

# Tubulin Hooks as Probes for Microtubule Polarity: An Analysis of the Method and an Evaluation of Data on Microtubule Polarity in the Mitotic Spindle

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**ABSTRACT** The structural polarity of cellular microtubules can be visualized in situ by lysing cells in special buffers containing tubulin. Under these conditions, the tubulin polymerizes to form curved sheets which attach to the walls of the endogenous microtubules. When such decorated microtubules are cut in cross section and viewed in the electron microscope, they appear to bear hooks curving clockwise or counter-clockwise. The direction of hook curvature is defined by the orientation of the decorated microtubule and thus serves as a probe for microtubule polarity. In this paper we describe a way to analyze the relative frequencies of hooks of different curvatures so as to measure the fidelity of the relation between hook curvature and microtubule polarity. The assumptions of the method are tested and found to be valid to a reasonable accuracy. The correlation between hook curvature and microtubule orientation is shown to be at least 0.98 for the spindles of PtK cells and *Haemaphysalis* endosperm at all stages of division and at all places in the spindle. The correlation is shown to be valid for each hook that forms, so the polarity of those microtubules that bear multiple hooks is specified with even better certainty than 0.98. This property of hook decoration is used to reinvestigate the possibility that some of the microtubules of the kinetochore fiber might be oriented with their plus ends distal to the kinetochore (opposite to the direction previously shown to predominate). Close analysis fails to identify such oppositely oriented microtubules. The scoring of tubules bearing multiple hooks also shows that individual interzone fibers at anaphase are constructed from clusters of antiparallel microtubules. The method for estimating the correlation between hook decoration and microtubule polarity is shown to be applicable to many structures and circumstances, but we find that the hook decoration assay for microtubule polarity is not uniformly accurate. We suggest that future studies using hook decorations should employ the method of data analysis presented here to assess the accuracy of the results obtained.

Microtubules are polar polymers, as seen both by high resolution structural methods (1) and by biochemical probes for tubulin assembly asymmetry (2–4). This polarity is thought to be physiologically significant, since microtubules may play a vectorial role in motile machines such as flagella (5) and the mitotic spindle (6, 7). Assembly asymmetry based on structural polarity may also contribute to the differential stability of microtubules in cells (8). Therefore, good methods for determining microtubule orientations in cells are needed.

Of the methods available for displaying microtubule polar-

ity (3, 4, 9, 17), the one used most to date is that based on a decoration of cellular microtubules with hook-shaped appendages made of tubulin. In the presence of 0.5 M PIPES and 2.5–5% DMSO, pure tubulin will assemble into curved sheets attached to the walls of pre-existing microtubules, forming “hooks” whose direction of curvature is defined by the structural polarity of the original microtubule (9). The form of assembly that predominates makes a clockwise-curving hook when the decorated microtubule is viewed looking from its plus (fast-growing) end toward its minus (slow-grow-

ing) end. With this assay the polarity of microtubules has been determined relative to significant cellular landmarks, such as the centrosome, in several biologically interesting systems (10–15).

In any such study, however, there is an important question concerning the fidelity of the relationship between the polarity probe (e.g., the direction of hook curvature) and the true structural polarity of the decorated microtubule. Our efforts to measure this fidelity have previously been limited by the need to assume that all of the microtubules in a given test structure are of known polarity. For example, the method has been applied to ciliary axonemes and the microtubule bundles that form the axopodia of a heliozoan (11). Essentially all the hooks that form curve one way, so it is plausible that all the component microtubules are of one polarity. The frequency of oppositely curving hooks can then be used to estimate the fidelity of the hook decoration method. Results from these studies encourage the view that the correlation between the direction of hook curvature and the structural polarity of the decorated microtubule is >99% (11). Analogous studies on the mitotic aster, however, show a uniformity of hook curvature no better than 90% (9), leading to the question are the spindle microtubules in some way different so they do not decorate with equivalent fidelity? Or, is the spindle itself structurally more complex, containing some microtubules of both polarities, even in an aster where one might expect that all the microtubules should be similarly oriented relative to the centrosome? There is reason for concern about fidelity because we know that the hook decoration assay is not perfect: one occasionally finds a single microtubule bearing both a clockwise and a counter-clockwise curving hook.

In the mitotic spindle, the fraction of microtubules bearing clockwise curving hooks varies considerably from one place to another (15). In all work so far, we have assumed that the fidelity of the assay is constant for a given spindle, so variation in the fraction of hooks with one direction of curvature can be used to determine the change with respect to position in the fraction of microtubules with a particular polarity. However, to test this assumption, it would be necessary to have an assay for the fidelity of the relation between hook curvature and microtubule polarity that is independent of microtubule orientation. In this paper we present a way of collecting and analyzing data on the frequency of microtubules bearing different arrangements of hooks so as to determine the probability that a given hook curvature means a given microtubule polarity. The method is based on those infrequently occurring microtubules that bear hooks of both curvatures. We then use the method to analyze data from our previous studies and refine our conclusions concerning the polarities of mitotic spindle microtubules.

## The Theory

**MICROTUBULES OF UNIFORM POLARITY:** Consider a set of parallel microtubules viewed from their plus towards their minus ends. Let  $p$  be the probability that when a hook forms on such a microtubule, it will curve clockwise.  $p$  will then specify the fraction of all one-hook microtubules that bear a clockwise curving hook, and  $(1 - p)$  will be the fraction of one-hook microtubules showing a counter-clockwise curving hook. If the presence of one hook does not bias the chances of forming a second hook of either curvature, then the fraction of two-hook microtubules having two clockwise

hooks is  $p^2$ , while the fraction having two counter-clockwise hooks is  $(1 - p)^2$ . The fraction of two-hook microtubules having one hook of each curvature is  $2p(1 - p)$ . A similar set of expressions can be generated for  $n$  hooks.

**MICROTUBULES OF MIXED POLARITY:** In a bundle of aligned microtubules with mixed polarities, let  $x$  be the fraction with plus end toward the observer and  $(1 - x)$  be the fraction with plus end away. Let  ${}^jF_n^i$  be the fraction of microtubules bearing  $n$  hooks,  $i$  of which curve clockwise and  $j$  of which curve counterclockwise. Now with  $p$  as defined above,

$${}^0F_1^1 = px + (1 - p)(1 - x),$$

where  ${}^0F_1^1$  is the fraction of one-hook microtubules bearing a clockwise hook.

${}^1F_1^0 = (1 - p)x + p(1 - x)$ , where  ${}^1F_1^0$  is the fraction of one-hook microtubules with a counter clockwise hook.

If we again assume that the presence of one hook has no effect on the direction of curvature of a second, then

$${}^0F_2^2 = p^2x + (1 - p)^2(1 - x)$$

$${}^2F_2^0 = (1 - p)^2x + p^2(1 - x)$$

$${}^1F_2^1 = 2p(1 - p)x + 2(1 - p)p(1 - x),$$

where  ${}^0F_2^2$ ,  ${}^2F_2^0$ , and  ${}^1F_2^1$  are the fractions of two-hook microtubules bearing two clockwise hooks, two counter clockwise hooks, and one clockwise and one counter-clockwise hook, respectively. These equations yield two expressions that are independent of the orientation of microtubules within the structure, i.e., expressions from which  $x$  has been eliminated:  ${}^1F_2^1 = 2p(1 - p)$  and  ${}^0F_2^2 + {}^2F_2^0 = p^2 + (1 - p)^2$ . The physical reason why these expressions are independent of microtubule polarity is that they are both constructed to be symmetric with respect to microtubule orientation.  ${}^1F_2^1$  is intrinsically symmetric and the sum  $({}^0F_2^2 + {}^2F_2^0)$  becomes symmetric by combining two equivalent, asymmetric terms. However, since  ${}^0F_2^2 + {}^2F_2^0 + {}^1F_2^1$  must equal one, these expressions are not independent and do not give separate measures of  $p$ .

Similar simple expressions may be constructed for  $n$  hooks, subject to the assumption that  $p$  for each hook is independent of the other hooks present. Table I diagrams the structures generated by multiple hook addition and defines the fractions  ${}^jF_n^i$ . For each  $n$ , we present combinations of the various  ${}^jF_n^i$ 's that are independent of the orientation of the component microtubules. Simple solutions for  $p$  in terms of the  ${}^jF_n^i$ 's are given; the other explicit solutions of  $p$  in terms of the  ${}^jF_n^i$ 's are better treated by numerical methods. We include Fig. 1 which presents all the useful relationships in graphic form.

These graphs may be used to estimate  $p$  from any observed  ${}^jF_n^i$ . Furthermore, the form of the curves is instructive concerning the variation of each expression with  $p$ . It is straightforward to show that all the expressions in Table I show either a maximum or a minimum at  $p = 0.5$  and are symmetric about that line. This is why the abscissa of the graph in Fig. 1 is double labeled.

Our theory is constructed in such a way that we never have to consider the absolute probability that a hook will form. Such a construction is necessary because the oblique orientation of some microtubules and the bizarre morphology of some hook decorations (see below) obviate their identification as belonging to a particular class. Thus the absolute frequen-

TABLE I  
Structures Generated by Multiple Hook Addition

No. of hooks per microtubule	Sample image	No. of ways to build images*	Notation for this fraction	Relations between the ${}^iF_n^j$ 's that are independent of microtubule orientation	$\rho_n$ expressed as a function of ${}^iF_n^i$ where possible*
1		1	${}^0F_1^1$	—	—
		1	${}^1F_1^0$		
2		1	${}^0F_2^2, {}^2F_2^0$	${}^0F_2^2 + {}^2F_2^0 = p^2 + (1-p)^2$	$\rho_2 = 1/2(1 \pm \sqrt{1 - 2{}^1F_2^1})$
		2	${}^1F_2^1$	${}^1F_2^1 = 2p(1-p)$	
3		1	${}^0F_3^3, {}^3F_3^0$	${}^0F_3^3 + {}^3F_3^0 = p^3 + (1-p)^3$	$\rho_3 = 1/2[1 \pm \sqrt{1 - 4/3({}^1F_3^2 + {}^2F_3^1)}]$
		3	${}^1F_3^2, {}^2F_3^1$	${}^1F_3^2 + {}^2F_3^1 = 3p(1-p)$	
4		1	${}^0F_4^4, {}^4F_4^0$	${}^0F_4^4 + {}^4F_4^0 = p^4 + (1-p)^4$	
		4	${}^1F_4^3, {}^3F_4^1$	${}^1F_4^3 + {}^3F_4^1 = 4p(1-p)[p^2 + (1-p)^2]$	
		6	${}^2F_4^2$	${}^2F_4^2 = 6p^2(1-p)^2$	
5		1	${}^0F_5^5, {}^5F_5^0$	${}^0F_5^5 + {}^5F_5^0 = p^5 + (1-p)^5$	$\rho_5 = 1/2[1 \pm \sqrt{1 - 2/5[10({}^2F_5^3 + {}^3F_5^2)}]^{1/2}}$
		5	${}^1F_5^4, {}^4F_5^1$	${}^1F_5^4 + {}^4F_5^1 = 5p(1-p)[p^3 + (1-p)^3]$	
		10	${}^2F_5^3, {}^3F_5^2$	${}^2F_5^3 + {}^3F_5^2 = 10p^2(1-p)^2$	
6 <sup>§</sup>		1	${}^0F_6^6, {}^6F_6^0$	${}^0F_6^6 + {}^6F_6^0 = p^6 + (1-p)^6$	
		6	${}^1F_6^5, {}^5F_6^1$	${}^1F_6^5 + {}^5F_6^1 = 6p(1-p)[p^4 + (1-p)^4]$	
		15	${}^2F_6^4, {}^4F_6^2$	${}^2F_6^4 + {}^4F_6^2 = 15p^2(1-p)^2[p^2 + (1-p)^2]$	
		20	${}^3F_6^3$	${}^3F_6^3 = 20p^3(1-p)^3$	

\* In scoring the hooked microtubules, we have made no distinctions based on either the spatial or temporal order of hook addition. Therefore, each class of image shows a degeneracy described by the binomial expansion.

\* The subscript  $n$  is assigned to each  $p$  determined in recognition of the fact that the values of  $\rho_n$  so calculated are based on different data and all depend on the assumption that  $p$  is independent of  $n$ .

§ The analysis is carried only through six hooks because we have seen seven hooks on a single microtubule only occasionally.

cies of each class cannot always be determined with accuracy. We think that the relative frequencies of each class within a given value of  $n$  are reasonably accurate because there is no reason to expect one class to be lost more frequently than any other. It will be seen, however, that the frequencies with which one observes microtubules with hooks of both curvatures are low. Hence  $p$  is calculated from small numbers and may not be so precise as one would like.

## MATERIALS AND METHODS

Several microtubule-containing structures have been decorated with polarity-revealing hooks by detergent lysis in neurotubulin buffered with 0.5 M PIPES at pH 6.9 and containing 2.5% DMSO, 1 mM guanosine triphosphate, and 1 mM of either EGTA or EDTA. Preparations of the specimens used in the present study are described in previous publications: ciliary axonemes and heliozoan axopods (11); mitotic spindles of mammalian cells (10, 12); and mitotic spindles of *Haemanthus endosperm* (15).

Each micrograph was scored to determine  ${}^iN_n^j$ , i.e., the number of microtubules bearing  $n$  hooks,  $i$  of which are clockwise and  $j$  of which are counter clockwise. Micrographs at  $\sim 50,000\times$  were scored by using a five-channel counter to record the number of microtubules bearing two hooks, three hooks, etc. When a tubule showing hooks of both curvatures was seen, a hash mark was placed in an appropriate bin on a score sheet. Each microtubule was marked on the print as it was scored to prevent double counting.  ${}^iN_n^j$ 's from several micrographs were then pooled in various ways and used to calculate the relevant  ${}^iF_n^j$ 's for different structures or regions of structures.

Usually, the pattern of hook decoration on a given microtubule allows its classification into one of the categories defined in Table I. On some microtubules, however, the hooks grow long enough to close on themselves or on another microtubule, generating an ambiguity about their direction of curvature. In other situations the hooks themselves grow hooks. Elsewhere, some hooks are too short to score with confidence. We adopted several conventions to deal with these and other cases. Fig. 2 shows the important ambiguities and what we did about them.

## RESULTS AND DISCUSSION

### Tests of the Model

Axopodia of the heliozoan *Actinosphaerium* are bundles of parallel microtubules (11). We have scored 4,133 hooks on 1,384 axopodial microtubules and found 4,110 (0.994) curving clockwise when the microtubules were viewed looking toward the cell center, suggesting a high dependability to the assay. The microtubules of these micrographs were classified to test the theory presented here. From the microtubules that could be classified by the criteria of Fig. 2, the numbers observed were:  ${}^0N_7^7 = 4$ ;  ${}^0N_6^6 = 14$ ;  ${}^0N_5^5 = 30$ ;  ${}^0N_4^4 = 56$ ;  ${}^4N_4^0 = 1$ ;  ${}^0N_3^3 = 91$ ;  ${}^0N_2^2 = 186$ ;  ${}^1N_2^1 = 2$ . Thus  ${}^1F_2^1 = 0.0106$  and  $p = 0.995$ , in fortuitously good agreement with the "correct" hook frequency, based on the assumption that axopodial microtubules are oriented with their plus ends distal to the cell body. If we assume that the value  $p = 0.995$  can be used to predict  ${}^1F_3^2 + {}^2F_3^1$ , the 91 three-hook microtubules predict  ${}^1N_3^2 + {}^2N_3^1 = 1.6$ , so the absence of microtubules in this class is not surprising.  ${}^2F_4^2 = 0.0002$ , so the absence of microtubules here is predicted, as is the absence of microtubules in most of the higher order mixed categories.

An essential assumption of the simple theory presented above is that  $p$  must be independent of the number of hooks already present. This assumption can be tested directly by comparing the values of  $p$  calculated from the frequencies,  ${}^iF_n^i$ , for each value of  $n$ , considering first microtubules with two hooks, then microtubules with three hooks, etc. One data set used for this study came from 20 micrographs of PtK cells lysed at metaphase. The sections were all cut within a few

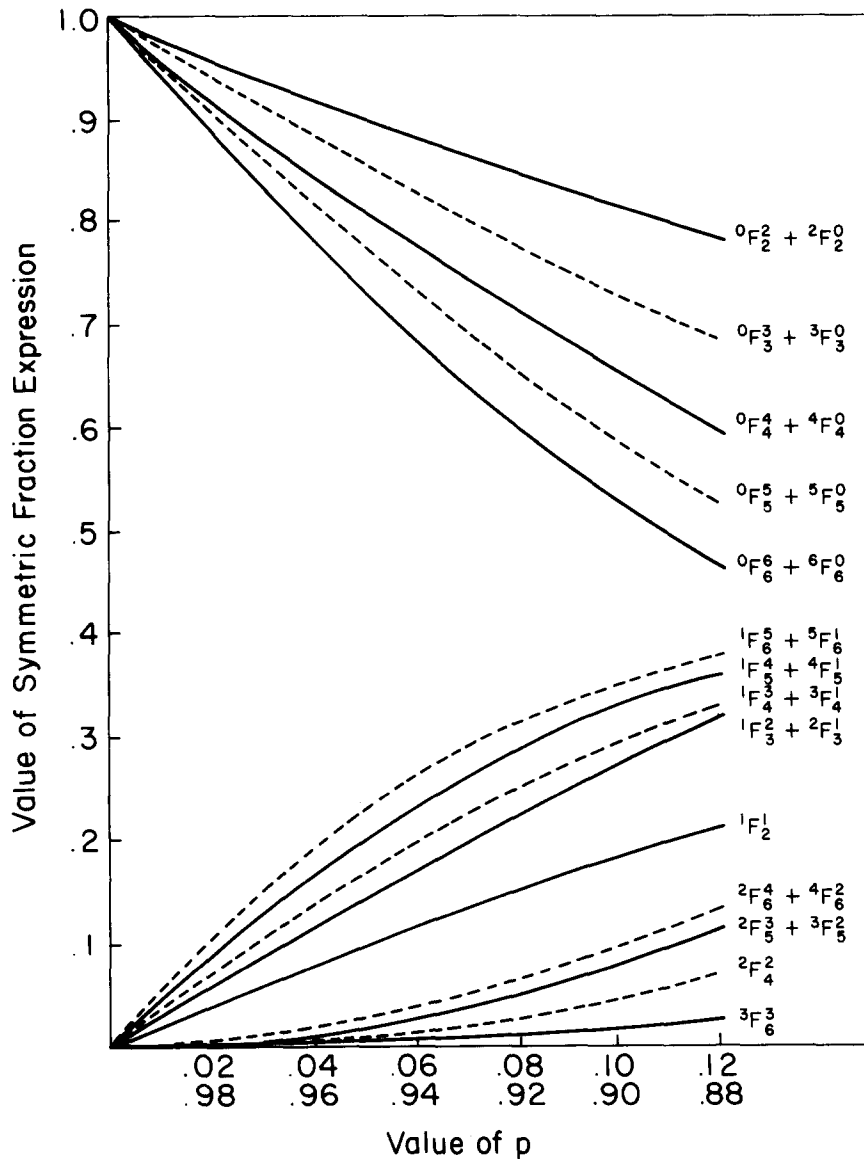


FIGURE 1 Some of the symmetric expressions defined in Table I graphed versus  $p$  for small and large values of  $p$ . These expressions are also symmetric with respect to  $p$ , so either value of  $p$  shown on the abscissa yields the same value for the fractions graphed. Since the initial slopes of the expressions vary widely, some expressions are more sensitive measures of  $p$  than others, but as will appear in the other figures, the frequencies of occurrence of the different morphologies vary too, so the utility of each fraction depends very much on the amount of data available to calculate it.

micrometers of a spindle pole, so the fraction of microtubules bearing clockwise-curving hooks (as seen by an observer looking toward the nearby pole) was 85–96%. A total of 1,931 microtubules was scored, and the  ${}^jF_n^i$ 's were calculated for each micrograph. Mean values and standard deviations of the symmetric fractions ( ${}^0F_2^2 + {}^2F_2^0$ ,  ${}^0F_3^3 + {}^3F_3^0$ , etc.) were determined by pooling data from all the micrographs, and the corresponding mean values of  $p$  for each number of hooks,  $p_n$ , were obtained. For up to three hooks per microtubule, the solutions were found analytically. For four, five, and six hooks, the  $p_n$ 's were found by linear interpolation from the tables used to construct Fig. 1. Standard deviations of  $p_n$  were estimated using conventional methods for following the propagation of errors ( $\sigma_p^{(dF/dp)} = \sigma_f$ ). The resulting values are  $p_2 = 0.979 \pm .031$ ,  $N_2 = 380$ ;  $p_3 = 0.986 \pm .021$ ,  $N_3 = 591$ ;  $p_4 = 0.983 \pm .013$ ,  $N_4 = 564$ ;  $p_5 = 0.989 \pm .022$ ,  $N_5 = 323$ ;  $p_6 = 0.994 \pm .017$ ,  $N_6 = 73$ . These means are obviously similar and show only slight variation with  $n$ . While a comparison of  $p_2$  with  $p_6$  by Student's  $t$  test shows a significant difference at the 99% confidence level, the small systematic variation in  $p_n$  with  $n$  and the fact that all the means are closer to one another than the smallest of the standard deviations suggest that the

apparent dependence of  $p_n$  on  $n$  may have little meaning. The weighted mean value of  $p$  for all these data is  $p = 0.984 \pm .021$ .

A similar study of 45 micrographs from eight anaphase PtK cells has produced similar results. 1,749 microtubules were scored in the neighborhood of the spindle poles where between 85 and 100% of the hooks observed curved one way. For these micrographs  $p_2 = 0.977 \pm .041$ ,  $N_2 = 611$ ;  $p_3 = .984 \pm .024$ ,  $N_3 = 597$ ;  $p_4 = 0.972 \pm .030$ ,  $N_4 = 349$ ;  $p_5 = .990 \pm .024$ ,  $N_5 = 153$ ;  $p_6 = 0.986 \pm .019$ ,  $N_6 = 39$ . Again, all the means are well within one standard deviation of one another, and there is no clear tendency in the variation of  $p_n$  with  $n$ . The mean of all these data, weighted by the frequency of each individual mean, is  $0.980 \pm .032$  which differs by less than one-fifth of one standard deviation from the metaphase mean. We have therefore combined the anaphase and metaphase data to obtain a larger set with which to examine the variation of  $p_n$  with  $n$ . The 3,680 microtubules summarized in Fig. 3 provide sufficiently representative means that one can see a small trend toward increasing  $p$  with increasing  $n$ . The slope of the regression line through these points is 1.003 with a correlation coefficient of 0.8159.

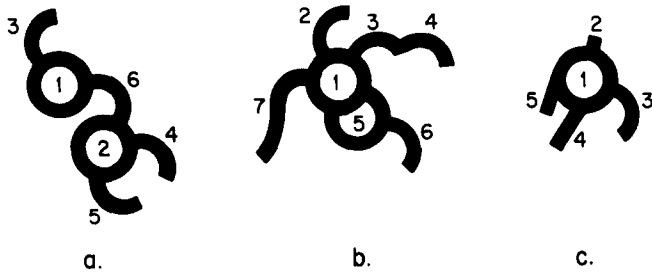


FIGURE 2 (a) An ambiguous structure. 1 and 2 are microtubules, 3-5 are clear hooks, but 6 is either a clockwise hook on 1 or a counter-clockwise hook on 2. In the first case, this would be one microtubule in the  ${}^0F_2^2$  category and one in the  ${}^1F_2^1$ ; in the second case it would be one in the  ${}^0F_1^1$  category and one in the  ${}^2F_3^1$ . Because of the ambiguity, this sort of image was not scored. (b) 1 is a microtubule, 2 and 3 are clockwise hooks. 4 is a hook on a hook and is not scored. 5 is a hook that has closed. It is of ambiguous direction of curvature and therefore is not scored. 6 is a hook on a hook and is not scored. 7 is a counter-clockwise arching hook which bends to become clockwise. Such hooks are scored on the basis of their direction of curvature at the microtubule surface. This structure is therefore in the  ${}^1F_3^2$  category. (c) 1 is a microtubule. 2 is a hook so short that its curvature is not visible, and hence it cannot be counted. 3-5 are hooks of different lengths and handedness which would be scored. This microtubule would therefore be in the  ${}^1F_3^2$  category.

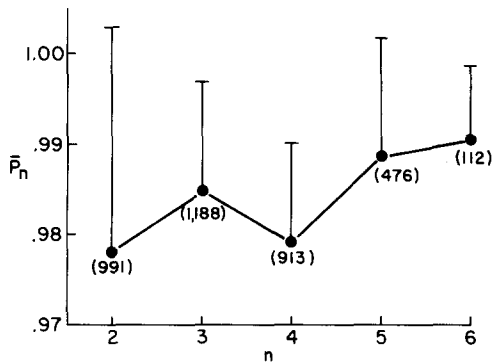


FIGURE 3 A graph of the average  $p$  for each number of hooks ( $\bar{p}_n$ ) vs. number of hooks ( $n$ ). The error bars shown above the means represent the standard deviations. The numbers in parentheses are the number of microtubules scored for each data point.

A different way to study this trend is to assume that the presence of an existing hook with a given curvature biases the tendency of the next hook to be clockwise or counterclockwise. This bias can be expressed by a parameter  $b$  defined in such a way that if  $p$  is the probability the first hook is clockwise,  $bp$  is the probability that the second one is also clockwise. Under these circumstances, if the first is counterclockwise, then  $(1 - p/b)$  is the probability that the second hook is also counterclockwise. Thus,

$${}^0F_2^2 = p^2bx + (1 - p)(1 - p/b)(1 - x)$$

$${}^2F_2^0 = (1 - p)(1 - p/b)x + p^2b(1 - x).$$

So  ${}^0F_2^2 + {}^2F_2^0 = p^2b + (1 - p)(1 - p/b)$

and  ${}^1F_2^1 = p\{(b + 1)/b - [(b^2 + 1)/b]p\}.$

Clearly these expressions reduce to the corresponding equations shown in Table I when  $b = 1$ . Similar expressions

constructed for microtubules with more hooks are cumbersome in detail, but when both  $p$  and  $b$  are as close to 1 as they are above, direct calculation shows that the expressions may be approximated to 0.01% as  ${}^0F_3^3 + {}^3F_3^0 = p^3b^2$ ,  ${}^0F_4^4 + {}^4F_4^0 = p^4b^3$ ,  ${}^0F_5^5 + {}^5F_5^0 = p^5b^4$ , and  ${}^0F_6^6 + {}^6F_6^0 = p^6b^5$ . If one assumes that (a) the  $p$  of the equations is independent of the number of hooks, (b)  $p$  is best determined from the  ${}^1F_2^1$  class, and (c) all the observed variation in  $p$  with  $n$  can be described by the "bias"  $b$ , then one can solve for  $b$  by comparing the  $({}^1F_n^1 + {}^1F_n^1)$  observed for each  $n$  with that calculated from the above expressions. The mean  $b$  so calculated for the 3,680 microtubules in the PtK sets above is 1.0095. If, on the other hand, one thinks that using just the two-hook data to estimate  $p$  is too restricting, and instead one uses the weighted mean value of  $p$  from all the data to calculate expected  ${}^1F_n^1$ 's and compares them with the observed  ${}^1F_n^1$ 's, then one obtains the best fit with  $b = 0.9991$ . The three approaches (linear regression and two ways of modeling a bias on  $p$ ) all suggest that the variation of  $p$  with  $n$  is  $<1\%$  per hook, and so we conclude that for practical purposes,  $p_n$  may be assumed to be independent of  $n$ .

Our model for the frequencies of hooks of different curvatures can be tested more extensively by determining a best value for  $p$  from a given data set, using that value to predict an "expected" frequency for each class of hook-decorated microtubules, and then comparing the expected frequencies with those observed using the Chi square test for goodness of fit. We have used the metaphase PtK cell data discussed above for the first study. Because the occupancy of all the classes involving mixed hooks is low, we have pooled these classes into a single category. The resulting analysis shows that values chosen by chance would be worse than those predictions from the model only  $\sim 80\%$  of the time. On the other hand, when micrographs whose hooks were particularly clear were selected from the PtK data and these were scored as a separate set, the Chi square test for goodness of fit showed that one would do worse by chance 95% of the time. We conclude that with the complex build-up of hooks that sometimes occurs (see Fig. 2), it is difficult to place each microtubule in the correct category. Probably the small departures from true transverse orientation contribute significantly to the difficulty in categorizing microtubules with hooks of both hands. Detailed use of the model to predict the frequencies of each class of decorated microtubule is thus warranted only with especially clear decoration. The use of the model to estimate  $p$ , on the other hand, is likely to be valid for most data sets because it is straightforward to distinguish  ${}^0F_n^n$  and  ${}^nF_n^0$  from the mixed categories. Since it is not always easy to know the particular mixed category to which an anomalous image belongs, determinations of  $p_n$  are best made with the  ${}^0F_n^n + {}^nF_n^0$  expressions. These include, in a single expression, information about the frequency of all the aberrant classes of hook decoration for a given value of  $n$ , because all the frequencies with a particular  $n$  must sum to 1. We have used this approach throughout the current paper, unless otherwise stated.

The symmetry of the mixed expressions shown in Table I predicts that the values of  $p$  should be independent of the orientation of the decorated microtubules used to make the measurements. We have tested this expectation directly by determining the  ${}^1F_n^1$ 's from micrographs of sections near the spindle equator where about half the microtubules are of each orientation. We have scored 1,255 microtubules on seven micrographs from the equatorial region of PtK metaphases.

The fraction of microtubules bearing hooks of one curvature on these sections varied from 51–66%. For these data,  $p_2 = 0.995 \pm .015$ ,  $N_2 = 250$ ;  $p_3 = 0.986 \pm .020$ ,  $N_3 = 351$ ;  $p_4 = 0.988 \pm .012$ ,  $N_4 = 338$ ;  $p_5 = 0.994 \pm .030$ ,  $N_5 = 234$ ;  $p_6 = 0.994 \pm .065$ ,  $N_6 = 82$ . The weighted mean for these numbers is  $0.990 \pm .025$ . While this mean value for  $p$  is slightly higher than the ones observed near the poles, the two metaphase means differ by less than one quarter of a standard deviation, and we conclude that the assay for the fidelity of hook curvature as a measure of polarity is indeed independent of microtubule orientation.

We have applied the assay of hook fidelity to micrographs of the spindle in *Haemaphysalis* endosperm to ask if the values of  $p$  observed differ substantially for spindles in different organisms. We have scored 2,129 microtubules on 31 micrographs and find  $p = 0.982 \pm .021$ , consistent with the mean values from PtK cells.

### The Utility of Known Values of $p$

The consistency of the values of  $p$  for mitotic microtubules in different circumstances suggests that  $p = 0.98$  is a good estimate of the fidelity of the hook decoration assay applied to spindles. Further,  $p$  is essentially independent of the number of hooks present. These observations lead to a useful property of the method. Most decorated microtubules bear more than one hook. If a microtubule has two hooks curving the same way, the probability of knowing the polarity of the microtubule correctly is  $1 - (1 - p)^2 = 0.9996$ . With three hooks going the same way, the probability is  $0.999992$  and so on with more hooks. If a microtubule falls in one of the mixed classes, the approximate independence of  $p$  on pre-existing hooks allows the use of a simple subtraction to work out the confidence with which the microtubule's polarity is known. For example, a microtubule in the  $^1F_4^3$  class has two more

hooks going clockwise than counter-clockwise. The microtubule's polarity is known to a confidence of 0.9996 which is sufficiently certain to classify its polarity for most purposes. In general, the probability  $P$  that a microtubule is oriented plus end toward the observer, given a particular decoration pattern, is  $P = 1 - (1 - p)^{i-j}$ , where  $i$  is the number of clockwise hooks,  $j$  is the number of counter-clockwise hooks, and  $p$  is  $\sim 0.98$ . The small increase in  $p$  with  $n$  described above would change this confidence only slightly.

### Application of the Model to Spindle Microtubule Polarity

**KINETOCHORE MICROTUBULES:** We have previously shown that the majority of microtubules between chromosomes and poles are oriented with their plus ends distal to the nearby pole (12, 15). An independent investigation using exogenous dynein as a probe for microtubule polarity suggests a similar conclusion (17). In light of the above treatment, we have reexamined our micrographs to see if the number of hooks curving so as to suggest microtubules oriented with their plus ends proximal to the poles is large enough to exceed the 2% error in the assay. For this test, the microtubules in seven PtK cells treated to form hooks were classified into five categories. A microtubule bearing two or more hooks curving clockwise when viewed looking toward the nearby pole was interpreted as a microtubule "certainly" oriented plus end distal to that pole, the orientation we have previously identified as predominant. Microtubules with one hook clockwise were classified as likely to be of the same orientation. An equal number of hooks in both directions of curvature marked a microtubule as ambiguous. Microtubules with one hook counter-clockwise were called likely antiparallel, and those with two or more hooks counter-clockwise were classed as "certain" antiparallel, i.e., plus end proximal to the pole.

TABLE II  
Classification of PtK Microtubules in the Space between Chromosomes and Poles

	Approx. distance from pole $\mu m$	"Certain" plus end distal	1 hook clockwise	Ambiguous	1 hook counter-clockwise	"Certain" plus end proximal
<u>Cells treated with cold</u>						
Metaphase	2.0	78	23	1	1	0
	2.5	140	46	3	2	2
Early anaphase	2.5	237	83	3	2	0
	3.0	248	105	2	2	1
	3.5	198	90	3	1	0
Mid anaphase	1.5	190	100	6	14	3
	2.5	216	140	6	10	2
	3.0	31	29	0	0	0
Mid anaphase	2.5	259	97	11	8	3
<u>Cells not treated with cold</u>						
Early anaphase	2.5	245	29	4	3	10
	3.5	250	32	4	3	9
	4.5	340	48	6	8	19
Mid anaphase	1.0	167	160	1	1	2
	1.5	271	127	5	4	7
	2.0	239	156	3	0	5
Late anaphase	1.5	158	89	5	4	0
	2.5	190	91	7	16	4
	3.5	105	51	5	8	2
	4.5	105	46	4	5	3

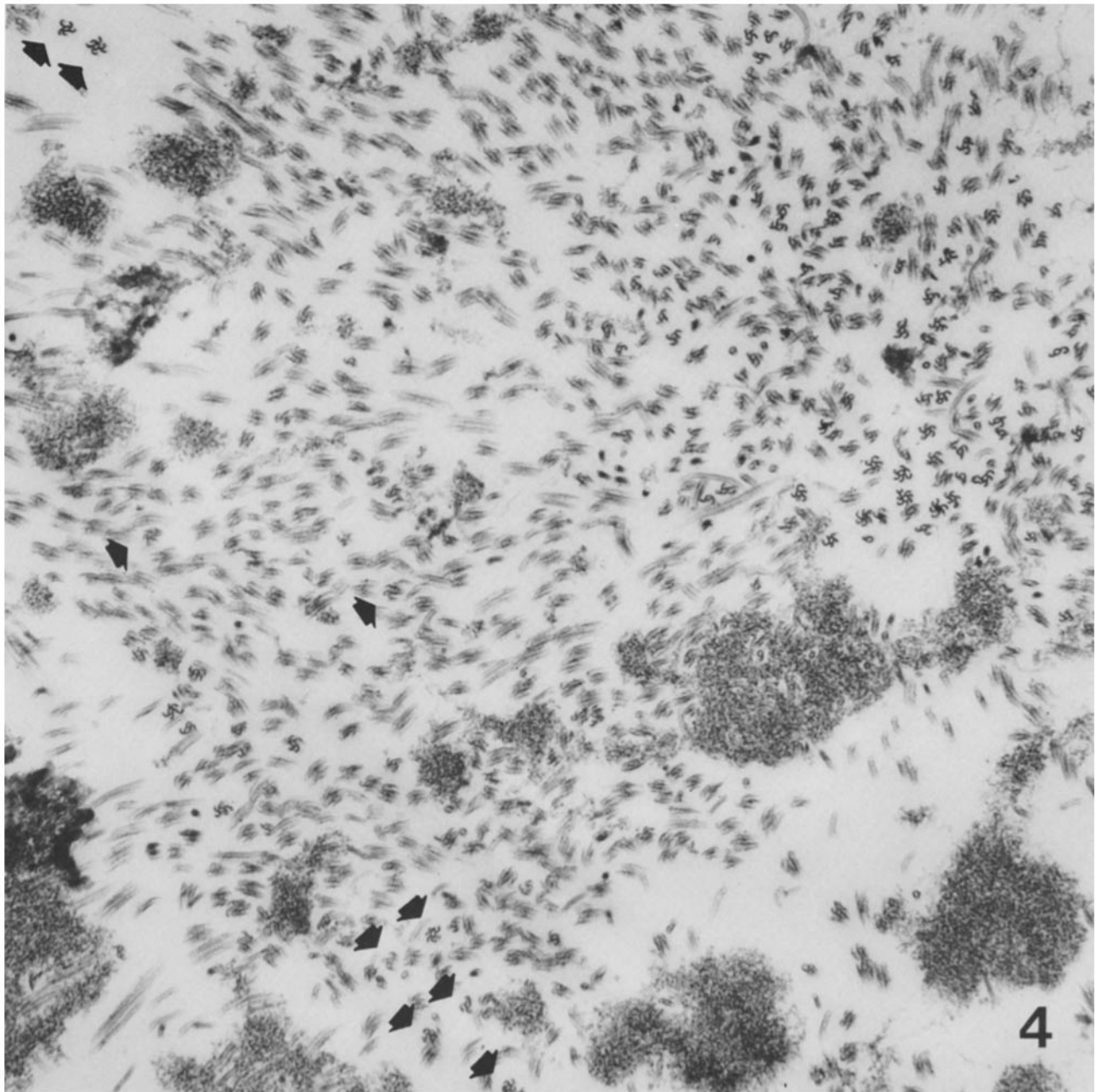


FIGURE 4 A PtK cell at early anaphase lysed without precooling. Most of the microtubules bear clockwise-curving hooks in this view looking toward the pole of a section between chromosomes at poles,  $\sim 2.7 \mu\text{m}$  from the pole. A few "certain antiparallel" microtubules have been marked with arrows. They all lie near the periphery of the spindle.  $\times 38,000$ .

Table II summarizes our observations on these micrographs.

Some of the cells were cooled before lysis in hook-forming buffer to dissolve the majority of the microtubules not in the kinetochore fiber bundles. We scored about 4,750 hooks on 2,439 microtubules in the cooled cells. A 2% error in hook orientation would mean that as many as 95 hooks might curve counter-clockwise. The total number of counter-clockwise hooks observed was 73, so there is no statistically significant evidence for antiparallel microtubules at the positions scored in the cold-treated cells (Table II). The presence of 11 microtubules in the "certain" category is, however, 10 more than expected by chance with a 2% error. (See Table I for the equations to estimate the frequency of the "certain" category of microtubule from a given value of  $p$ .) It appears that  $\sim 0.5\%$  of the microtubules in these cells are oriented antiparallel to the majority. For cells lysed into hook-forming buffer without

precooling, the situation is slightly different. We scored about 7,500 hooks on 3,076 microtubules. A 2% error in hook orientation would mean 150 counter-clockwise curving hooks. The number observed was 282, suggesting that some antiparallel microtubules exist in the half spindles of cells lysed without cooling. The significant rise in the number of microtubules in the "certain antiparallel" category is consistent with this numerical finding (Table II).

We have kept track of where the "certain" antiparallel microtubules are found in the cross-sections of the decorated PtK spindles to ask whether any of these lie among the kinetochore microtubules. We find that without exception they lie at the periphery of the spindle, distinctly removed from the bundles of microtubules that constitute the kinetochore bundles (Fig. 4). Our evidence is thus completely consistent with the idea that the microtubules of the kinetochore

bundle are all oriented with their plus ends at the kinetochore.

The bunching of microtubules into the kinetochore bundles in *Haemaphysalis* endosperm is more sensitive to our hook-forming lysis conditions than is the similar clustering in PtK cells. Nonetheless we have been able to score four kinetochore bundles from *Haemaphysalis* in the fashion of Table II. We find a total of 24 certain parallel (two hooks), 39 likely parallel (one hook) and two ambiguous (one hook of each curvature) in these bundles. No evidence for antiparallel microtubules is found.

**INTERZONAL MICROTUBULES:** In mid-to-late anaphase the microtubules of the interzone cluster into bundles. The hook decoration assay shows that as one approaches the spindle equator, the number of microtubules bearing hooks of clockwise curvature becomes approximately equal to the number bearing counter-clockwise hooks. For example, a section at the middle of a late anaphase showed 22, 14, 2, 17, and 20 microtubules in the five columns of Table II. More important, individual microtubule bundles are composed of fibers with both orientations, showing that the fibers of the interzone are formed by the co-mingling of microtubules from both poles.

**SPINDLES WITH MANY MALORIENTED MICROTUBULES:** Some metaphase half spindles contain an unusually high fraction of hooks with the less common or "wrong" direction of curvature (e.g., metaphase cells numbered 1 and 6 in Fig. 4 of reference 15). We have used the method described here to determine whether these cells have a lower value of  $p$ . We have scored 795 microtubules on five sections of a cell whose maximum percentage of "correctly" oriented microtubules in the half spindle examined was 80%. The resulting mean value of  $p$  is 0.988, showing that increased error in the assay is not the reason for the abnormally high

fraction of microtubules that appear to run the wrong way.

**SPINDLES WITH POOR HOOK DECORATION:** Sometimes a particular effort at hook decoration will go badly. When too few hooks form, increasing the concentration of DMSO, tubulin, or detergent will usually help, and longer incubation times before fixation can increase hook frequency. At other times the hooks are too numerous and long, forming honeycomb structures composed of multiple hooks that have closed (Fig. 5). We have analyzed images from such a decoration by relaxing our customary policy not to score hooks-upon-hooks. From microtubule-hook clusters where hooks could be scored, we have measured  $p = 0.96$ . Though this value is reasonably high, the scoring is much more difficult, and images such as those seen in Fig. 5 are to be avoided.

One cell, a cold-treated *Haemaphysalis* anaphase, gave anomalous results each time it was scored: each bundle of cold-stable microtubules contained significant numbers of hooks of both curvatures, unlike the majority of cells studied. A hook "fidelity" study of this particular cell gave a  $p = 0.926 \pm .031$  ( $N = 1002$ ). This is the only cell that has given such a low value of  $p$ . The reason for the anomaly is unknown, but it serves as a caution that  $p$  should be measured when hooks are to be used for determining microtubule polarity.

## SUMMARY AND CONCLUSIONS

This paper presents an analysis of the hook decoration method for determining microtubule polarity. A model is presented by which the analysis of microtubules bearing hooks of both curvatures (the certain evidence that hook curvature direction is not absolutely correlated with microtubule polarity) can be used to measure the actual fidelity of the assay. The assumptions of the model are tested and found valid to a reasonable

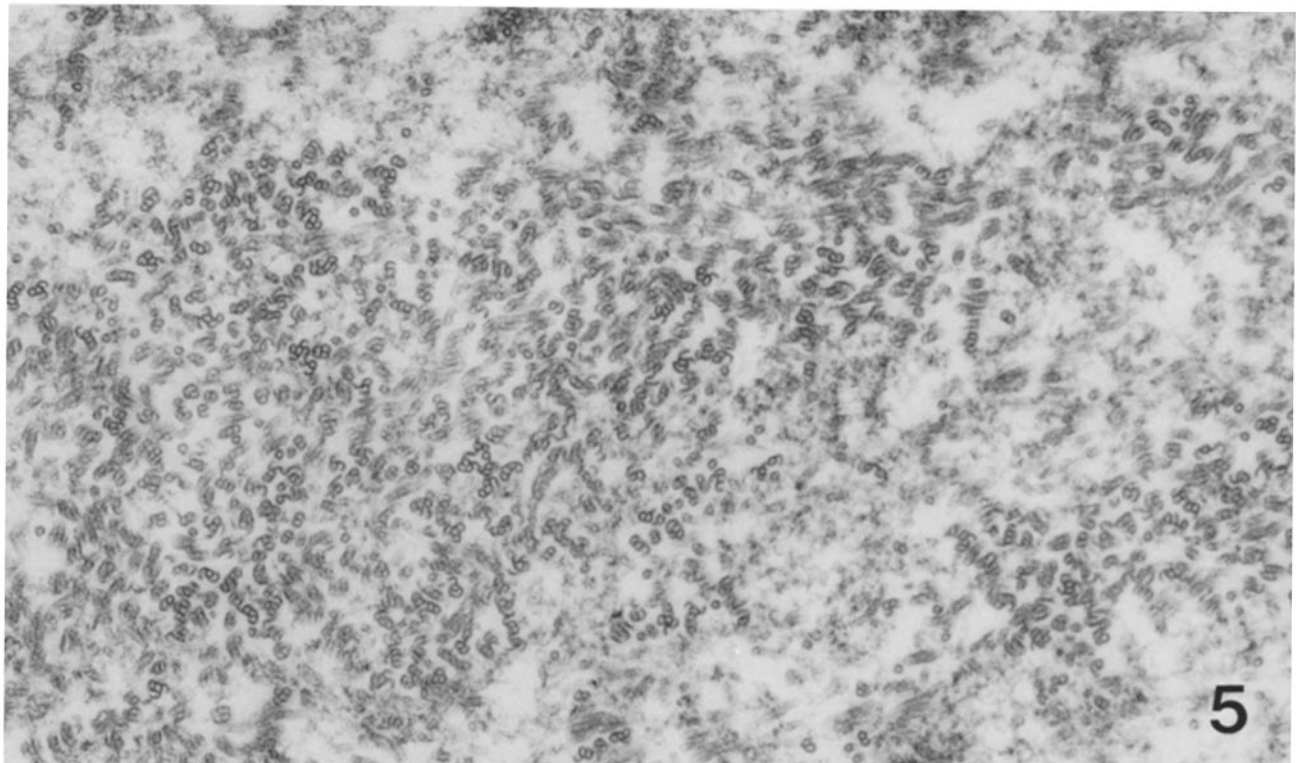


FIGURE 5 A poorly decorated *Haemaphysalis* anaphase cell which shows the problems characteristic of excessive hook formation.  $\times 41,000$ .



accuracy. Thus a technique is now in hand to assess the correlation between the direction of hook curvature and microtubule polarity in any system of interest without foreknowledge of the microtubule polarity in the array under study.

Application of this method to the axopodia of a heliozoan shows an accuracy of  $\sim 0.994$ , while the average accuracy observed in spindles is 0.982. The encouraging feature of these observations is that the assay is rather good in both cases. However, it is clear from our data that the correlation of hook curvature with microtubule polarity varies, even within one cell type, and a serious use of the hook decoration method should include a determination of the parameter  $p$  for the system under study. It is not clear why  $p$  varies, but without question it does.

The approximate independence of  $p_n$  from  $n$  described here ( $b \approx 1$ ) is a useful feature of the hook decoration assay, because it opens the possibility of increasing the certainty of polarity assignment for those microtubules that bear multiple hooks. This new way of looking at hook-decorated microtubules has increased our confidence in the assertion that kinetochore microtubules are oriented with their plus ends at the kinetochores. However, we still cannot exclude the possibility that some microtubules of opposite orientation are present in vivo, since some tubules with their plus ends distal to the kinetochores might dissolve during the hook decoration lysis.

The significance of the few "certain" antiparallel microtubules in the region between chromosomes and poles is presently unknown. They may represent long nonkinetochore microtubules that penetrate far enough into the opposite half spindle to be scored near the pole, even in anaphase. The larger number of such tubules found in early anaphase compared with late anaphase, when the poles have moved farther apart, is consistent with this notion. On the other hand, they may reflect the population of microtubule fragments that inhabits the spindles of some higher organisms (18–20). Tippit et al. (21) have shown that microtubule fragments may tentatively be assigned a polarity based on the known polar associations of microtubules that are their nearest neighbors. Using this criterion, the polarity of fragments has been shown to have no correlation with fragment position along the spindle axis. Fragments of antiparallel orientation might therefore be expected to contribute a level of "polarity noise" to the overall bipolar symmetry of the spindle. Such noise might be expected to decrease during anaphase, because the number of fragments and short microtubules generally decreases at this time (21, 22).

The observation that a *Haemanthus* metaphase half spindle can contain as many as 20% microtubules oriented antiparallel to the majority (see Results and Discussion) suggests that spindles can function even when some microtubules are awry. We cannot know, of course, whether a particular cell would have segregated its chromosomes successfully, had it not been fixed during metaphase, but the frequency of nondisjunction in endosperm is not high. We expect, therefore, that the mechanism for mitosis is one that can tolerate a modest fraction of antiparallel microtubules.

The presence of microtubules of both polarities in the

interzone fibers of anaphase is hardly surprising in the light of the structure of the metaphase spindle. This observation serves, however, to support the idea that the interzone fibers of anaphase are the result of interdigitating microtubules from the half spindles, and that they are intermediates in the formation of the midbody.

In summary, this work provides a quantitative basis for estimating the confidence of the hook decoration assay for microtubule polarity. Analysis of hook data from several kinds of spindles confirms the simplicity of spindle design suggested earlier (10, 12, 15, 17). It remains to be seen how the cell uses this simplicity to achieve reliable chromosome movement.

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