of these latter words as employed in current usage explicitly describes the structure with which we are dealing.

4. Research career development awardee, USPHS, grant No. AI-K3-18,403. The isolation of the culture and preliminary observations were done while I was a visiting investigator at the Friday Harbor Laboratories, University of Washington.

5 February 1964

Morphology of Microtubules of Plant Cells

Abstract. *The microtubules present in the cortices of plant cells are found to have a wall made up of slender filamentous subunits, probably 13 in number, which have a center-to-center spacing of about 45 angstrom units. Thus the micromorphology of these tubules is similar to that reported for the 9 + 2 fibrils of rat sperm flagella. This structure can be used to account for a primary function of motion attributable to the microtubules.*

In a recent paper we reported the existence of microtubular elements in the cytoplasm of cells of a few higher plants (*1*). These structures as seen in thin sections by electron microscopy have an outside diameter of 230 to 270 Å and are especially prominent in the cortices of meristematic cells (that portion subjacent to the plasma membrane) during interphase (Fig. 1). They are similar in size and appearance to the so-called fibrils common in the mitotic spindle and also resemble the filaments or fibrils (or microtubules) which make up the 9 + 2 complex of flagella and cilia. In the same report we mentioned several other locations where apparently homologous structures are found and suggested that microtubules thus defined are cytoplasmic structures of wide occurrence in the cells of both plants and animals. In order to provide further support for such a generalization, we have now sought to characterize them in greater structural detail.

It was noted in the earlier report that the limiting dense wall of the plant cell microtubule seems to be made up of subunits which appear circular in transverse section. The evidence, however, was admittedly poor, and whether this substructure existed or not remained a question which became especially important when, within the same year (1963), Pease (*2*) and André and Thiéry (*3*) reported that the "fibrils" or microtubules of the 9 + 2 complex are constructed of 10 or 11 filamentous subunits, bundled together to form the tubular structure. Here it seemed was a detail of fine structure that could be used to define these and other cytoplasmic tubules of this size as probably homologous and as possessing similar functions.

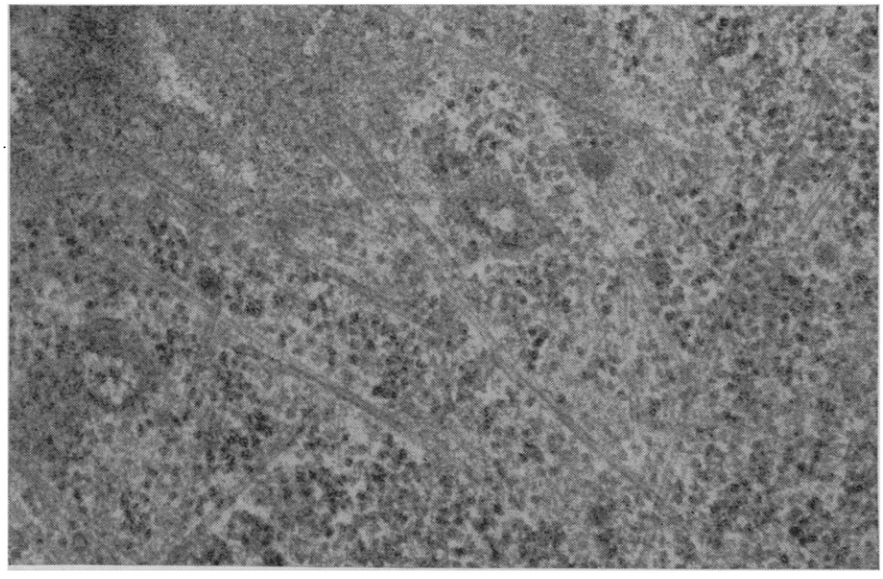

Fig. 1. Electron micrograph of section cut nearly parallel to and including a portion of the end wall (gray area at upper left) and adjacent cell cortex from root tip of *Phleum pratense*. The long, slender microtubules, unlike those found in parallel array along the side walls, here show an apparently random orientation within the cell cortex adjacent to the end wall (× 64,000).

For a further investigation of the problem it was found that certain meristematic cells in the root tips of *Juniperus chinensis* L. and cells from the nectaries of *Euphorbia Milii*, Ch. de Moulins, offered some advantages over materials previously used. Thin sections of these cells were prepared by techniques described earlier (*1*). These were stained only lightly for 10 minutes in uranyl acetate (*4*), washed briefly and then placed in basic lead citrate for 5 minutes (*5*). Longer periods of staining obscured some of the fine structure.

A single microtubule as it appears in this material is shown in cross section in Fig. 2. It is circular in form and about 230 Å in diameter, and has the characteristic "hollow" or low-density center. The wall, which is about 70 Å thick, is apparently constructed of a number of subunits also circular in outline. The center-to-center spacing of these subunits is about 45 Å. This structure, and most especially the clearly demonstrated subunits, have been encountered so far only in particular cells of *Juniperus* and *Euphorbia* which display after staining an overall greater density in many components of the cytoplasm. It appears that some substance in the protein-containing structures of these dark-staining cells greatly increases their affinity for uranyl and lead salts and therefore increases their density as seen in the electron microscope. The ribosomes, for instance, are extraordinarily prominent and the cytoplasmic membranes in which the three-layered structure is usually hard to resolve here show this structure with outstanding clarity. This unusual staining extends as well to the wall of the microtubules and introduces a problem of interpretation. We decided tentatively that the distinctly clear halo, which was noted earlier (*1*) as surrounding the microtubule, had in these cells stained to give the darker, more prominent image of the tubule. The effect of this, according to the interpretation, is to provide a natural "negative staining" of the structural elements in the wall, elements which now appear as circles of low density.

To test this interpretation we have compared images of transverse sections of ordinarily and "negatively" stained tubules as encountered in light- and dark-staining cells of *Euphorbia*, separated by only a cell wall. The dimensions and relative densities of these

two types of tubule images were measured from tracings made with an automatic microdensitometer. In all, six images of each of the two types selected for clarity and roundness were compared. From these tracings it was found that the outer limit of the circle of low-density units of the "negatively" stained image coincides in diameter (at about 230 Å) to the outside diameter of the microtubule as seen in the ordinary image. Further, it was noted that the density of the ring of subunits in the "negatively" stained image reaches a minimum at a diameter of 180 Å. However, this minimum density in the "negatively" stained images was in all cases greater than the maximum density of the wall in the ordinary image, supporting the idea that the subunits are visible as they are in the darker image only because of a negative staining effect provided by the dense staining of the surrounding substance.

It is, however, unusual with this material, even under the conditions of natural negative staining, to obtain micrographs in which the units are clearly defined around the entire circumference of the microtubule. This fact makes the determination of the exact number of units involved very difficult. The best that can be said is that the tubules are radially symmetrical with the subunits of structure regularly spaced within the wall. Fortunately, this is the type of structure which lends itself to analysis by Markham's rotation method (*4*), and images of transverse sections of four microtubules from *Juniperus* and eight from *Euphorbia* were therefore selected for this analysis on the basis of roundness and evenness of density around the wall.

Figure 3*a* shows one of the microtubules from *Juniperus* used for the Markham test. The pictures from this test are shown in Figs. 3*b*, 3*c*, and 3*d* and represent, respectively, instances where $n = 12$, 13, or 14 equal arcs of a complete circle used in making multiple exposures of the image in Fig. 3*a*. In this and nine other microtubules tested for $n = 10$ through $n = 16$, the strongest reinforcement of the ordered pattern occurred when $n = 13$. Although two of the *Euphorbia* microtubules showed the clearest pattern at $n = 12$, considerable reinforcement at $n = 11$ and 13 indicated these images were more irregular in density than the others. One can conclude, then, that either there are 13 subunits in the wall of the tubule, or the spacing between units is at an average of 1/13 of the circumference, with vacant spaces intervening between some of the subunits. Nothing is known of the constancy of the number of units among various species since we have been able to examine the microtubules of only two plants in this way. However, the fact that we have found the identical number of units in two such diverse species (one gymnosperm and one dicotyledonous angiosperm) suggests that this is a consistent feature of microtubule structure, at least in higher plants. Further, the size range of microtubules is rather narrow among the few species studied (*1*) and so only small deviations from the 13 units found here would be expected.

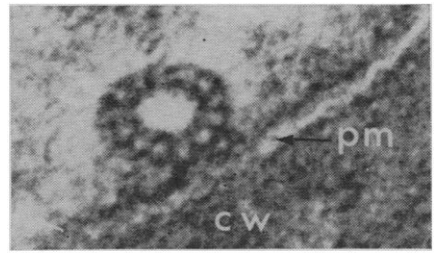

Fig. 2. Transverse section of microtubule from cortex of *Juniperus chinensis* root-tip cell, showing circular subunits which compose the wall. (pm, plasma membrane; cw, cell wall). Electron micrograph, × 740,000.

As already mentioned, the images of the microtubule show one side as made of distinct and equally formed parts while the other side is frequently indistinct. This suggests that the tubule may be slightly twisted in the depth of a section with the clear side having elements exactly normal to the plane of sectioning while on the other side they are slightly askew. This raised the question of whether the rotation method would be able to determine the correct number of units in such an arrangement. Therefore, a model of this type was constructed and tested, and despite the indistinctness of some of the subunits, the correct number of units was clearly discerned by the method used, although it was difficult to determine the number in the model by inspection. Regardless of the exact number of units involved, it is plain that such longitudinal elements exist, and that they must be quite straight in order to be seen at all in sections 50 to 100 mμ thick.

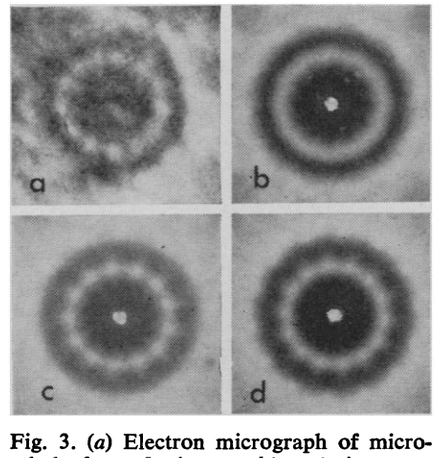

Fig. 3. (*a*) Electron micrograph of microtubule from *Juniperus chinensis* in transverse section used in multiple exposures where (*b*) $n = 12$, (*c*) $n = 13$, (*d*) $n = 14$ for n equal arcs of a circle. The greatest reinforcement of the image of repeating units is obvious in (*c*) (× 740,000).

The probable relationship of the plant cell microtubules to the "fibers" or "filaments" of cilia and flagella was mentioned earlier (*1*). It has recently been shown that the flagellar fibrils of rat and human sperm tails are composed of longitudinal filaments which are spaced 55 to 60 Å center-to-center (*2*, *3*). The fact that the units within the plant microtubules reported here compare favorably with those of the tail fibrils of rat sperm lends strength to the proposed homology between microtubules from these diverse sources. The significance of the differences in number 13 here, and 10 or 11 in cilia, is not at once evident.

From what is known of the distribution of microtubules so far, it seems that they are primarily associated with portions of the cytoplasm which move. This is evident in flagella and the mitotic "spindle fibers." In the cortex of the meristematic plant cell the microtubules are favorably disposed for participation in cyclosis. The functional advantage of a tubule made of longitudinal units is obvious. If the microtubules are to bend in order to set up an undulatory motion, the required bending could result from the differential shortening or elongation of the longitudinal elements. The same construction could reasonably be supposed to endow them with enough rigidity and elasticity to maintain a single direction over relatively long expanses of intracellular space.

We have observed that where the microtubules are quite close to the

plasma membrane, there is a connecting substance between them and the inner dense layer of the membrane. This point of attachment may anchor a portion of the microtubule so that any undulations or other displacement forces along its length would induce a streaming motion within the surrounding ground substance of the cytoplasm.

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17 February 1964

Tetrasporic Embryo-Sac Formation in Trisomic Sectors of Maize

Abstract. *Nondisjunction in mitotic divisions occurs spontaneously at a low frequency in somatic and germinal tissue in maize and results in sectors of trisomic cells. When this happens with chromosome 3 and in germinal tissue the embryo sac development is changed from the normal monosporic type to a tetrasporic type which is common in some species but not in maize.*

Under normal conditions the embryo sac in maize develops with eight nuclei from three divisions of a single basal megaspore. The position of these nuclei is such that two polar nuclei join to produce a $2n$ fusion nucleus, one becomes the egg nucleus, and the other five become the antipodal and synergid nuclei. Under these conditions and with certain rare exceptions the endosperm and embryo of a developing seed have the same genes.

In an experiment designed to test the effect of crossing-over on mutable loci, an apparent deviation from this scheme was observed. To determine the nature of factors taking part in mutable behavior at the A_1 locus on chromosome 3, a heterozygote composed of two distinguishable mutable alleles but lacking their respective mutators was prepared. One segment (α-*a-sh*) carried α (dilute aleurone), *a* (*Dt* responding, colorless aleurone), and sh_2 (shrunken endosperm) all within a map distance of 0.25 crossover units. The segment (a^m-*Sh*) carried either a^m-*1* (*Dt* responding), a^{m-3} (*Ac* responding), a^{m-4} (*Ac* responding), or a^s (nonresponding), depending on the experiment and Sh_2. The heterozygote was crossed by a recessive male stock (a^s-*sh*, *Dt*).

Progeny from this cross included the expected parental types and a variety of crossovers depending on the particular allele in the a^m-*Sh* segment (*1*). Among the α-a^m-*Sh* and α-*a*-*Sh* crossovers were some which on test had noncorresponding embryos of three types, namely, trisomic, (α-*a*-*sh*/a^m-*Sh*/a^s-*sh*) and both types of parentals (α-*a*-*sh*/a^s-*sh* or a^m-*Sh*/a^s-*sh*). The cultures carrying the alleles a^m-*1*, a^{m-4} and a^s all had a considerable number of both crossover and noncrossover cases but in the heterozygote carrying a^{m-3} all of the apparent crossover cases of the α-a^m-*Sh* and α-*a*-*Sh* types turned out to be noncorresponding cases.

The data (Table 1) clearly show these differences and also reveal that while the frequency of crossovers varies between alleles the frequency of noncrossover embryos of all three types is surprisingly constant (compare a^m-*1* and a^{m-3}, Table 1). Of further significance is the fact that several of the parent ears in this experiment had groups of apparent crossover seeds which on test were found to be noncorresponding and to include one or more of the embryo types already mentioned. A good example of this is presented in Fig. 1 where the position of the cases on the ear and the condition of the embryos for each case is indicated. The proximity of the α-*a*-*Sh* seeds and the low frequency of single occurrence of such cases in the rest of the population make it almost certain that all five have a related origin, an origin which precedes meiosis.

In seeking a reasonable explanation for the noncorresponding cases the expression of both chromosomes of the parent heterozygote in the endosperms, the occasional occurrence of these cases in groups or sectors, and the fact of noncorrespondence between endosperm and embryo in a large portion of these cases must be accounted for.

The first of these is fairly easy to explain as being due to nondisjunction with the production of an endosperm having both the α and a^m chromosomes. The occurrence of some of these in sectors (Fig. 1) is accounted for if we assume that nondisjunction may occur at various mitotic divisions including those preceding meiosis. Accounting for the third fact is more difficult. If nondisjunction has provided the megasporocyte with an extra chromosome thereby allowing (n + 1) nuclei carrying α and a^m as meiotic products, normal development of the megaspore into an eight-nucleate embryo sac will provide polar nuclei with the extra chromosomes which will give the observed type of endosperms. However, the egg from such development should also have an extra chromosome and therefore should generally produce a trisomic embryo. Since only one-third of the embryos are trisomic some other explanation is needed.

Additional nondisjunction in the three megaspore divisions could provide noncorrespondence. Unequal distribution of the extra chromosome (α or a^m) at the first megaspore division would provide one polar nucleus with two extra chromosomes (α α or a^m a^m) and the other polar nucleus with a normal complement. The fusion nucleus would be composed of one of each, and would be either α, α a^m a^m, or α α a^m, a^m; both would give the observed endosperm phenotype. The egg nuclei would be either α a^m a^m, α α a^m, α, or a^m. Fertilization of these would produce two tetrasomics; one α parental and one a^m parental. Nondisjunction at the sec-

Table 1. Distribution of all the dilute nonshrunken cases from the heterozygote α-*a*-*sh*/a^m-*Sh* pollinated by a^s-*sh*.

Allele	Seeds examined (No.)	Crossovers			Noncrossovers			Lost
						Parentals		
		α a^m *Sh*	α *a Sh*	α-*Sh*	Trisomic	α *a sh*	a^m *Sh*	
a^m-*1*	312,057	20	35	61	13	11	15	96
a^{m-3}	131,448	0	0	5	10	5	8	4
a^{m-4}	40,501	0	5	6	3	5	5	2
a^s	307,090	0	43	68	22	19	14	121