# THE MITOTIC APPARATUS

## Fine Structure of the Isolated Unit

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ABSTRACT

The fine structure of the mitotic apparatus isolated from the sea urchin egg has been investigated. The isolation was accomplished by lysis of metaphase eggs in a 1 M solution of hexanediol, buffered at pH 6. The fine structure of the isolated apparatus was studied after fixation with osmium tetroxide directly in the isolation medium. The spindle is composed of fine fibrils, approximately 20 mμ in diameter, which appear tubular. Similar fibrils, radially oriented, are found in the aster. If the isolated mitotic apparatus is exposed to water at pH 6 before fixation, the structure is considerably modified. The most pronounced effects are an increase in the number of large membrane-bounded vesicles and in the amount of free granular material present. The conditions necessary for the fixation of the mitotic apparatus in dividing cells are discussed in the light of these observations on the isolated unit.

### INTRODUCTION

The development of methods for the isolation of cell organelles has generally required the use of the electron microscope, since most organelles are near or below the limit of resolution of the light microscope. The mitotic apparatus (MA) presents an unusual case, for in many cells it is of sufficient size to be well within the range of light microscopy. The apparatus was studied in fixed and stained cells before the end of the last century, although mass isolation was not achieved until the experiments of Mazia and Dan in 1952 (1). The unstained, isolated MA has little contrast in normal bright field, but the use of phase contrast optics allows the observation of the main structural features.

Critical evaluation of isolation methods must be based on studies at the fine structure level since changes in the structure of the apparatus may occur during isolation which are not detectable by phase contrast observation. The task of determining the effects of isolation on the fine structure of the MA has been complicated until recently by the lack of general agreement concerning the structure of the intracellular MA. Early observations of dividing cells with the electron microscope (2–4), using preparative methods similar to those for light microscopy, showed the presence of very coarse fibers in the mitotic apparatus. Rozsa and Wyckoff (5, 6) studied the effects of various fixatives on the structure of the spindle and concluded that such fibers were aggregation artifacts of acid fixation. They believed that fixation in neutral formalin, which caused the spindle region to appear completely homogeneous, gave a better representation of the true situation. Shortly after this, however, the polarization optical studies of Inoué (7) demonstrated the presence of spindle fibers in the living cell. Following the general adoption of buffered osmium tetroxide as a fixative for electron microscopy, fine fibrils, often

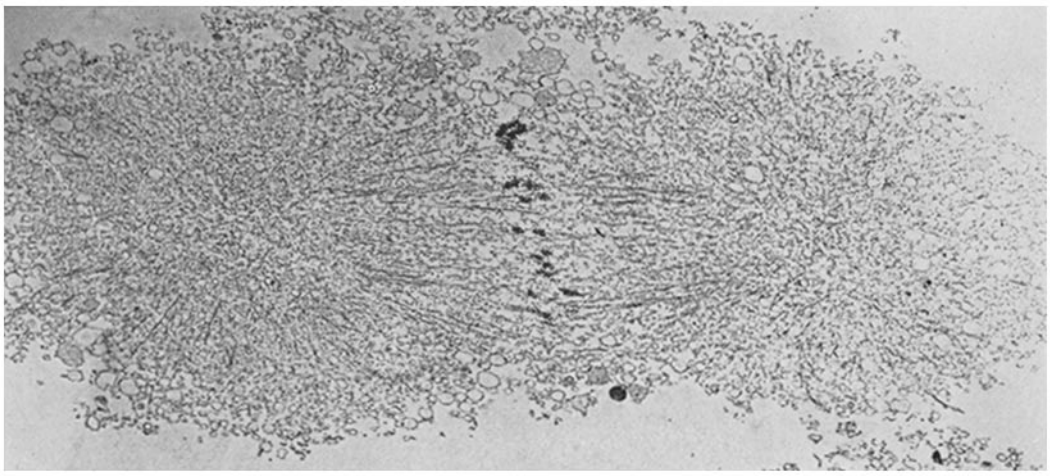


FIGURE 1

Survey view of isolated mitotic apparatus after fixation in the presence of hexanediol. × 2760.

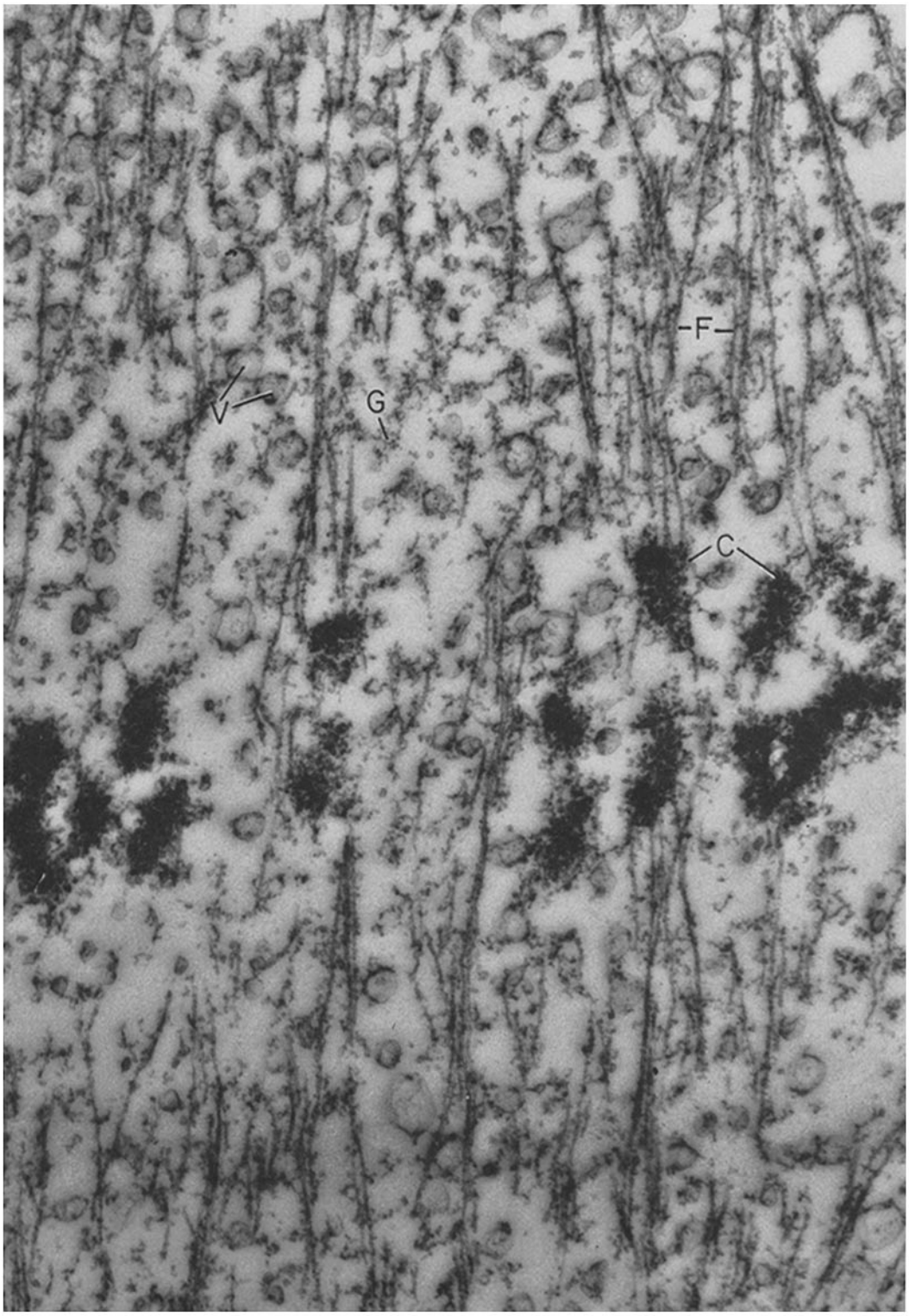
described as canalicular, were observed in dividing cells by a number of investigators (8–14). It was generally accepted that these fibrils formed the basis of the spindle, although Lehmann (15, 16) and Sato (17) insisted that fixatives other than osmium were required for spindle preservation. More recently, Roth and Daniels (18), studying dividing cells of the ameba, *Pelomyxa carolinensis*, have provided much new and detailed information concerning the fine structure of the spindle. These investigators obtained excellent fixation of the spindle fibrils by the addition of divalent cations to the buffered osmium tetroxide. In the absence of these ions the fibrils are not obvious, while in cells fixed in solutions containing divalent ions the spindle can be seen to be composed of fibrils with a diameter of 14 mμ, having a dense cortex and a less dense center. Continuous and chromosomal fibrils can be identified and have a similar structure.

Studies of mitosis in the sea urchin egg have passed through similar stages. In the experiments of Kurosumi (19) the eggs were fixed in $OsO_4$ in sea water and the embedding material was removed before examination of the sections. He observed the presence of coarse canalicular fibrils in the aster and spindle, which he believed to arise from "microsomal" granules. Gross *et al.* (20) found no evidence of fibrils in the spindle region of eggs which had been fixed in $OsO_4$ in acetate-veronal–buffered sea water but did observe 20 mμ canalicular fibrils in the spindle of cells which had been treated before fixation with the ethanol solution used in Mazia's original isolation procedure (1). These investigators concluded that osmium fixation dispersed the fibrils which are present in the living cell. More recently Harris (21) has observed the presence of filaments in the spindles of cells of the 6 hour sea urchin embryo which had been fixed with osmium tetroxide in buffered sea water and embedded in Araldite. She concluded that the asters had a different structure and were formed by vesicles and tubules of the endoplasmic reticulum.

The only electron microscopic study of the isolated mitotic apparatus is that of Mazia (22), using MA isolated by the original subzero 30 per cent ethanol procedure. These micrographs are not of sufficient resolution to allow the fine structure of the fibrils to be determined. Mazia

FIGURE 2

Spindle region of an MA fixed in hexanediol solution, showing chromosomes (*C*), fibrils (*F*), associated granular material (*G*), and irregular vesicles (*V*). × 23,000.


F
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*et al.* (23) have since described a method for the direct isolation of the mitotic apparatus, based on the disulfide compound dithiodiglycol, but no electron micrographs of such MA have been presented.

The author has recently developed a method for the direct isolation of the mitotic apparatus which involves only the lysis of metaphase cells in a suitably buffered solution of long chain glycol. In the present investigation the fine structure of such isolated mitotic apparatuses has been investigated and compared with the fine structure of the intracellular MA as described by recent authors.

## MATERIALS AND METHODS

These observations were made on mitotic apparatuses isolated from dividing eggs of the sea urchins *Arbacia punctulata* and *Strongylocentrotus purpuratus*. The isolation procedure has already been presented in detail (24). It is sufficient to note here that the final step in isolation involves lysis of the cells in a 1 M solution of 2,5-hexanediol, buffered at pH 6.0. Isolations were timed so as to yield MA in metaphase or early anaphase; the preparations were checked for stage by phase microscopy before fixation. The isolated MA were fixed for 30 minutes at 0°C in 1 per cent osmium tetroxide in 1 M hexanediol or in water, buffed at pH 6 with 0.005 M potassium phthalate. After fixation they were dehydrated in a graded ethanol series and embedded in a mixture of 80 per cent butyl and 20 per cent methyl methacrylate containing 0.2 per cent benzoyl peroxide as catalyst. 1 per cent phosphotungstic acid was included in one change of 100 per cent ethanol. The capsules were polymerized at 60°C for 24 hours. Sections were cut on an LKB Ultrotome at settings of 200 to 400 A, and picked up on formvar- and carbon-covered grids. They were examined with a Siemens Elmiskop I at 60 kv using a 200 $\mu$ condenser aperture and a 50 $\mu$ objective aperture.

The MA were randomly oriented in the capsule and most sections contained a number of MA cut at varying angles. Only those cut parallel or perpendicular to the long axis were studied in detail. Lead staining was carried out by the procedure of Watson (25), using the apparatus devised by Peachey (26).

## OBSERVATIONS

### *Mitotic Apparatuses Fixed in the Isolation Medium*

The mitotic apparatuses were isolated by lysis of metaphase eggs in 1 M 2,5-hexanediol at pH 6 and washed in several changes of the same solution to reduce contaminants. They were then fixed in 1 per cent osmium tetroxide in 1 M 2,5-hexanediol, buffered at pH 6. The apparatuses were thus exposed only to hexandediol solutions from isolation through fixation.

The basic structural organization of the isolated mitotic apparatus is seen most clearly in longitudinal sections through the MA at low magnification (Fig. 1). The chromosomes are recognizable as a row of very dense bodies lying across the center of the MA. The spindle is composed of fine fibrils, which converge from the chromosomes towards the asters. Similar fibrils, radially oriented, are seen in the asters, which also contain vesicular and granular material. The fibrils of the spindle and asters do not come to a distinct focus and centrioles are generally not seen in the isolates. Some contamination is present at the margins of the MA in the form of lipid droplets and swollen yolk granules.

At higher magnification (Fig. 2) the chromosomes appear as aggregates of very dense granules, without a limiting membrane. Individual spindle fibrils, which usually appear to be tubular, can be followed for long distances on such micrographs. Particulate material associated with the fibrils gives them an irregular outline. Aggregates of similar granules, unattached to fibrils, are also present. Small, irregular membrane-bounded vesicles are scattered throughout the spindle. The fibrils often appear to attach to chromosomes, but when such a region is examined at higher magnification (Fig. 3) no special differentiation can be seen at the junction. In cross-sections through the spindle after lead staining (Fig. 4), the spindle fibrils appear as dense rings, with an over-all diameter of approximately 20 m$\mu$. The particulate material is of similar size, suggesting that it may be ribosomal in nature.

### *Mitotic Apparatuses Fixed in Water*

The stability of the isolated mitotic apparatus is controlled chiefly by the pH of the suspending medium and is relatively insensitive to its composition. At pH 6 the MA is stable in water, dilute salt solutions, and a number of non-electrolytes. To determine the effects of such solutions at the fine structure level, the structure of the MA after exposure to water at pH 6 was investigated. Its appearance in phase contrast does change

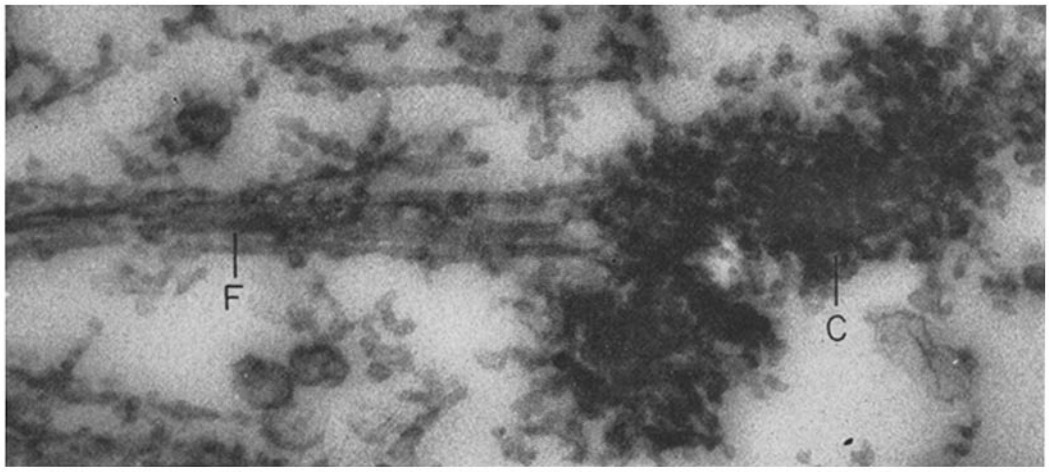



FIGURE 3

View of chromosome (*C*) and attached fibrils (*F*). No distinctive structure can be identified at the junction. × 69,000.

somewhat in this solution, the apparatus becoming granular and more fragile.

After isolation in hexanediol the MA were centrifuged out of the solution and washed in several changes of water buffered at pH 6. Fixation was carried out in 1 per cent osmium tetroxide in water with similar buffering. The appearance of longitudinal sections of such MA at low magnification (Fig. 5) differs considerably from that of those fixed in hexanediol (Fig. 1). The main structural features are similar, but the general density is increased and the entire MA is filled with membrane-bounded vesicles.

The chromosomes are much less distinct in this preparation as compared to those fixed in the presence of hexanediol. A section through the chromosome region at higher magnification (Fig. 6) shows that the loss of contrast of the chromosomes has two causes. The chromosomes are quite dispersed and there is a considerable increase in the small particulate component throughout the spindle. It is, in fact, rather difficult to sharply localize the chromosomes, as they appear to be only slightly more dense aggregations of particles similar to those forming the general background. Comparison of Fig. 6 with Fig. 2 illustrates the increase in the number of large vesicles present. These vesicles are uniformly circular in outline, while those in hexanediol are often irregular. The tubular appearance of the fibrils is more evident after fixation in water (Fig. 7) and the fibrils seem to have fewer granules associated with them than do those fixed in hexanediol solution (Fig. 3).

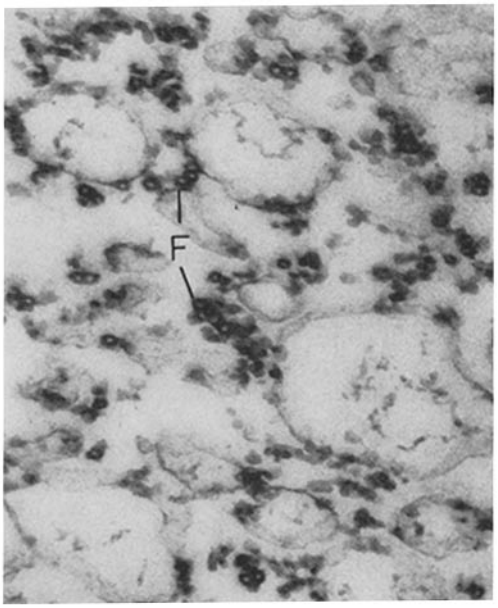



FIGURE 4

Cross-section through the spindle showing the tubular outline of the fibrils (*F*). Lead-stained. × 69,000.

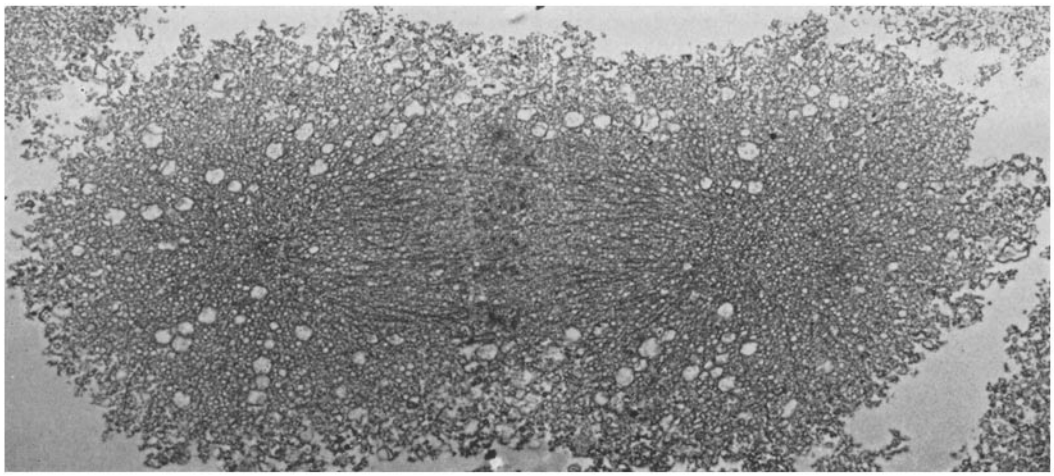


FIGURE 5

Survey view of an isolated mitotic apparatus fixed in water. × 2760.

## DISCUSSION

The first aim of any study of the fine structure of isolated cell organelles is to determine how closely the structure of the isolated unit corresponds to that of the intracellular organelle, since this provides a basis for the evaluation of the isolation procedure. In the case of the mitotic apparatus, the information concerning the intracellular unit is quite limited, the most detailed study being that of Roth and Daniels (18) on dividing cells of *Pelomyxa carolinensis*. Since cleavage in these cells is anastral, no comparisons of astral structure can be made, but the structure of the spindle in these cells is similar in most respects to that of the isolates studied here.

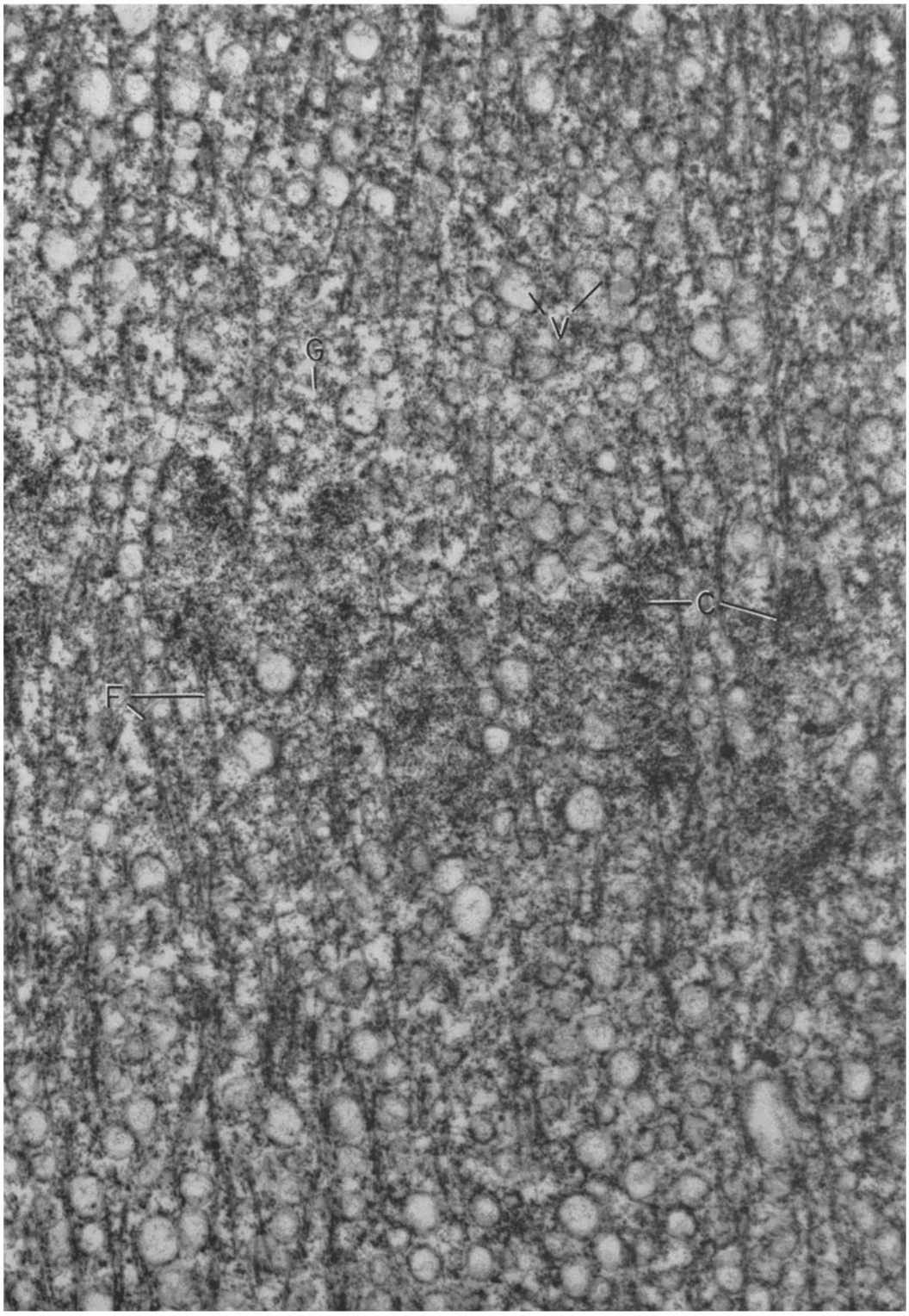
The spindle of the mitotic apparatus isolated from the sea urchin egg and fixed in the isolation medium is composed of long, straight fibrils, which appear tubular in micrographs. These fibrils exist as both continuous and chromosomal fibrils; aggregates of such fibrils presumably form the coarser fibers visible with the light microscope. The cause of this tubular appearance is not known, and it is unchanged by the common EM staining procedures.

The granules associated with the fibrils in the isolates are intermediate in size between the two types of granular material seen in the spindle of *Pelomyxa* by Roth and Daniels (18). These authors observed the presence of fine material in the 2 to 6 mμ range and also larger particles, resembling ribosomes, with a diameter to 30 to 40 mμ. The granules present in the isolated MA are similar in diameter to the fibrils, that is, approximately 20 mμ. These granules occur both free and associated with the fibrils, and in the latter case the granules do not appear to be attached in a periodic fashion. The granules forming the chromosomes of the isolates are somewhat larger than those seen in the ameba by Roth and Daniels, but it is of interest that these investigators were also unable to see any evidence of a specialized differentiation at the junction of the fibrils and the chromosome.

The study of Harris (21), although not so detailed, is particularly relevant since it is concerned with mitosis in the sea urchin embryo. Harris observed the presence of fine filaments in the spindle of these cells, which appear similar in structure to the fibrils discussed above. The micro-

FIGURE 6

Spindle region of an MA fixed in water, showing fibrils (*F*), highly dispersed chromosomes (*C*), and the great increase in the granular material (*G*) and in the number of large vesicles (*V*). × 23,000.


V
G
C
F

graphs of Harris also show evidence of a dense region at the junction of the fibril and the chromosome, which is not evident in the isolated MA. The chromosomes themselves, however, are much less dense than the background and appear as negative images. This is the reverse of the situation in the isolates, where the chromosomes are the densest component present. Thus if a kinetochore region existed in the isolates, it would be much more difficult to detect.

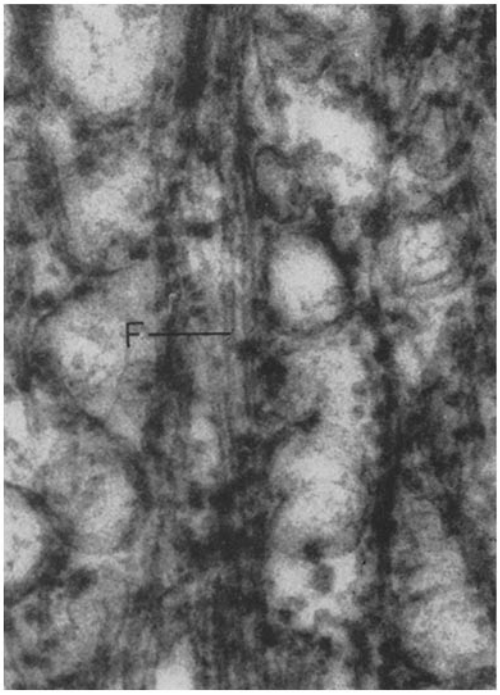



FIGURE 7

Longitudinal section through the fibrils (*F*) at higher magnification. × 69,000.

Harris (21) concluded from her study that two different components could be distinguished in the mitotic apparatus. One was the fine fibrils which were observed in the spindle and which were also found associated with the centriole; the other was the coarse material, formed by the endoplasmic reticulum, which made up the bulk of the asters. This distinction is not evident in the isolated mitotic apparatus. Both the spindle and the asters of the isolates are composed of fine fibrils which appear similar in structure. The asters of the isolated MA fixed in the presence of hexanediol contain slightly more vesicular material than the spindle, but its origin cannot be determined from such micrographs.

Comparison of the micrographs of the mitotic apparatus fixed in the presence of hexanediol with those of MA fixed in water shows that the fine structure has been considerably modified. The most striking changes are an increase in the amount of free granular material present and an increase in the number of large membrane-bounded vesicles seen throughout the apparatus. The great increase in the amount of free granular material present after fixation in water is difficult to explain, as the number of granules attached to the fibrils of the MA fixed in hexanediol seems insufficient to account for it. However, the dispersal of the chromosomes may provide another source of these granules. It is also possible that a greater extraction of material occurs during fixation in the presence of hexanediol, thus accounting for the loss of these granules in MA fixed under such conditions.

The many large vesicles present in the MA fixed in water may arise through the swelling of the small irregular vesicles visible in the micrographs of MA in hexanediol. Since these small vesicles are presumably in equilibrium with the hexanediol solution, they may swell osmotically when exposed to water. If one further assumes that they are not elastic, they would retain this larger size after a new equilibrium is established in water.

These observations may aid in explaining the importance of osmotic effects during isolation of the mitotic apparatus. Isolation of the MA in water is possible only at pH values sufficiently low to coagulate the cytoplasm, while isolation can be obtained at pH values approaching physiological in the presence of 1 M hexanediol (24). The small size and irregular shape of the vesicles present in hexanediol solution indicate that equilibrium was attained without swelling, while transfer of the MA to water causes considerable swelling of these vesicles. If a similar, and possibly more extreme, swelling occurs when the cell is cytolyzed in water and the MA thus transferred rapidly from its normal intracellular environment to water, such vesiculation may render the MA too fragile to withstand the dispersal of the cell.

The conditions necessary for the fixation of the mitotic apparatus in dividing cells have remained unsettled for some time, as reproducible results have not been obtained by all investigators. Roth

and Daniels (18) concluded, on the basis of their study of *Pelomyxa*, that the presence of divalent cations in the fixation mixture was of critical importance. They obtained excellent fixation of the spindle in osmium tetroxide buffered at pH 8, containing 0.002 M calcium, magnesium, or strontium chloride. This conclusion is supported by the studies of the author on the properties of the isolated mitotic apparatus (24), in which the disintegration of the MA which normally occurred in water buffered above neutrality was prevented by the addition of 0.001 M calcium or magnesium chloride.

Such divalent ion effects do not explain the differing results obtained by various investigators with regard to the fixation of the spindle of the sea urchin egg. In all cases, fixation was carried out in sea water, which contains a high concentration of both calcium and magnesium. The Araldite embedding used by Harris (21) can also be ruled out as a relevant factor, since both Roth and Daniels (18) and the author have obtained adequate embedding of the spindle in methacrylate. Some additional factor, as yet unknown, must be responsible for these differing results.

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