STRUCTURE OF THE MITOTIC SPINDLE IN L STRAIN FIBROBLASTS

AWTAR KRISHAN, Ph.D., and ROBERT C. BUCK, M.D.

From the Department of Anatomy, University of Western Ontario, London, Canada

ABSTRACT

The mitotic spindle of L strain fibroblasts, fixed with glutaraldehyde followed by osmium tetroxide, contains many 150- to 180-A tubules. They appear first in the cytoplasm. They extend from the centrospheres to the kinetochores, and from one centrosphere to the other. Only occasionally can points of continuity between the spindle tubules and the tubules of the centrioles be observed. The chromosomal insertion is by a means of a thin dense plate of the kinetochore. The total number of continuous spindle tubules is between 500 and 600. Occasionally, tubules appear paired. At anaphase, short lengths of individual spindle tubules possess a coating of a substance of high density midway between the poles. These parts of the spindle tubules aggregate to form irregular groups, comprising the stem-body, and, by becoming aligned into a plate, they form the mid-body.

INTRODUCTION

The observation of Bernhard and de Harven (1958), that a component of the spindle of mitotic cells consists of fine canaliculi, has been confirmed by a number of workers studying mitotic cells in such diverse organisms as amoeba (Roth and Daniels, 1962); sea urchin (Harris, 1961, 1962, Kane, 1962); cultured mouse fibroblasts (Dales, 1963); and root tips of various plants (Ledbetter and Porter, 1963). It is now apparent that the failure to visualize the features of this spindle component in earlier work on mitosis was largely the result of poor preservation. A number of methods have been used to improve fixation, including the use of divalent cations in low pH osmium tetroxide fixative (Roth and Jenkins, 1962, Harris 1962), and the use of osmium tetroxide buffered to low pH with potassium phthalate (Kane, 1962). Recently it has been found that fixation with glutaraldehyde followed by osmium tetroxide (Dales 1963, Ledbetter and Porter, 1963) provides good preservation of the spindle.

There remain, however, many questions concerned particularly with the connections of the spindle. Little seems to have been accomplished in defining the relationship between the spindle and the centrioles or the kinetochores. The morphological basis for the origin of the spindle remains obscure. Although the dissolution of the central part of the continuous fibers and the formation of the mid-body was studied by Buck and Tisdale (1962), the cells were fixed only by conventional methods now known to be inadequate for this purpose.

The aim of this paper is to provide further information on the morphology and connections of well preserved spindle fibers (or tubules) studied at various stages of mitosis in Earle's L strain fibroblasts by a method that permits correlation of the electron and light microscopic appearance.

MATERIALS AND METHODS

Earle's L strain fibroblasts, obtained from Dr. Ian Walker of the Cancer Research Laboratory, University of Western Ontario, were grown in medium 1066 supplemented with 5 per cent horse serum and 50 to 100 units of penicillin per ml.

Two methods were used for obtaining cells for study. In the first method, the cells were grown in

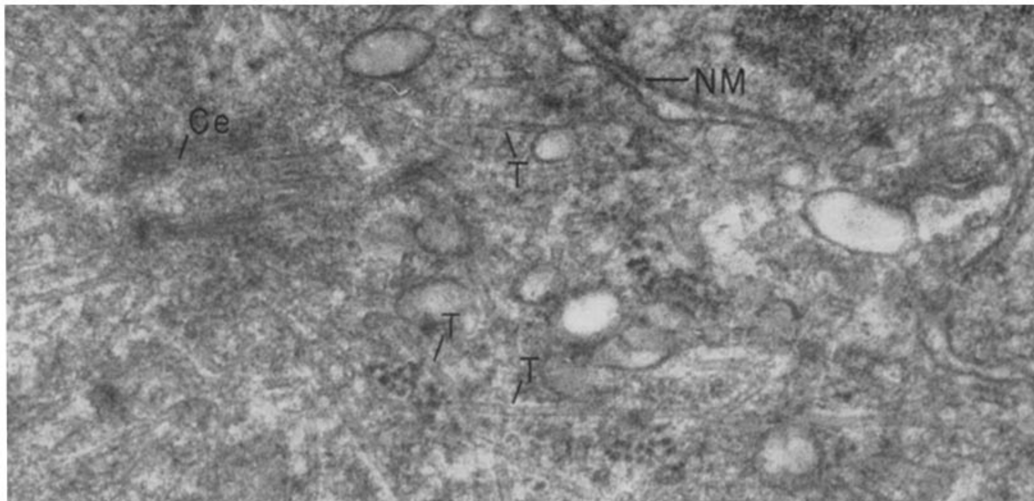

FIGURE 1 Centriole (*Ce*) sectioned obliquely, and spindle tubules (*T*) in prophase cell before breakdown of nuclear membrane (*NM*). × 77,500.

8- or 12-ounce medicine bottles, and large numbers of mitotic cells were obtained by gently washing the surface of the culture with a stream of medium from a bent pipette (Terasima and Tolmach, 1963). Such cells were fixed and centrifuged to form a pellet.

The other method was to grow the cells on coverslips in Leighton tubes and to fix, dehydrate, infiltrate, and embed them *in situ*. Then by examining the preparation in a phase contrast microscope, it was possible to select cells in a particular phase of mitosis and of suitable orientation for thin sectioning in a known axis. The selected cells were photographed and marked with a diamond circle marker attached to the microscope. The preparation was then chilled on the freezing microtome to free the embedding from the coverslip. Usually, cells were sectioned in their long axis, parallel to the coverslip surface, but they could also be cut at right angles to the plane of the coverslip by gluing the preparation to a block in the correct orientation.

A number of fixatives were tried, beginning with phosphate-buffered and veronal-buffered OsO_4, neither of which preserved the spindle well, even after the addition of divalent cations. Rather better preservation was obtained with potassium phthalate–buffered OsO_4 (Kane, 1962) and with a fixative described by Afzelius (1959), consisting of 40 per cent OsO_4 dissolved in carbon tetrachloride.

The best fixation was obtained with glutaraldehyde (Eastman, 25 per cent in water), added directly to the tissue culture medium to produce a final concentration of about 2 per cent. The fixative was allowed to remain for about 5 minutes at room temperature, after which the cells were washed 3 times with 0.1 M phosphate buffer at pH 7.0. This was followed by 1 or 2 per cent OsO_4, with or without buffer, for 1 hour. The cells were then washed with 30 per cent alcohol, dehydrated in graded alcohols and acetone, and embedded in Vestopal W (Martin Jaeger, Geneva).[1]

Sections were cut on a Porter-Blum microtome, stained with lead citrate (Reynolds, 1963), and examined in an R.C.A. E.M.U. 3D electron microscope at 50 kv.

OBSERVATIONS

A. *The L Cell In Interphase*

The cells show no remarkable general characteristics. After fixation with glutaraldehyde, the cytoplasm is well preserved and one is impressed by the retention of much more material in the cell than after osmium tetroxide fixation. The mitochondrial matrix is so well preserved that the cristae are often somewhat obscured by it. The cells have moderately short and sparse membranes of the endoplasmic reticulum and rather large numbers of free ribosomes. A small number of tubules of the type found in the spindle of mitotic cells were sometimes observed in the vicinity of the centrioles. (The term "spindle tubules" will be used henceforth in referring to the structures that have commonly been called spindle fibers.) Frequently none

[1] Epon could not be used in the coverslip method because it would not cleave from the glass when the preparation was chilled.

FIGURE 2 Centriole (*Ce*) and spindle in prophase cell. Tubules radiate towards the nucleus, and they are visible also in the deep fold of the nuclear membrane (*NM*). × 59,000.

of these tubules could be found. The possible significance of the phases of the interphase period in relation to the appearance of the tubules has not yet been determined.

B. *Cells In Mitosis*

1. PROPHASE: Prophase cells could be recognized in electron micrographs by the presence of dense but discrete chromosomes in nuclei, the

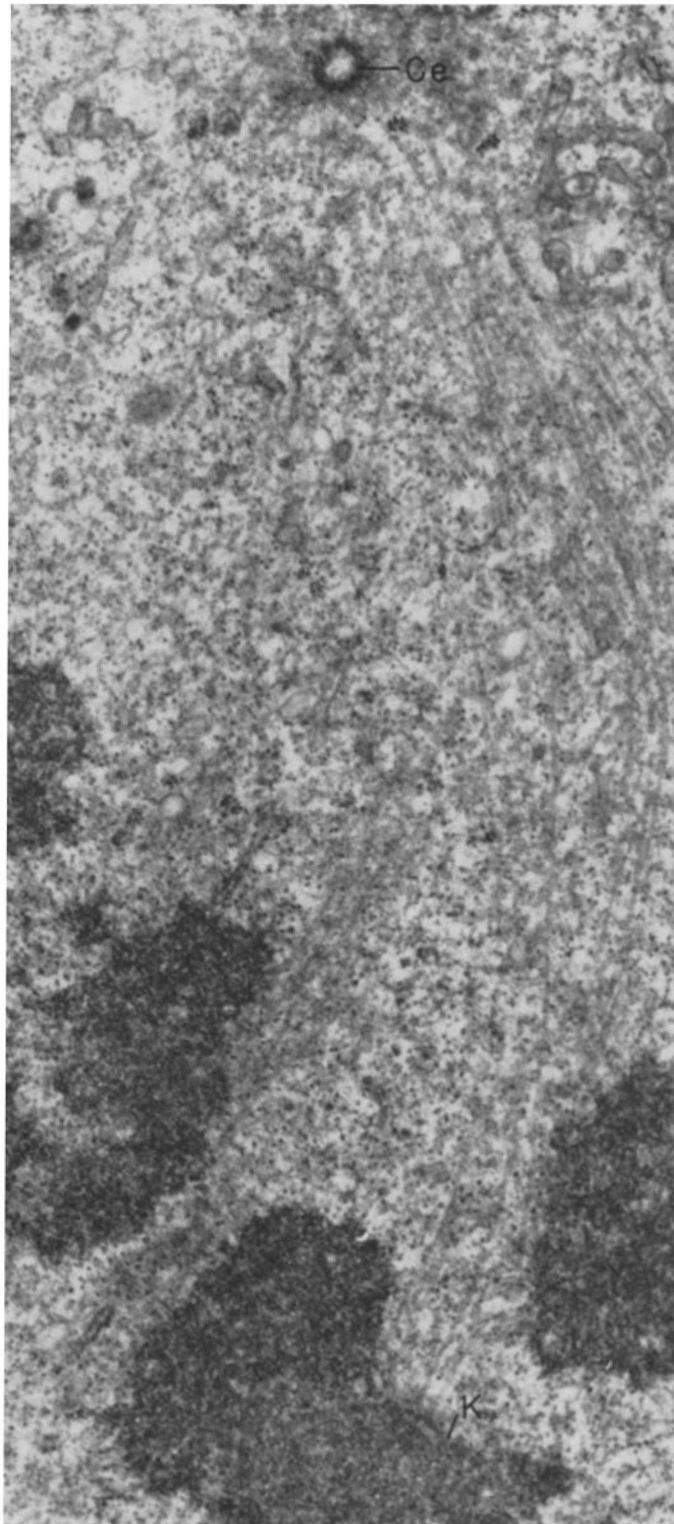

FIGURE 3 Metaphase. Chromosomal spindle tubules passing from the region around the centriole (*Ce*) to a kinetochore (*K*), here seen as a dense plate. × 40,000.

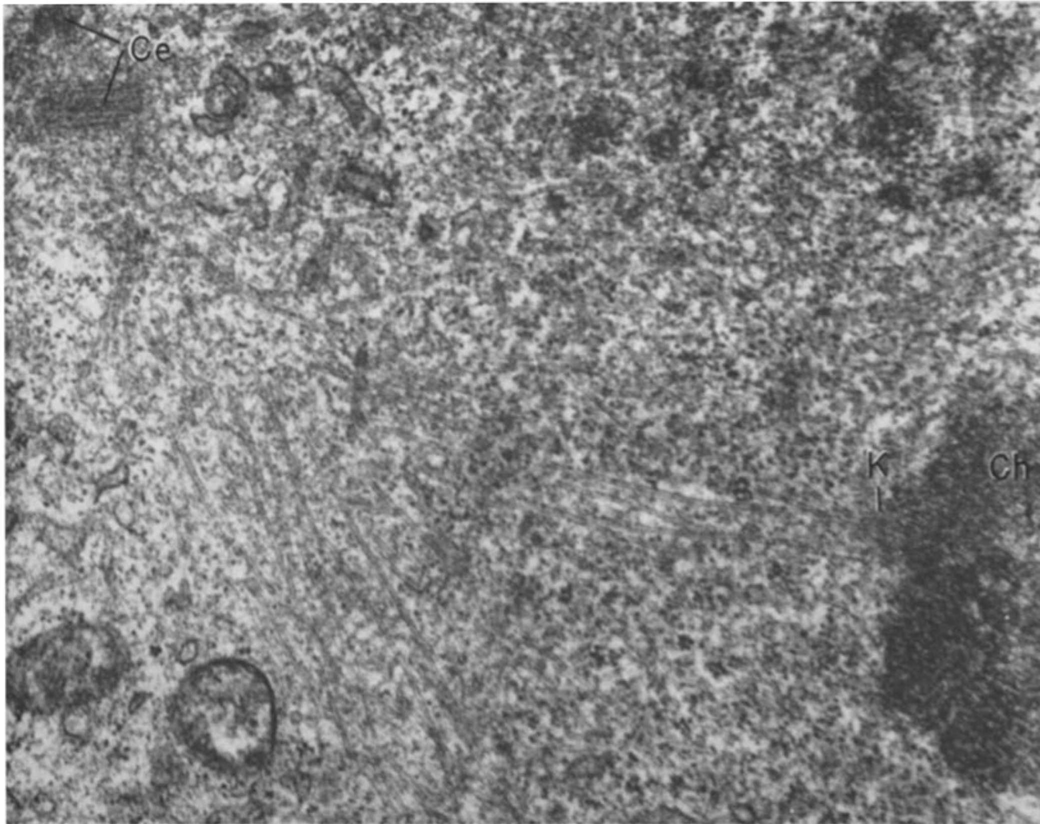

FIGURE 4 Spindle tubules in metaphase cell running to kinetochore (*K*). The kinetochore has the appearance of a plate of dense points separated from the chromosome (*Ch*) by a lighter band. Centrioles at *Ce*. × 43,000.

nuclear envelopes of which were irregular in contour and in the degree of separation of the two membranes. Since the orientation in relation to the plane of sectioning could not be determined with the light microscope, prophase cells were studied mainly in pellets rather than in monolayers.

Great numbers of tubular elements of the spindle were seen before the breakdown of the nuclear membrane, and in such cells the tubules were confined to the cytoplasm. An example of a prophase cell showing the centriole with radiating spindle tubules is shown in Fig. 1. The centriole, lying in an indentation of the nucleus, is the focus of tubules radiating in all directions. Some are sectioned transversely, others obliquely, and others run for several microns in the plane of the section. The tubules measure about 150 to 180 A in outside diameter and have a wall thickness of only about 30 to 40 A, considerably less than that of the unit membranes of adjacent structures. The nuclear termination of the tubules in prophase has not been determined. They show no obvious continuity with other structures such as the nuclear membrane. However, in some cases, the tubules run for long distances into folds of the nuclear membrane, and their high quantity in these folds suggests a preferential concentration there (Fig. 2). With the dissolution of the nuclear membrane in late prophase, the spindle tubules pass into the nuclear substance. They seem to disappear amid the granular material of the prophase chromosomes.

2. METAPHASE: The spindle tubules are visualized running in a straight or slightly arched course extending from the centrioles to the kinetochores, and also, appearing to pass through the metaphase plate as continuous tubules (Fig. 3).

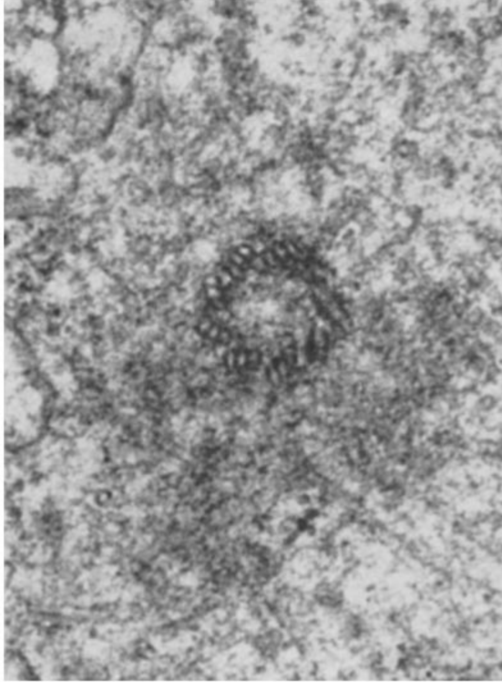

FIGURE 5 Cross-section of a centriole at metaphase. Some of the centriolar tubules are found in groups of 4. × 109,000.

Many ribosomes are scattered between them. Occasionally, a favorably sectioned kinetochore can be found (Figs. 3 and 4). It consists of a thin, dense plate separated from the substance of the chromosome by a lighter band several hundred angstroms in width. Into the dense plate a number of spindle tubules are inserted. A similar, but slightly more elaborate, plate-like form of the kinetochore was described by Nebel and Coulon (1962) in pigeon spermatocytes.

Some images suggest that the dense part of the plate is formed by the close apposition of a number of spindle tubules converging on this point. The tubules appear to become massed together through the short length represented by the dense plate.

Except for the ribosomes and a few smooth-surfaced membranes of the ER, virtually no other formed structures are seen in the metaphase spindle.

3. CONNECTIONS OF THE SPINDLE TUBULES WITH THE CENTRIOLES: The tubular components of the centriole, described by Bernhard and de Harven (1958), are well preserved by glutaraldehyde fixation. The centrioles measure about 0.3 μ in length and 0.15 μ in diameter, the lighter core being about 0.08 μ across. The nine arrays of tubules are arranged in the usual spiral manner. Sometimes each array consists of 4 tubules (Fig. 5). The lumen of each tubule of the centriole corresponds in size with that of the spindle tubules. In favorably sectioned material, particularly when the centriole is cut longitudinally, it is occasionally possible to observe what appear to be points of continuity between the tubules of the centriole and those of the spindle (Fig. 6).

4. ANAPHASE: The feature of the continuous spindle tubules of the anaphase cell is the consistent finding of short lengths of increased density

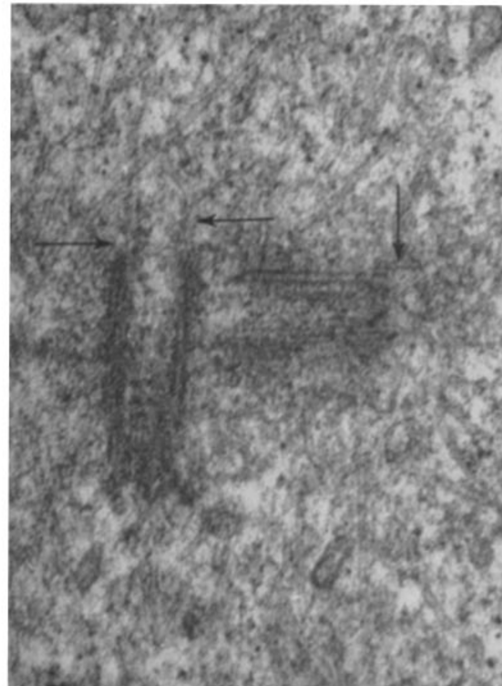

FIGURE 6 Longitudinal section through a pair of centrioles and surrounding spindle tubules at metaphase. Spindle tubules that appear as short profiles lie at various angles with respect to the plane of the section, and, therefore, to the plane of the long axis of the centrioles. Of those tubules which do lie in the plane of the section, most appear to approach the centrioles from angles that would make continuity with the tubules of the centrioles seem unlikely. However, at points indicated by the arrows, continuity is observed between the two types of tubules. Since the section thickness is less than that of the centrioles, the appearance of continuity cannot be due to the centriole and spindle tubules lying in different planes within the section. × 67,000.

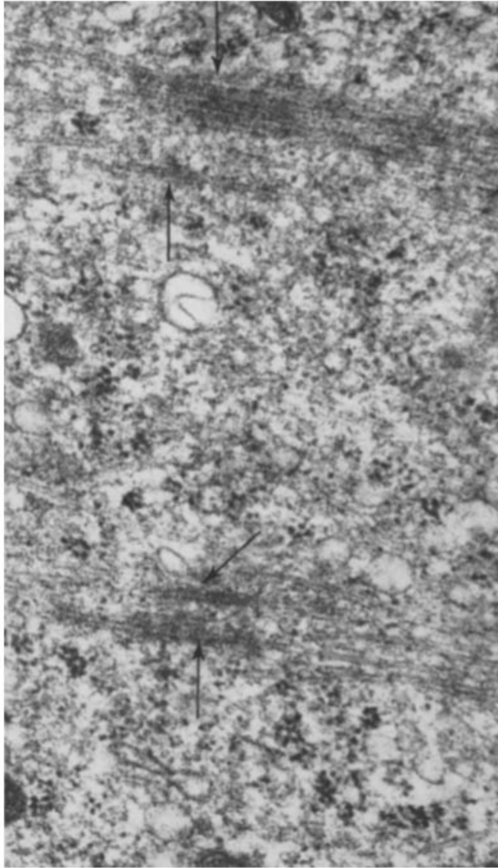

FIGURE 7 Midpoint of the continuous spindle tubules in anaphase cell. Individual spindle tubules display short lengths of increased density (arrows). These represent an early stage in the development of the stem-body. × 60,000.

at a point equidistant from the poles (Fig. 7). Dense material, resembling that surrounding the tubules of the centriole, is applied to the wall of the tubules over a length of about 0.5 to 1 μ. However, the spindle tubules are not arranged in the form of a hollow cylinder. Transversely sectioned anaphase or telophase cells show aggregations of the tubules embedded in the dense material in a rather random fashion (Fig. 8). The profile of the transversely sectioned equatorial region of the anaphase cell is approximately oval, with the flattening in the plane of the coverslip. The densities form an irregular pattern through the oval, being absent only in the peripheral parts. As Hughes-Schrader (1931) had reported that the number of densities on the spindle in the coccid testis was equivalent to the number of chromosomes, we were interested in trying to count them in our cells. However, there was no possibility of estimating their number because, although the densities appear circumscribed in longitudinal sections, they are found to be diffuse and even continuous with each other in transverse sections.

In transverse sections of the interzonal region, the number of individual spindle tubules can be estimated. In one early telophase cell, we counted between 500 and 600 tubules in a single cross-section of the whole cell.

If the cells are sectioned transversely at a level other than that of the stem-body, many of the profiles of the tubules are arranged in pairs (Fig. 9). Usually, the walls of the tubules are slightly flattened on the contacting surfaces, but sometimes the members of a pair lie a short distance apart and are connected by a fine dense line.

5. TELOPHASE: The continuous tubules of the spindle proceed in an almost perfectly parallel arrangement over considerable lengths (Fig. 10). The dense parts of the tubules have become massed

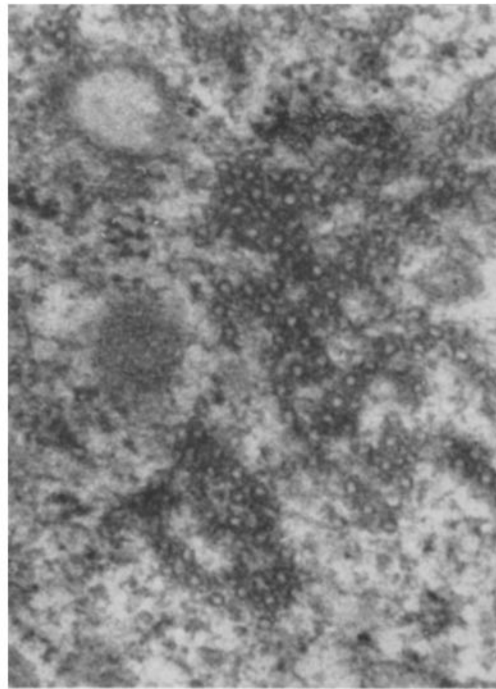

FIGURE 8 Transverse section through the stem-body of a cell in early telophase. Plane of section at right angles to that represented in Fig. 7. Groups of spindle tubules are embedded in dense material. × 96,000.

together, forming the mid-body, and additional dense material seems to lie between them. In telophase, some mitochondria are often found lined up between the spindle tubules. At the poles, the spindle tubules persist, radiating towards the centrioles which are still on the polar side of the nucleus. Unfortunately, we have not been able to determine whether continuity still exists at this stage between the tubules of the central part of the spindle and those on the polar side of the newly formed nuclear membrane.

An interesting arrangement of the spindle tubules in a tripolar mitosis is shown in Fig. 11. Here a Y-shaped density on the mid-body lies between the 3 sister cells. Each arm of the Y shows tubules running to 2 cells. Thus, each cell shares it continuous spindle tubules with the other 2 cells.

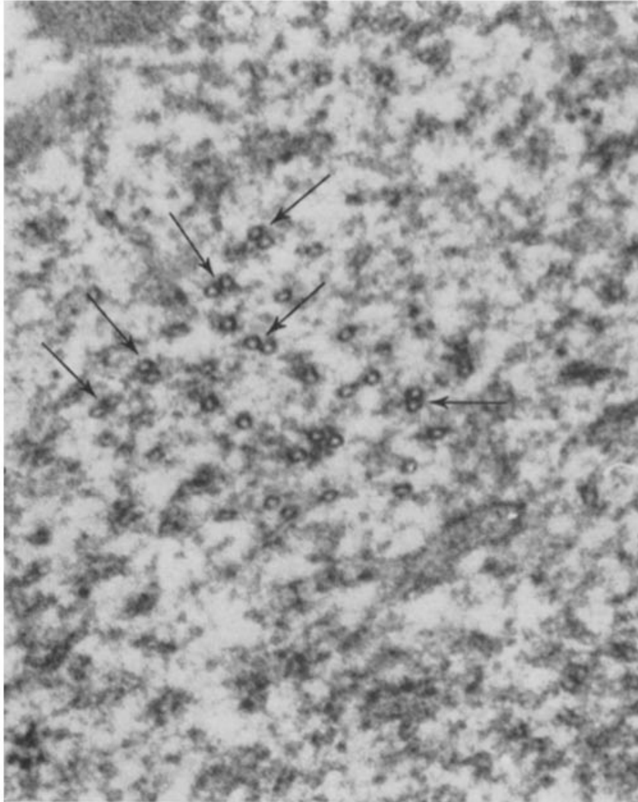

FIGURE 9 Transverse section through the spindle tubules of a cell in anaphase. The section is cut at a level that does not pass through the stem-body. There appears to be a tendency toward pairing of the tubules (arrows). × 100,000.

DISCUSSION

The observations of Bernhard and de Harven (1958) on the spindles of a variety of mammalian species included a description of the spindle tubules. They were said to be long canaliculi with a diameter of 150 to 200 A. No difference was observed between the chromosomal spindle fibers, the continuous ones, or those of the aster. Roth *et al.* (1960) and Roth and Daniels (1962) described similar tubules in the spindle of the ameba. The tubules, 140 A in diameter, were apparently attached directly to the chromosomes, no kinetochores being visible. At the polar end, the tubules seemed to be attached to fragments of the nuclear membrane.

Similar structures have been observed in sea urchin eggs by Harris (1961 and 1962) and by Kane (1962). Dales (1963) demonstrated that, after fixation with glutaraldehyde and postosmication, the tubules were seen in the spindle of dividing Earle's L cells. The dimensions were given as 180 to 230 A, with walls 30 to 40 A thick.

In a study of the dividing cells of the root tips of a number of plants, Ledbetter and Porter (1963)

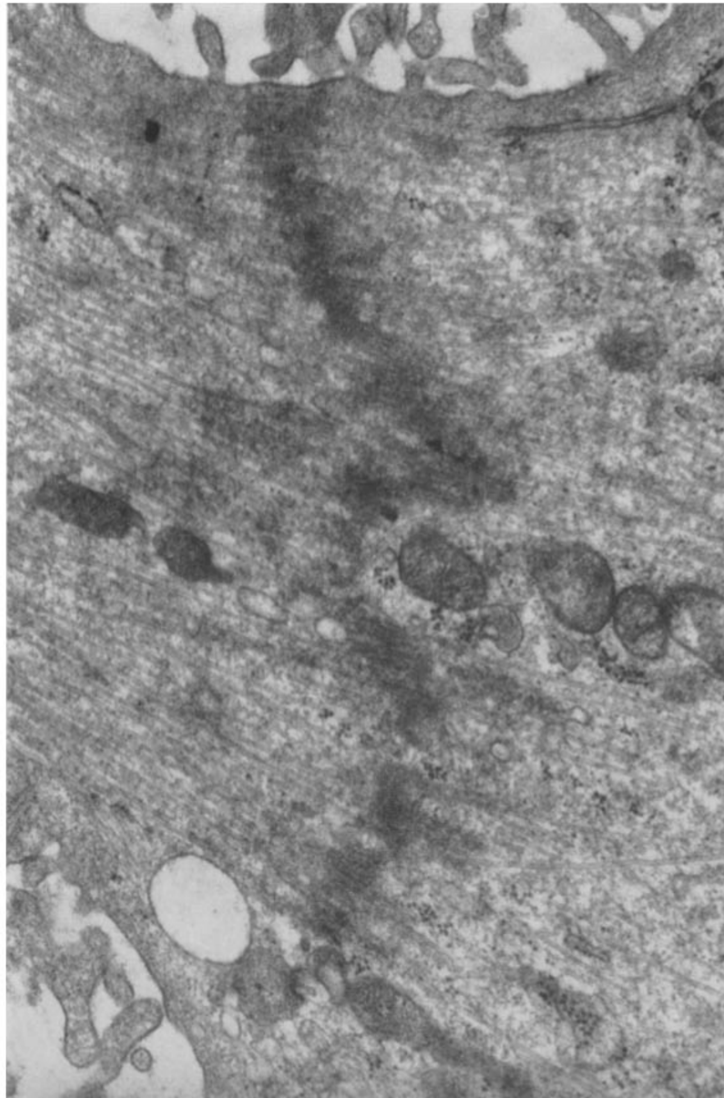

FIGURE 10 Longitudinal section through the mid-body in late telophase. The densities of the spindle have aggregated to form a plate extending across the bridge between the daughter cells. × 38,000.

described spindle tubules of a diameter of about 200 A, particularly abundant in the interzonal region of anaphase and telophase cells.

Aside from the tubules of the spindle, microtubules varying in diameter from 150 to 250 A have been described in a large variety of animal cells, including: interphase root tip cells (Ledbetter and Porter, 1963); myo-epithelial junctions (Auber, 1963); interstitial cells of Hydra (Slautterback, 1963); axons and dendrites of neurons (Gray, 1959, and Robertson *et al.*, 1963); caudal sheath or manchette in spermatocytes (Burgos and Fawcett, 1955); and the inner membrane of the nuclear envelope in spermatocytes (Meek and Moses, 1961). A detailed review of such microtubules is given by Slautterback (1963).

Bradke (1963), describing the origin of microtubules in the spermatocytes of earthworm, records that soon after the last meiotic division, tubules 160 A in diameter are seen near the outer

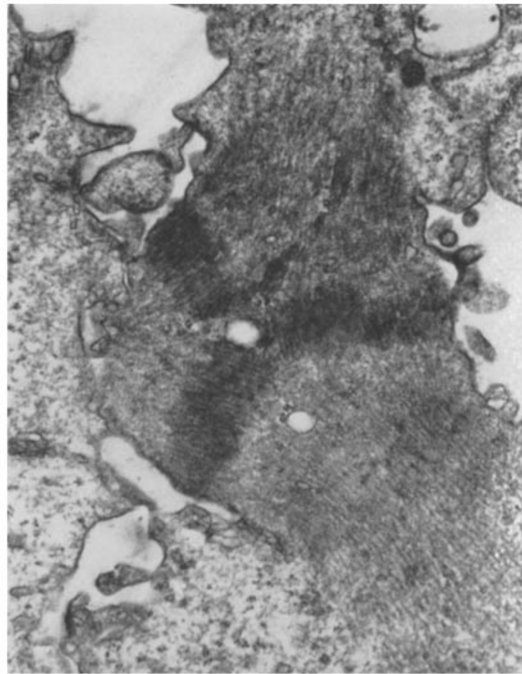

FIGURE 11 Form of the midbody in a tripolar mitosis. × 23,000.

nuclear membrane, some of them originating from what will become the distal centriole. Early in the spermatid maturation their diameter increases to 200 A, and they form the manchette by coming close together around the nuclear membrane.

There seems to be some real variation in the reported diameter of microtubules from various sources, but, as Ledbetter and Porter (1963) have indicated, comparisons are difficult to make because of the variety of techniques used in the preparation of the tissues. It seems generally agreed, however, that the wall thickness of the tubules is less than that of the "unit membrane." For this reason, it is probably a mistake to consider the spindle tubule as a cylindrical membranous structure in the sense that the term "membrane" is used. Ledbetter and Porter (1963) suggested that the wall may be composed of longitudinally arranged subunits. If this is their true structure, we may expect an unusual mechanism to be responsible for their formation.

Our observation that there are apparently only occasional points of continuity with the centrioles does not seem to support the belief that a very great number of tubules might be produced by a "spinning-out" process from the tubules of the centrioles. Moreover, the spindle tubules seen in the centrosphere are running in all directions, and there are many more than one would expect if each were continuous with one or the other end of a centriolar tubule. Assuming that in each centriole there were 9 groups, averaging 3 tubules in each group (we have seen 4 in some), a total of only 108 free ends of centriolar tubules would be available for "spinning" spindle tubules in one centrosphere. We have counted more than twice this number in a micrograph that included only part of a transverse section near the pole of an anaphase cell.

It is possible that spindle tubules bud off from those of the centriole in a side-by-side manner, short lengths of spindle tubules being generated at the periphery of the centriole. Perhaps our observation of the occurrence in some centrioles of 4, rather than 3, tubules in each of the 9 sets may be interpreted as evidence favoring such a mechanism.

It is also possible that the spindle tubules are self-reproducing units, once they are set free from the centriole. Whether their tendency to appear as pairs in cross-sections of the spindle has any significance for self-reproduction remains obscure. One is tempted to compare them with the paired peripheral tubules of the cilium, a question already

discussed by Ledbetter and Porter (1963) in whose plant material, however, pairing was not evident. On the other hand, Fig. 4 in the paper by Kane (1962), illustrating a cross-section of tubules, shows a number of pairs. He did not mention this appearance in the text.

The dense material at the midpoint of the spindle was observed in electron micrographs by Buck and Tisdale (1962). It apparently corresponds to the stem-body of Bělar (1927) during the period of anaphase and early telophase, and to the mid-body in late telophase. The nature of the dense material remains to be studied. From the present study, it is now apparent that, at the time of its first appearance, it is applied to the individual spindle tubules. In late anaphase or early telophase, the tubules become clumped together at the midpoint, the dense material then forming the matrix in which the tubules are embedded. The present incomplete state of our knowledge of the chemistry of this region precludes meaningful speculation on the significance of the dense material.

This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada.

The authors are grateful to Messrs. William Daniels and Charles Jarvis for technical assistance.

Received for publication, March 25, 1964.

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