A "MICROTUBULE" IN PLANT CELL FINE STRUCTURE

M. C. LEDBETTER, Ph.D., and K. R. PORTER, Ph.D.

From the Biological Laboratories, Harvard University, Cambridge, Massachusetts

This paper reports an electron microscope examination of the cortices of some plant cells engaged in wall formation. Previous studies of similar material fixed in OsO_4 alone have disclosed discontinuities in the plasma membrane and other evidence of inadequate fixation. After glutaraldehyde, used as a fixative in this present study, the general preservation of cortical fine structure is greatly improved. This is shown, for example, by the first evidence of slender tubules, 230 to 270 A in diameter and of indeterminate length, in plant cells of this type. They have been found in the cortical regions of cells of two angiosperms and one gymnosperm, representing all the material so far studied following this method of fixation. The tubules are identical in morphology to those also observed here in the mitotic spindles of plant cells, except that the latter have a somewhat smaller diameter. It is noted that the cortical tubules are in a favored position to govern cytoplasmic streaming and to exert an influence over the disposition of cell wall materials. In this regard it may be of some significance that the tubules just beneath the surface of the protoplast mirror the orientation of the cellulose microfibrils of the adjacent cell walls.

INTRODUCTION

The relation of cytoplasmic organization to wall formation in plant cells is a problem of long standing interest for botanists and students of cell differentiation. One asks whether in the cytoplasm there is a visible organization of any components which anticipates and influences the deposition of specific patterns of thickenings in the secondary walls of plant cells? The question was examined some years ago by Sinnott and Bloch (23) in a study of regenerating vascular elements in internodes of *Coleus*. They noted, in primordial vascular cells, the development of reticulate patterns of granular cytoplasm over which, in subsequent stages, lignified bands of wall material appeared. Evidently the strands represented thicker bands of cytoplasm containing mitochondria, dictyosomes, and possibly unusual concentrations of ribosomes and elements of the endoplasmic reticulum (ER). Observations similar to those of Sinnott and Bloch were made nearly a century earlier by Cruger (1855) (3). The subsequent deposition of cellulose over those parts of the peripheral cytoplasm containing the richest collections of organelles is perhaps not surprising. But it remains to discover what elements of the cell are involved in determining these initial cytoplasmic patterns—*i.e.*, in interpreting and transmitting genetic information bearing on wall patterns, on cell polarity, and on mitotic asymmetries (1, 24).

In a preliminary investigation of certain of these questions in plant cells a few years ago, it was observed that elements of the endoplasmic reticulum were disposed in a definable relationship to the developing band pattern in the walls of primitive tracheids (14, 16). Whether this reflected a significant primary patterning in the ER or was a secondary accompaniment of wall formation was not indicated. However, the picture of the ER as a determiner or primary initiator of

cell form and such patterns as those in cell walls has always been marred by the apparent lability of the system—even though it is obviously patterned—and by the fact that several cells develop and form asymmetries of impressive complexity with a very meagre ER, and even that apparently discontinuous.

More recently, in a continuing examination of the cortices of plant cells engaged in wall formation, we have tried new combinations of fixing reagents featuring aldehydes and, more specifically, glutaraldehyde (20). Contrary to earlier results with OsO_4 alone, where the plasma membrane and adjacent cortical substances usually showed discontinuities, the results with glutaraldehyde followed by OsO_4 were impressive in the wealth of materials retained. These included some tubular units of minute dimensions, which heretofore had been seen in similar locations only in animal cells and certain naked plant cells, and which, in this instance, are featured in the cell cortex and in the mitotic spindle. What follows is a brief description of these structures.

MATERIALS AND METHODS

Root tips of *Phleum pratense* L. seedlings, whole plants of *Spirodela oligorrhiza* Kurtz, and root tips from cuttings of *Juniperus chinensis* L. were fixed for 1½ hours in 6 per cent glutaraldehyde, washed for 3 hours in 3 changes of phosphate buffer, and post-fixed in 2 per cent OsO_4 for 1 hour. The fixing and washing solutions contained 0.1 M Sorensen phosphate buffer at pH 6.8. The plant parts were dehydrated in a graded series of acetone: water mixtures and embedded in Epon by the method similar to Luft's (11).

Sections were cut with a diamond knife and stained, with uranyl acetate (26) and basic lead citrate (17), prior to examination in the electron microscope.

RESULTS

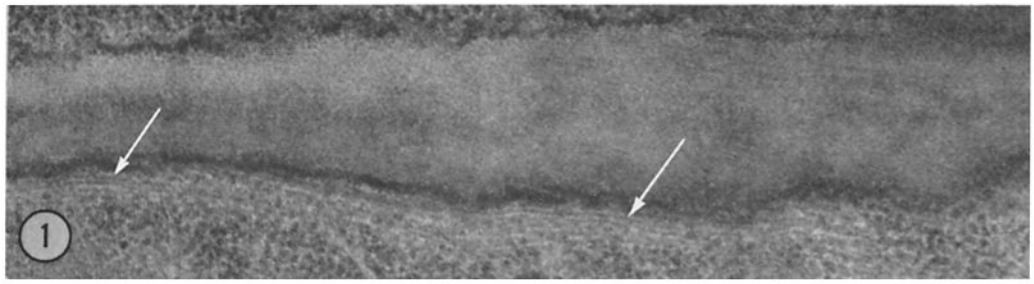
General Observations on Fixation

In most of its general features, the electron microscopic image of plant cells fixed in glutaraldehyde and OsO_4 is similar to that of cells fixed in OsO_4 alone or, even in some respects, to that of cells fixed in $KMnO_4$ (15, 27). The mitochondria show similar patterns of fine structure; the dictyosomes or Golgi components are stacks of laminate vesicles; the profiles of the ER have the long slender configurations observed after permanganate. It is in the finer and more subtle features of structure that the striking differences appear. The instant impression is that more of the cell's contents has been preserved. There are few or no discontinuities or vacant areas. The cytoplasmic ground substance apart from ribosomes is filled in with a finely divided material of low density. Ribosomes are not so markedly clumped as after $OsOs_4$ alone. Oddly, the membranes of the ER are difficult to discern and the limits of the cisternae are frequently more clearly defined by ribosomes than by anything else. It is especially peculiar, in this regard, that the plasma membrane is sharply depicted with the characteristic 3 layers, whereas, by comparison, the mitochondrial membranes are thin and faintly evident, and the ER membranes can hardly be seen. Finally, the image after glutaraldehyde + OsO_4 fixation differs from

FIGURE 1 Micrograph of a small segment of the cell wall and adjacent cytoplasmic cortex belonging to a meristematic root cell of *Phleum*. Microtubules marked by arrows can be seen subjacent to the cytoplasmic surface. They lie in the plane of this transverse section and so are circumferentially oriented around the lateral walls of the cell. × 45,000.

FIGURE 2 Part of the lateral wall (*cw*) of *Phleum* root cell as it appears in longitudinal section of the cell. The tubules appear in cross-section (end on) as circles with prominent limiting lines around centers of low density. In both cortices included in the micrograph, the circular profiles are within 0.1 to 0.2 μ of the protoplast surface. × 87,000.

FIGURE 3 This depicts part of a section which cuts longitudinally and tangentially through the wall (*cw*) and cortex of a *Phleum* cell. Dark regions in the image on the left and right represent grazing and oblique sections through two side walls (*cw*) of the cell. Microtubules in the cell cortex stay within the plane of section over distances of 2 to 3 microns. They are parallel in groups of 5 or 6 units, and all bundles are roughly circumferential to the cell's long axis, like hoops around a barrel, although a few diverge from this orientation at small angles. Ribosomes in large numbers fill the ground substance between the bundles of tubules. A vesicle of the endoplasmic reticulum is identified at (*er*). × 45,000.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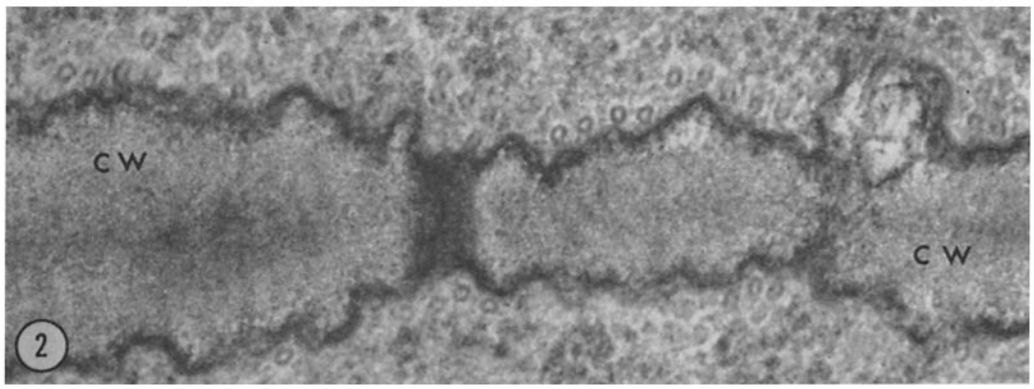
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others in showing large numbers of thin but uniform filaments which we shall refer to as "tubules," "microtubules," or "cytotubules."

Character and Distribution of "Cytotubules" in the Interphase Cell

The elements referred to under this name are 230 to 270 A in diameter and of undetermined length. Some have been traced for several microns in a single section, and it was not evident that the ends were contained in the section. In longitudinal section they appear as two parallel lines, sharply defined on the outer side but grading off into a lighter grey zone between the lines (Fig. 4). In cross-section they appear as hollow cylinders or tubules, limited by a dense wall about 70 A thick (Figs. 2 and 7). The center or lumen of the tubules is about 100 A in diameter and is devoid of content after these methods of preparation.

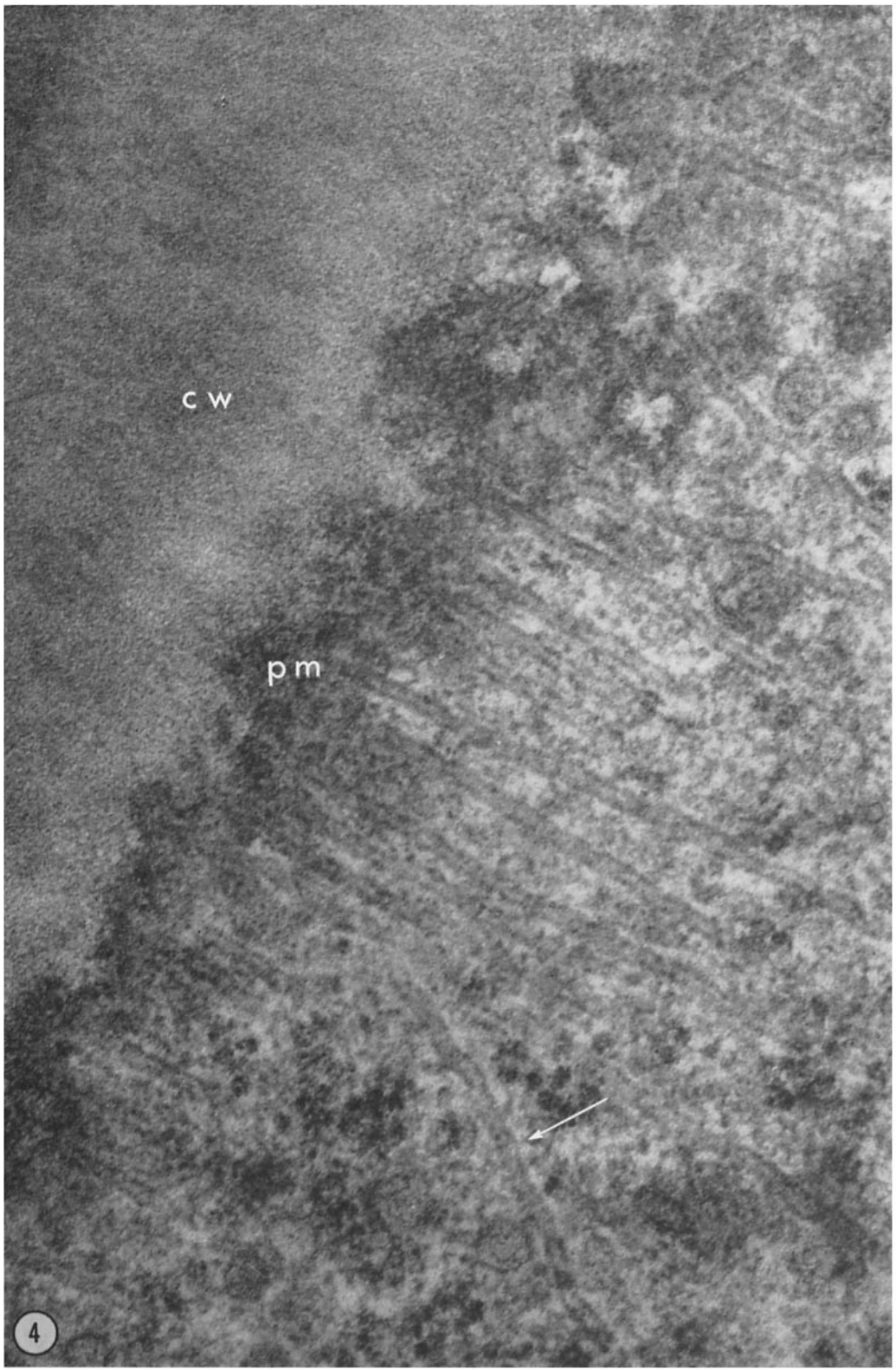
In the material examined, structures of this type have been found in greatest numbers within that portion of the cell cortex subjacent to the plasma membrane (Figs. 1 to 4). Here they may appear lined up in parallel array. The center-to-center distance between adjacent tubules is never less than 350 A, though it may be much greater than this. The space separating the plasma membrane from the nearest tubule is difficult to determine because of variable orientation of section to plane of wall and consequent overlap, but, where the section is essentially normal to the protoplast surface, the tubules always seem to be separated from the plasma membrane by a distance which is not less than half the distance between adjacent tubules (175 to 200 A). The implication of these facts is that each tubule is surrounded by a zone or layer of material from which all major particulate and membranous elements of the cytopasm are excluded. Such zones can be seen around the tubules depicted in cross-section in Figs. 2 and 7.

Though these structures are most numerous just within the protoplasmic surface, they are not confined to this part of the cell cortex. Cross, and oblique sections of them have been identified at distances as great as 1.0 μ from the plasma membrane. Whether, in these interphase cells, they are present in the central regions of the cytoplasm has not been demonstrated, but it is expected that they will be found when a further search is made. If present there in relatively smaller numbers and randomly oriented, their identification would be difficult.

The organization and distribution of these tubules within the cortex require further comment. First of all, they lie parallel to the cell surface (Figs. 1, 3, 4, and 8). Along the side walls of the cell they are oriented circumferentially, *i.e.*, in planes normal to the long axis of the cell and parallel to one another. Along the end walls, where they are less numerous, they are parallel to the surface but otherwise randomly oriented, occasionally in bundles of 3 or 4 parallel tubules. In both situations, their disposition mirrors the orientation of cellulose fibrils in the secondary walls on the other side of the plasma membrane (Fig. 8). Observations made to date have shown as many as 3 layers of tubules in compact array beneath the cell membrane. The deeper tubules are, as a rule, not so clearly organized into parallel arrays. Except for the clear affinity these structures have for the cell surface, they show no striking structural relation to any other organelle or system in the cytoplasm of the interphase cell. Cisternae of the ER frequently parallel the surface and, as noted in an earlier study (16), show some reticulation in the cortical zone of the cytoplast. But the tubules here described are closer to the surface and essentially interposed between the ER and the surface. After glutaraldehyde fixation, free ribosomes seem very numerous in these meristematic cells and they crowd into the cortical zone and into the regions inhabited by the tubules. The disposition of the two might suggest an interrelation, but clear cut structural associations have not been established.

Evidence was presented earlier in support of the hypothesis that in wall production portions of the cell cortex are contributed essentially intact by the development of a new membrane behind the old one (14, 16). Illustrations of this process have

FIGURE 4 This shows at higher magnification a small part of the wall (*cw*) and cortex of a *Phleum* root cell. The section passes from the cell wall through the plasma membrane (*pm*) and into the cortical zone including numerous microtubules. For the most part these are parallel and are oriented circumferentially around the cell. A few, especially those deeper in the cell, are oriented at about a 30° angle to the others (arrow). × 128,000.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been seen only infrequently in this material. This may reflect the change in fixing reagents, or it may be that the process is, so to say, pulsatory and, therefore, found only infrequently in fixed material. It is probable, however, that any ecdysis of the protoplast surface would include these microtubules and call for their resynthesis and reorganization within the new cortex.

The limited scope of this study thus far has identified these tubular elements in meristematic interphase cells—or cells active in wall formation —in a few species only: namely, 2 Angiosperms, *Spirodela oligorrhiza* and *Phleum pratense*, and one Gymnosperm, *Juniperus chinensis*. It is anticipated that, with these procedures of preservation or with improvements later introduced, they will be found in all growing plant cells and especially where wall production is most active.

The "Tubules" in Dividing Plant Cells

Electron microscopy of animal cells in division has demonstrated in several instances the presence of fine "tubular" filaments (150 A in diameter) in the mitotic apparatus. These have been referred to as chromosomal filaments or fibrils, and as interzonal filaments. They have been assumed to play a role in chromosome movements (18, 8).

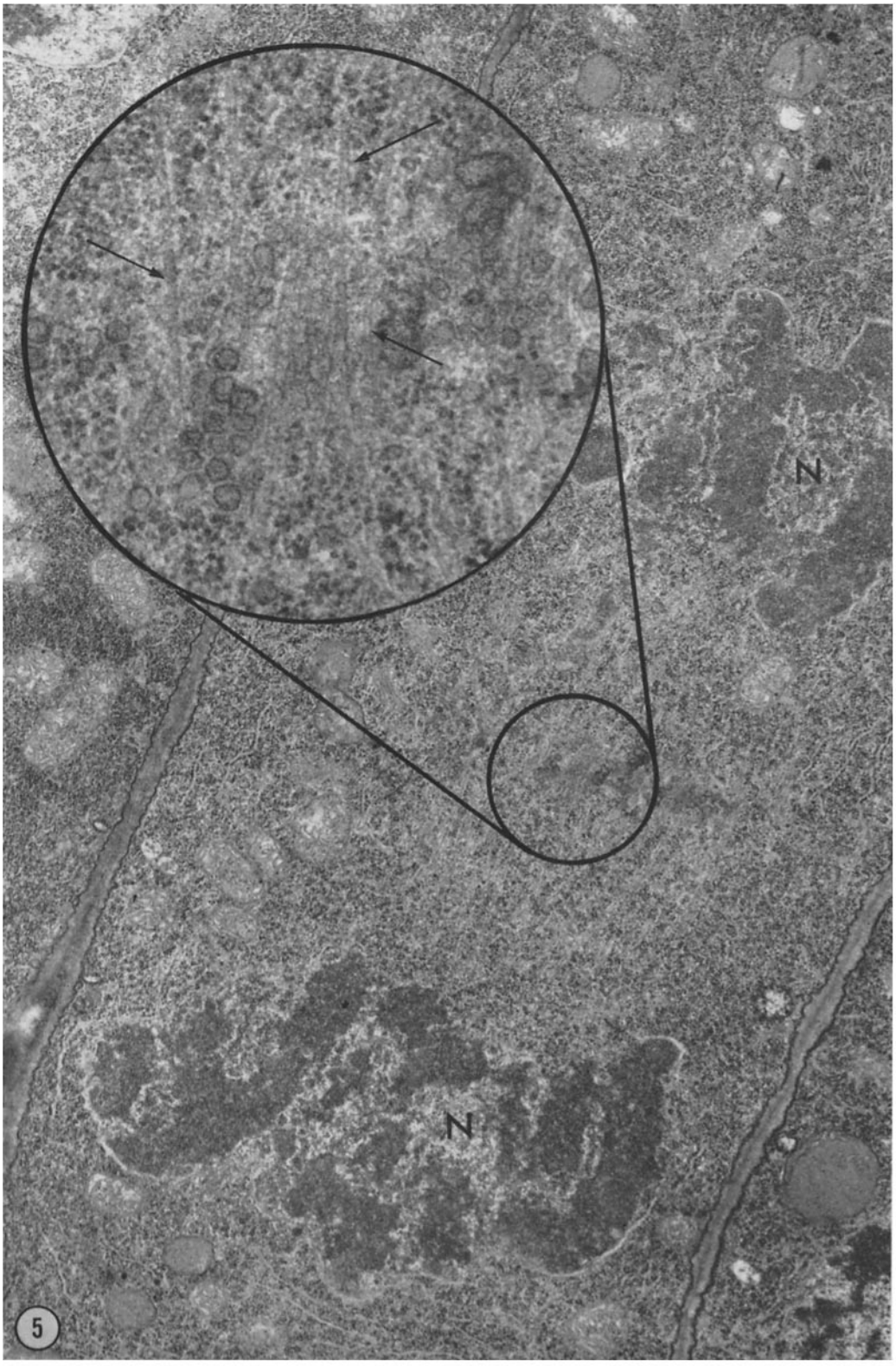
The same or similar fixations applied to plant cells, for some reason, have failed to preserve identical structures. Organizations of fibers and of fibrous material parallel to the long axis of the spindle have been noted (19, 22), but the fine structure image has been poorly resolved and fixation apparently inadequate. This latter is especially true after $KMnO_4$, which has failed to preserve anything except the membrane-limited components of the ER (19, 22).

After glutaraldehyde, the improvements in fixation, in terms of spindle elements and matrix materials in general, are impressive (Fig. 5). Cisternae of the ER and isolated organelles are more difficult to define than after permanganate or OsO_4, but smaller elements are shown more prominently. Particles of size and density resembling ribosomes are very numerous within the spindle. They are displaced only by large numbers of "tubules," and this displacement makes certain longitudinally oriented zones of the spindle appear less dense than the surrounding cytoplasm (Fig. 5). The "tubules" of the spindle have dimensions somewhat smaller than those which, in interphase, occupy the lateral cortices of the cell. For the most part, in the late anaphase spindle, they pack the interzonal regions and are oriented parallel to the long axis of the spindle (Fig. 5). At the level of the forming cell plate they intermingle with the pectin vesicles and elements of the ER, but retain at this stage an orientation normal to the plane of the plate. Some tubules within the section pass directly through the plate zone. And away from this zone they converge toward the telophase chromosomes. From a few counts of their numbers in thin sections, we estimate that there are more than 500 tubules in a single spindle. They appear in all parts but seem to thin out in number towards the spindle margins. In other parts of the *dividing* cell these "microtubules" are not encountered, even in the thin cortical layer subjacent to the plasma membrane which in the interphase cell is densely populated.

The Fine Structure of the "Microtubules"

In all materials examined, the tubules have shown a few features of fine structure which are worth recording. As mentioned earlier, they are 230 to 270 A in diameter in the cortex (200 A in the spindle) and of undetermined length. The wall of the tubules is about 70 A thick and the lumen is 100 A in diameter. The wall is not so uniformly dense as, for example, the lines in the adjacent unit membrane limiting the cell, and it does not show the 3-layered structure of the unit membrane. There is some evidence that it is made up of smaller filamentous units packed together to form the wall of the cylinder. Presumably, these "smaller units" which appear circular in cross-section represent the major macromolecular elements of which the tubules are constructed.

FIGURE 5 *Phleum* root tip cell in telophase of mitosis. The envelope is just forming around the daughter nuclei (*N*), and the phragmoplast and cell plate are evident at the spindle equator. Thin lines oriented normal to the cell plate representing the tubules, are apparent in the interzone of the spindle. They are shown at higher magnification in the insert (arrows). Long slender profiles of the endoplasmic reticulum occupy the cytoplasm peripheral to the spindle. × 7000. Insert, × 53,000.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DISCUSSION

These preparations of plant cells and the electron micrographs of them demonstrate the existence, in the cortices of interphase cells, of slender cylindrical units, uniform in size and structure. Their cross-sectional image shows them to have a center of low density and suggests that they are like small tubes. Even if the image were interpreted as describing a filament with a dense, perhaps osmiophilic or stainable, cortex, it would still be preferable to refer to them as tubules or microtubules in order to distinguish them from the several other filamentous and fibrous components of the cytoplasm. Thus far, these elements of fine structure have been observed in only two species of angiosperms and one of gymnosperms, which, however, is 100 per cent of all forms studied after glutaraldehyde + OsO_4 fixation. Thus, though the range of exploration is limited, the available observations make it seem probable that the tubules will be found quite generally in plant cells when the same or similar procedures are applied more broadly.

Evidence has also been presented to show that morphologically similar structures are present in large numbers in the mitotic spindles of cells of these same species. On the basis of size and structure there is reason to regard these tubules as essentially identical with those in the interphase cortex. In the spindle they are oriented parallel to its long axis or normal to the plane of the cell plate. Thus, they are disposed in such a way as to be responsible for, or at least related to, the birefringence characteristic of the spindle and the phragmoplast. Certainly, in the picture of spindle and plate fine structure that is currently available, they are the only well defined structures of a fibrous character whose distribution coincides precisely with that of the birefringence.

Accepting, then, the validity of this correlation, we have available for consideration a wealth of information on so-called spindle fibers, and on the physiology and biochemistry of the mitotic spindle. The tubules, for example, can be thought of as transient structures, at least in terms of location and organization. Whether they disappear as unit structures after mitosis is impossible to say. Since they are found in the cortices of interphase cells but not in the same regions of dividing cells, it may be that they migrate at the end of telophase from the spindle and phragmoplast locations to the lateral and end walls of the daughter cells. It is equally possible that they form anew in the cortex, just as they would appear to form in each mitotic spindle.

The distribution of the tubules in the interphase cell also takes on some interest when considered in relation to well known and important phenomena typically localized at or near the cell periphery. We have noted here that the tubules are disposed close to and parallel to the cell surface, and that around the lateral walls they are arranged circumferentially. They are, therefore, like hundreds of hoops around the cell. At, or just within, the end-

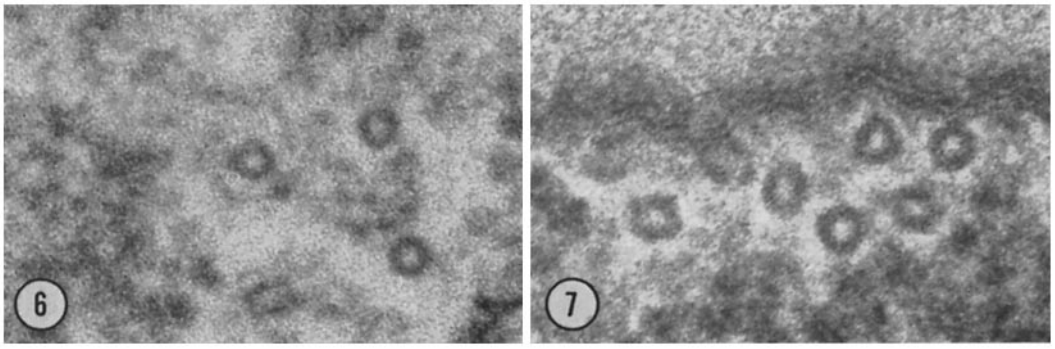

FIGURE 6 Cross-section through metaphase spindle of *Spirodela*, showing three tubules with an outside diameter of about 230 A. A small part of a chromosome is included at the left. × 240,000.

FIGURE 7 Cross-sections of tubules found in the cortex of a *Phleum* cell. They are about 270 A in diameter. It is evident that a halo of lower density material surrounds each tubule profile. × 240,000.

walls, their distribution is again close to and parallel to the surface, but their orientation is otherwise random. It will be obvious to students of cytoplasmic streaming in plant cells *a*) that the distribution of these tubules coincides with the plane, or thin peripheral zone, where streaming is most rapid, *i.e.*, in intimate proximity to the cell cortex; and (*b*) that the orientation of the long axes of the tubules parallels the direction of streaming. In the considered opinion of Kamiya (10), a displacement force is generated at the interphase between the endoplasm and the cortical gel layer which is responsible for the rotation of the whole endoplasm. This system is ATP-sensitive (21). In a more extended discussion of this correlation with streaming, further evidence and arguments could be produced to support it and to relate the cortical tubules to the development and orientation of this "displacement force." For present purposes, we would propose that this system of tubules be thought of as an elastic framework in the cortex of the plant cell, and in the spindle, along which (constantly or at certain short intervals) the adjacent cytoplasm or, more specifically, the continuous phase of the ground substance is in motion. Whether this flow is a product of undulating motions of the tubules or is a result of interactions at the surfaces of the stationary tubules cannot be decided at this moment. In this connection, however, there are some observations by Jarosch (9) with darkfield illumination which are pertinent. He has described, in the protoplasm of *Chara* after extrusion from the cell, some fine fibrillar structures that wave like flagella. Their

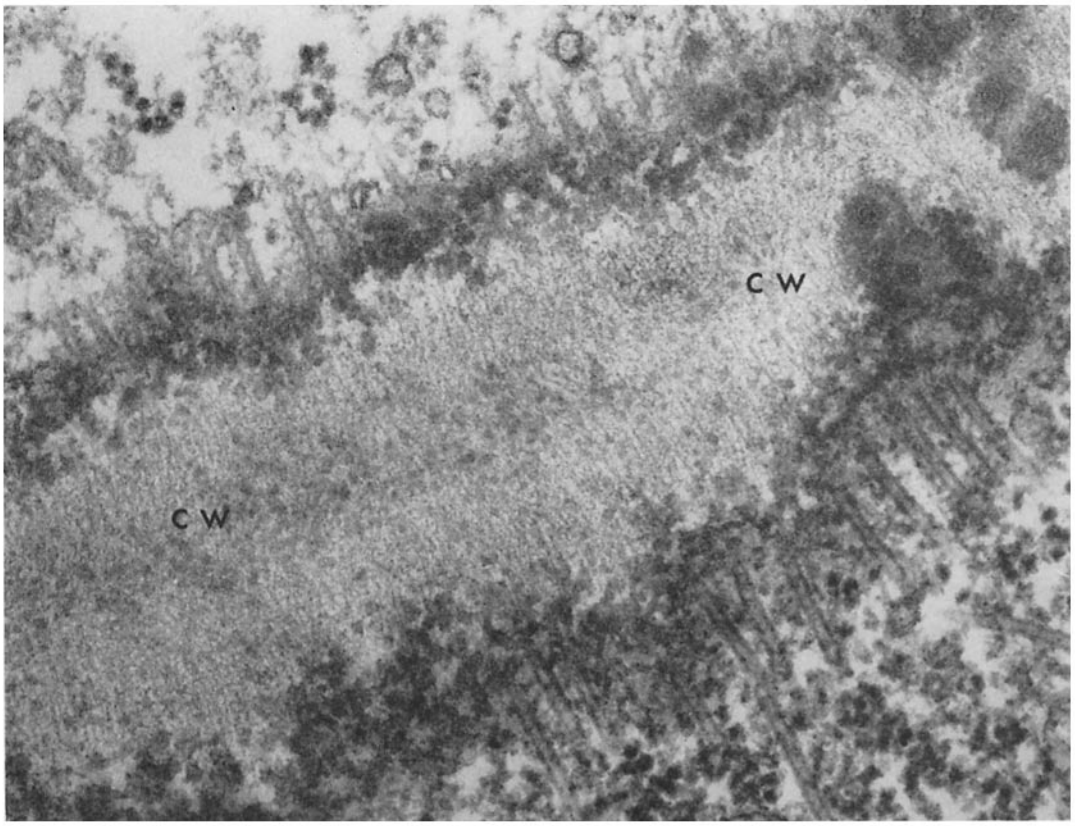

FIGURE 8 Micrograph depicting oblique section through lateral wall (*cw*) separating two cells of a *Juniperus* root tip. The microfibrillar structure of the cell wall, evident in this preparation, is organized with fibrils parallel to the tubules in the cell cortex. Here again, the tubules next to the cell surface are parallel, whereas those deeper in the cytoplasm are less well ordered. × 100,000.

size, form, and behavior would suggest that they are the "living" expression of the tubule of the EM images and that in relation to their surroundings they are capable of motion (see also Kamiya, 10).

In assembling information that may pertain to these tubules as found in plant cells, it is valuable to recall other instances where similar structures are found. Fortunately, the literature on animal cells already records a number of apparently related observations. For example, in a study of mitosis in the giant amoeba, *Pelomyxa carolinensis*, Roth and Daniels (18) found, after OsO_4 and Ca^{++} fixation, that the spindle fibrils possessed a tubular form. The reported diameter (140 A) is smaller than that of the tubules in these plant cells, but the morphology and disposition in the spindle are similar. Until their observations are repeated and extended in material fixed with glutaraldehyde, etc., it is possibly useless to be concerned about the size difference. More to the point in the present discussion is the observation, in the ameba material, that the spindle forms in the macronucleus before the breakdown of the nuclear envelope and so presumably from components of the nucleoplasm. Centrioles are apparently not essential to their development, for they are absent in *Pelomyxa* as in plant cells. Only slightly later, Harris (8) reported finding similar tubular units (150 A filaments) in dividing cells of the early sea urchin embryo (*Strongylocentrotus*).

In their form, if not dimensions, these structures of the mitotic spindle are not unlike the filaments which make up the 9 + 2 filamenture of the cilium and flagellum. Fawcett and Porter (5) described the filaments earlier as having a tubular form and a diameter of *ca.* 150 A. After more extensive and detailed examination of the same filaments in other material, Gibbons (6) reports the 2 central "fibres" as having an outside diameter of 240 A and the peripheral ones as measuring 260 A. Manton (12) has published similar measurements for the filaments in plant cilia. Thus, their dimensions match closely those of the cytotubules described here but obviously exceed those reported in the mitotic spindle of animal cells.

Any exploration of the literature for descriptions of similar structures will find several. In nucleated red cells, for example, there is a marginal band, associated with the asymmetric form of these cells, which comprises a bundle of 25 or 30 filaments found in section to be tubules (220 A diameter) (4). The developing spermatid, in many forms, shows a collar extending from the margins of the nucleus, and it is found, on close examination, to be made up of fibrous units (Burgos and Fawcett (2)) which, upon closer study, are microtubules. The cortex of unicellular flagellates frequently shows a net of filaments, arranged spirally around the cell body (13), which also are fine tubules in cross-section (25).[1] It has been the good fortune of the authors to be shown electron micrographs of similar structures in a wide range of animal cells. Frequently, but not always, they are located in the cortical zones; but in some few instances they are randonly disposed in the central cytoplasm. This kind of information makes it perfectly evident that if these cytoplasmic tubules may be classed together on the basis of size and appearance they represent a protoplasmic structure of wide occurrence.

In recent publications, Green has postulated that control of cell form resides in cytoplasmic elements near the growing side walls (7). He reasons, from indirect observations, that these elements have a long axis oriented parallel to the long axis of the cell and that the microfibrils of cellulose are put down at right angles to these ectoplasmic elements. It is obvious that the tubules described herein do not correspond to Green's hypothetical elements with orienting properties, and we have thus far failed to find any other resolvable fibrillar elements that might. Instead, therefore, of relating the orientation and deposition of cellulose microfibrils to hypothetical elements, we suggest that these tubules should be considered as strong contenders for a primary role in this activity of the plant cell. We are influenced in this proposal by the parallelism between the orientation of the tubules and that of the microfibrils, circumferential in the side-walls and random in the cross- or end-walls. It is true that the direc-

[1] When this paper was in press, our attention was directed to some observations by Manton (*J. Exp. Bot.*, 1957, **8**, 382; *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 413) on the fine structure of spermatozoids of *Sphagnum* and the bracken fern, *Pteridium aquilinum*. In each instance, the micrographs of thin sections show slender fibrous elements (200 A in diameter), parallel and evenly spaced in a single layer immediately beneath the body membrane. The individual units appear tubular especially in the bracken fern spermatozoids. And the author comments that the fibrous bands these units comprise "seem likely to be intimately connected with the over-all shape of the cell."

tion of cytoplasmic flow also parallels the orientation of the microfibrils. Which, therefore, is to be regarded as the primary determiner is in question and will be considered more fully in a later publication. The tubules seem, however, in some situations in which they occur, to adopt an orientation independent of, and in advance of, the more obvious phases of motion. Also, in some instances cellulose microfibrils and the direction of cytoplasmic streaming are not parallel. It would seem, therefore, that the more peripheral layers of tubules, immediately beneath the plasma membrane, alone can be said, on the basis of these limited observations, to parallel the orientation of the wall components. Possible in wall formation, as has been suggested previously, the outermost layer with membrane is cut off, and the tubules or subunits of them act as primers for cellulose deposition. These are questions requiring more penetrating study than time has thus far permitted.

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