases, the microtubule profiles usually were uninterrupted. We occasionally saw ends, but these may have been due to breakage (see below). Similar values were obtained from thin sections of fixed cells that had not been extracted, except that an occasional band with 15 or 16 microtubules was seen.

The quantitation of microtubules after chilling and rewarming was supported by biochemical measurements. Using detergent-extraction procedures that we have applied to the analysis of microtubule structure in cells (9), we demonstrated that <1% of the normal assembled tubulin in these cells remain after incubation in the cold, as assayed by staining of

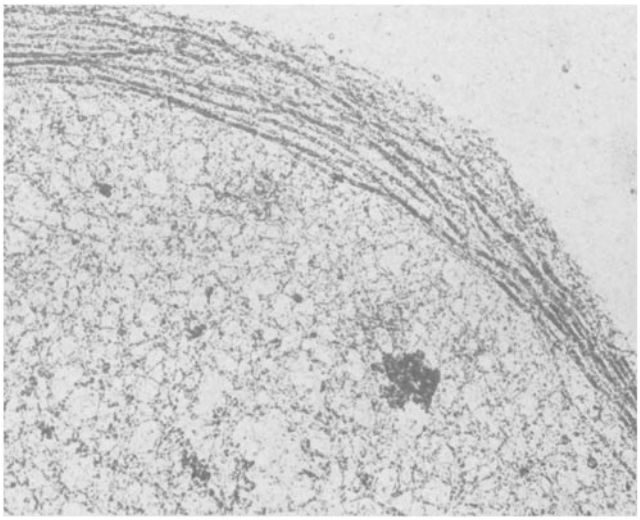

FIGURE 2 High magnification view of individual microtubules in the marginal band.

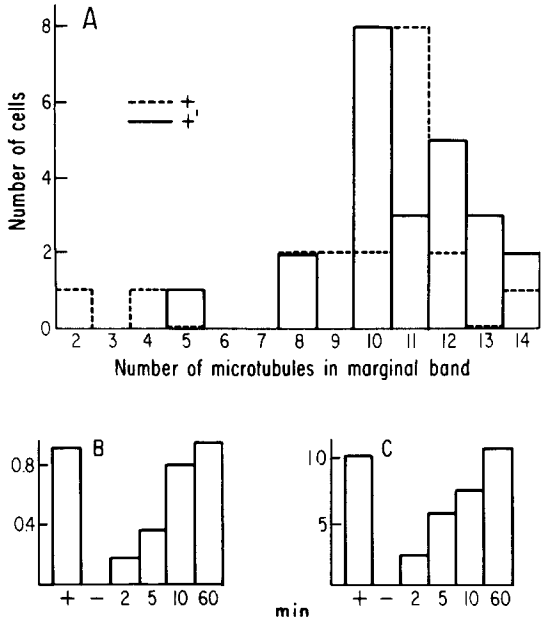

FIGURE 3 (*A*) Microtubule profiles visualized in whole-mount views of extracted cells. Two populations were scored: cells incubated at 39°C for 120 min (+, broken line) and cells incubated at 0°C for 60 min then shifted up to 39°C for 60 min (+′, solid line). (*B*) Fraction of cells containing one or more microtubule profiles in a complete circumferential structure in cells incubated at 39°C (+), 0°C (−), or incubated at 0°C and then shifted up for 2, 5, 10, or 60 min before extraction. (*C*) Number of microtubules in a complete circumferential structure. Categories are the same as those described under *B*.

SDS gels. The amount of tubulin in control samples and in chilled and rewarmed samples was identical (data not shown).

Intermediates in Marginal Band Reformation

To follow the time-course of marginal band reformation, cells that had been incubated for 90 min at 0°C were returned to a 39°C water bath. At various times, they were extracted and prepared for whole-mount electron microscopy. Fig. 4 shows a low-magnification view of a field of cells extracted 5 min after shift up. It was from fields like this one that the data in this study were drawn. The typical morphology of almost all of the cells survived the experimental handling; those that were grossly distorted or occluded by other cells were not counted.

Regrowth of the marginal band was asynchronous, so that after 5 min cells with complete, partial, or no marginal bands were seen (Fig. 4). Nevertheless, the reformation of the marginal band was clearly time dependent. Fig. 3*b* shows the proportion of cells displaying one or more microtubule profiles all of the way around the perimeter in cells held at 39°C (+), cells shifted down to 0°C (−), and at 2, 5, 10 and 60 min after shift up to 39°C. That proportion rose rapidly for 2–5 min after shift up and reached the level of the control cells 30–60 min later.

The number of individual microtubule profiles found in these circumferential structures also increased with time (Fig. 3*c*). In particular, we saw cells with a complete marginal band but with only one or a few microtubule profiles (Fig. 5). Observations from many cells show that the number of microtubule profiles in a cell rarely rose above two until a circumferential marginal band was complete (Fig. 6). Occasionally, examples of partial bands containing multiple microtubules were found at late times of reformation. However, they were also seen in untreated controls (Fig. 6). Almost always, the appearance of these cells suggested that such partial bands were produced by shearing during experimental preparation and probably did not represent true intermediates of regrowth.

Kinetics of Marginal Band Reformation

Fig. 7 shows a plot of total microtubule length achieved per cell as a function of time of regrowth. The contour length of the microtubules in each cell was determined with a planimeter. The average value at each time point is indicated. For the three time points studied before there was significant accumulation of completed structures—2, 5, and 10 min—those averages fall on a straight line that extrapolates through zero. The slope of the line does not increase with time, suggesting that the number of ends available for microtubule

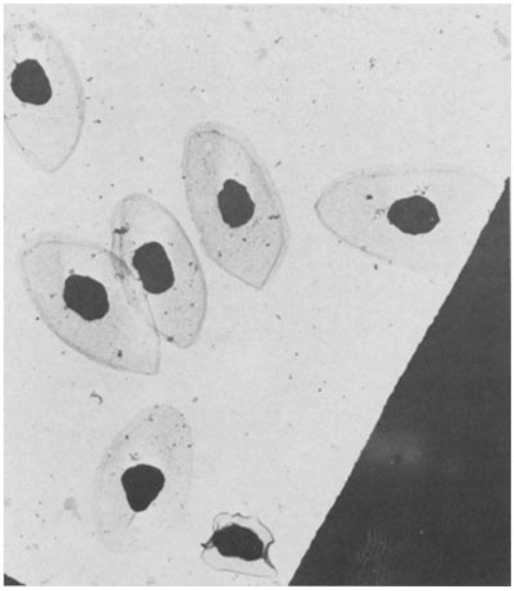

FIGURE 4 Low-power view of a field of cells extracted 5 min after shift up. Most cells show normal form. The extent of reformation of the marginal band is variable.

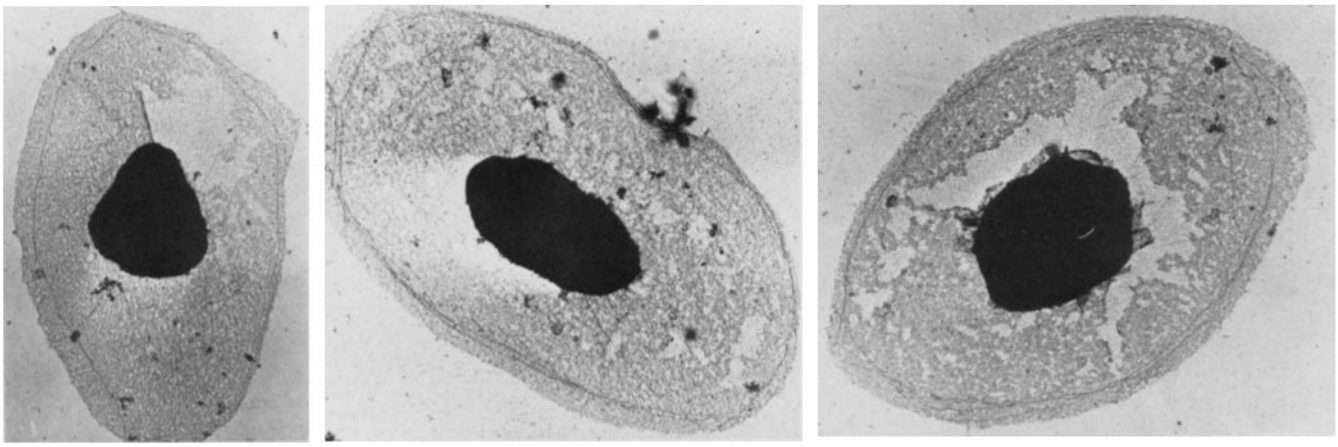

FIGURE 5 High-power view of cells extracted 5 min after shift up, all showing complete marginal bands but with only one or a few microtubule profiles.

elongation does not increase as reformation proceeds. In addition, although there is considerable scatter in the values found, the microtubule lengths in those cells that have complete bands but less than a full complement of microtubule number, such as those shown in Fig. 4, fall close to the average values. These results suggest that those early intermediates do not represent aberrant forms but rather lie on the main kinetic pathway of marginal band reformation.

From these data, and from other observations, it is possible to estimate the number of sites available for elongation during reformation of the marginal band. That number is a function of the observed rate of growth divided by the pseudo-first-order constant for microtubule assembly in vivo. The observed rate of growth is derived from the slope of the line through the average values shown in Fig. 7: 15.4 μm/min. The concentration of tubulin in the cell was calculated from estimates of tubulin on SDS polyacrylamide gels stained with Coomassie blue and compared with known standards and from calculations of the volume of the cytoplasm of these cells obtained by comparing the volume of unextracted and extracted cells. These manipulations produced a value of 6.0 mg/ml (J. A. Swan, unpublished results). Of course, the rate constant for assembly in vivo has not been measured. However, the second-order rate constant in an in vitro reaction using axonemal fragments to nucleate linear microtubule growth is 7.0 μm/min/mg/ml (10). From these considerations, the pseudo-first-order rate constant in vivo would be 12 μm/min, so the number of growing ends available per cell is 1.3, or between one and two per cell. Obviously, this number is not precisely arrived at. The value for tubulin concentration could be too high: Murphy and Wallis (11), who used different extraction techniques and different assays, arrived at 2.4 mg/ml as the correct value. Were this the case, the number of growing ends would have to be about three per cell to account for the observed rate. On the other hand, it is possible that the conditions in vivo are more favorable for microtubule assembly than those of in vitro assays and that the value of the second-order rate constant is too low. In that case the number of growing ends could be lower. The conclusion reached by the kinetic analysis fits well with the structure of the isolated intermediates available for reformation.

DISCUSSION

Workers in other laboratories have studied the mode of microtubule formation in various cell types after reversible depolymerization. In cultured fibroblasts and neuronal cells, reformation involves apparent regrowth of multiple microtubules from one or two sites, usually near the nucleus (12). In some cell types, multiple centers are seen, but each is capable of giving rise to many microtubules (13). By a combination of immunofluorescent and electron microscopy, the coincidence of these microtubule-organizing centers (MTOCs) with centrioles and associated dense material has been demonstrated (14). In experiments in our laboratory, neuroblastoma cells with multiple neurites have been found to have only one or two MTOCs, and their distribution in the cells bears no apparent relationship to the position of the neurites.

A similar mechanism may be involved in the reformation of the blood clam erythrocyte. These cells contain a centriole pair at the periphery, and during regrowth several microtubules extending part way around the cell can be seen (Fig. 9 in reference 15). Were such a scheme to pertain in the chicken erythrocyte, we also would have detected partial marginal bands with many microtubules, perhaps the normal complement or half that number. However, such intermediates were not detected in our studies. On the contrary, more than two microtubule profiles in a cell are rarely observed until a complete band is formed. On this basis, the results are not consistent with involvement of a typical MTOC in the reformation of the band. One could argue instead that an atypical MTOC is involved—one that nucleates microtubule assembly from just one or two points along its surface. Such spatially restricted assembly has been observed for special MTOCs, such as basal bodies, but those in animal cell cytoplasm generally give rise to a radially or spherically symmetrical pattern. In addition, we have been unable to find any evidence of a structural or functional MTOC in these cells. No centrioles have been visualized at the cell periphery or at the nucleus by whole-mount microscopy or by sectioning of unextracted, partially extracted, or completely extracted cells. Antisera that are known to detect centrosomes in other cell types gave no clear signal when applied to chicken erythrocytes (data not shown). Finally, only occasional microtubule profiles were found in regions of the cell not normally occupied by the marginal band. No obvious relationship exists between the positions of these fragments and the symmetry axes of the cell. It is possible that the fragments were artifacts produced during sample preparation, since similar structures could be found lying on the grid between cells. Unlike other

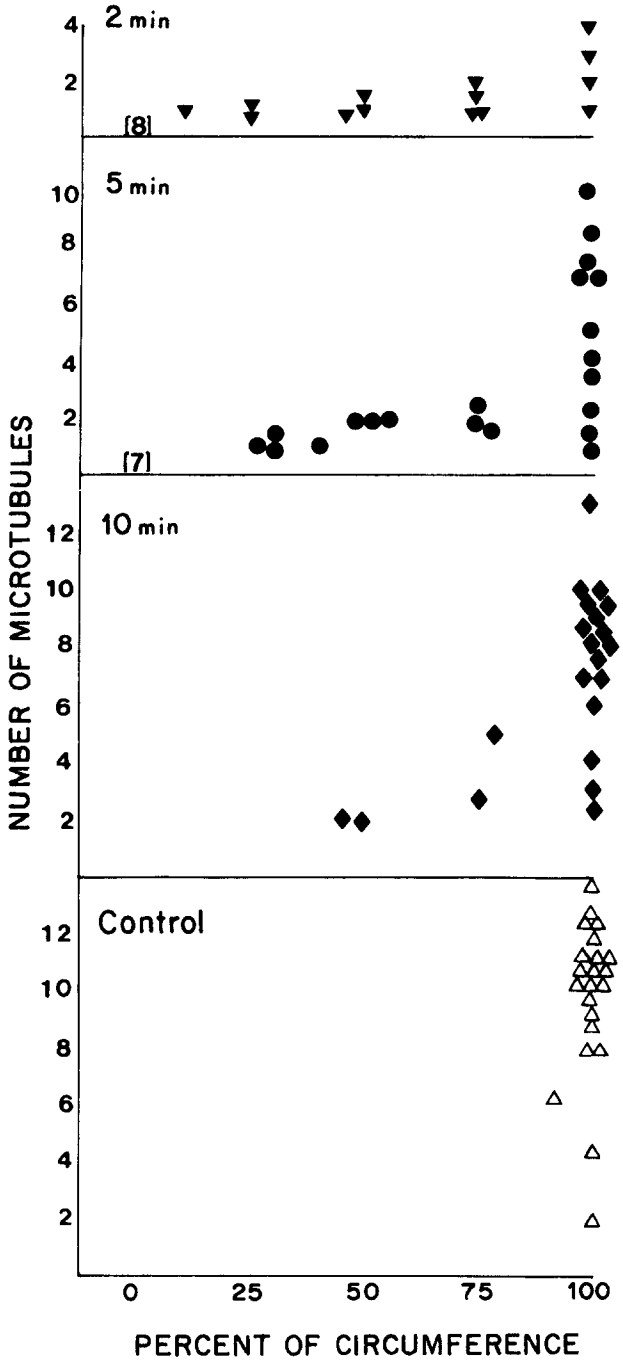

FIGURE 6 Number of microtubule profiles in individual cells, plotted with respect to percent of circumference occupied by marginal band. Panels show results from cells allowed to reform for 2, 5, and 10 min and for control cells. The numbers in brackets in the 2- and 5-min panels represent the number of cells that show no microtubule reformation.

cells, then, the microtubules of the chicken erythrocyte appear to originate only at the periphery and do not associate with a formal MTOC at any place in the cell.

Our findings are consistent with the spontaneous assembly of microtubules at the periphery. In an extreme version of this model, a single microtubule begins to assemble and then elongates at both ends until steady state is reached. The predictions of this scheme are met by the intermediates observed and by the predictions for the number of available growth sites calculated from the kinetics. However, that calculation is not sufficiently firm to rule out the participation of more than one microtubule fragment, each providing ends for elongation. A previous study of the in vitro assembly of microtubules from these cells produced relatively long microtubules, and led to the suggestion that few assembly sites were present (11).

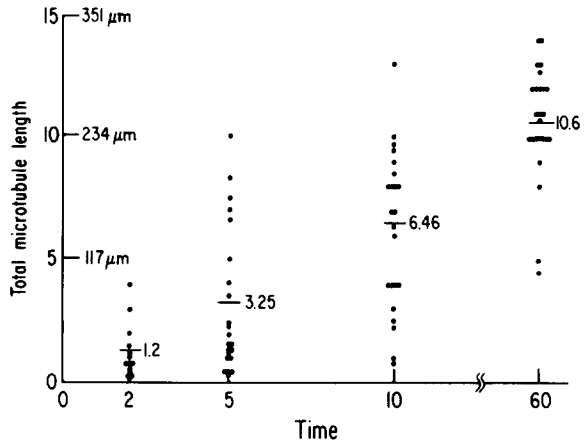

FIGURE 7 Total microtubule length achieved as a function of time (in minutes) of regrowth. The average circumference of the cells was 23.4 μm. The numbers next to the hash marks for each time point are the average numbers of microtubules per cell after 2, 5, 10 and 60 min of incubation.

Previous work performed in our laboratory and in that of Albrecht-Buehler (16) has provided evidence that individual cells contain determinants of their detailed asymmetry that result in their unique patterns of shape and movement. These studies, however, left unresolved the question of where in the cell the determinants lie. They also did not deal with a related problem: how does the information in different types of cell differ, so that neurons are distinguished from, for example, fibroblasts? In the present study, we found no evidence of the participation of an MTOC in the arrangement of cytoplasmic microtubules. Rather, we conclude that determinants of a particular microtubule organelle—the marginal band—can lie at the periphery of the cell and specify the orientation of the microtubules. The structural and molecular nature of these determinants is under study.

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