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ON THE ORIENTATION OF CENTRIOLES IN DIVIDING CELLS, AND ITS SIGNIFICANCE: A NEW CONTRIBUTION TO SPINDLE MECHANICS[1]

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The centriole is one of the most fascinating and baffling of the cell components. Despite the painstaking and inspired work done on this cell organelle during the last quarter of the nineteenth century, its very existence as a real structure was called into question within recent times (Fry, 1929). The idea that the centriole is a fixation artifact, without organic continuity, was refuted brilliantly by E. B. Wilson and his students, and the continuity of the centriole from one cell generation to another is now firmly established, for some materials at least (Wilson, 1930; Sturdivant, 1931; Wilson and Huettner, 1931; Johnson, 1931; Huettner, 1933). The observations on the centriole recorded by the older generation of cytologists, including Flemming, Boveri, Conklin, Lillie, Mathews, van Beneden, Heidenhain, Mead, MacFarland, and many others, were not only substantiated but actually reinforced by this controversy.

Despite all this interest, the actual function of the centriole in fertilization and cell division has remained essentially unelucidated, because of the anomalous situations existing in the *de novo* origin of centrioles during parthenogenesis, in the existence of primary and secondary centers during the normal fertilization of certain eggs, and in the well demonstrated existence of anastral and acentriolar mitosis in many forms, including the higher plants.

The great variation in form and behavior shown by this cell component, and by the centrosome, aster, blepharoplast and other structures related to it, is such, also, as to render controversial many of the hypotheses thus far made about the specific functions of the whole centriole-complex. The centriole itself may occur, in different materials, as a tiny dot-like structure; as a diffuse group of fine particles; as a definitive rod; as a distinct V-shaped body, with or without axial filaments and terminal vesicles; as a ring-shaped circlet; or there may be a complete absence of visible centrioles in certain anastral configurations. These variations present an array of manifestations which are, to say the least, confusing.

[1] Aided by a grant from the National Institutes of Health, RG-5328.

Recent electron microscope studies (on all too few materials, thus far) have revealed, however, that the centrioles examined to date by this means are cylindrical cage-like bodies composed of 9 rod-like or tubule-like structures embedded in a matrix. The tubules may be double or single. Presumably the dot-like centrioles as seen by visible microscopy are actually very short cylinders, the longer rods longer cylinders, and the V's composed of two attached cylinders. The rings may be very short, very broad cylinders. No electron microscope studies have been made, to my knowledge, of the more specialized types of centrioles or of the finely particulate centriolar areas.

If the majority of centrioles are actually rod-like structures, however, it seemed that it might be profitable to examine one of the few forms in which the rod-shaped centrioles are sufficiently large to be easily distinguishable in fixed material without the use of electron microscopy. One of the purposes of the present paper is to give a more complete description of the remarkable giant first cleavage spindle of the egg of *Polychoerus carmelensis* and its unusual centrioles. Certain comparisons will be made, also, between the resting first cleavage spindle and the non-resting maturation spindles and their centrioles, but a more complete description of the meiotic spindles and their chromosomes will be reserved for a later paper.

Materials and Methods

Polychoerus carmelensis is a small orange-colored marine acoel flatworm from the tidepools of Pescadero Point, Carmel Bay, California, described by Costello and Costello (1938a). It is similar in many respects to *Polychoerus caudatus,* named and described by E. L. Mark (1892) and once common from Great Egg Harbor, New Jersey, to Casco Bay, Maine. Representatives of this genus are fascinating little animals, which as early as 1865 attracted the attention of A. E. Verrill (see his 1892–1893 paper), of Joseph Leidy in 1874 (see Verrill, 1892–1893), of T. H. Morgan in 1890 (personal communication from Ross G. Harrison), and later of E. G. Gardiner (1895, 1898). They have several unique or unusual features, certain of which have been mentioned earlier, in papers (Costello and Costello, 1938b, 1939) or brief abstracts (Costello, 1937, 1946, 1960a, 1960b, 1960c).

Since the egg of *Polychoerus* contains quantities of several types of orange pigment granules, yolk spheres, lipoid droplets, and nuclear remnants of incorporated vitellogenic cells, it is far too opaque for study of the living spindle. Only the dumbbell-shaped outline of the large amphiastral figure can be made out in living material. The primary oöcytes are fertilized and undergo maturation within the body of the hermaphroditic worm, after the worms have reciprocally mated, and there advance to the metaphase of the first cleavage division, at which stage they remain until the eggs are laid and enter sea water. Hence, an ample supply of fixed material, at stages up to and including the resting metaphase, can be obtained by examining the living adults with a hand-lens, and fixing those containing large oöcytes or ova visible through the body wall. The adult worms are quite hardy, and live well in a laboratory aquarium with running sea water, crawling about on the glass sides or wooden walls or on *Ulva* placed in the tank. They may also be kept in fingerbowls if the water is changed at intervals.

A variety of fixatives was used, including Heath's polyclad fixative, Worcester's, Boveri's picro-acetic, Flemming's, Lillie's modification of Meves' fluid, B-15, 20%

formalin, Champy's, Carnoy and Lebrun's, Regaud's #1, and Gilson's fluids. The majority of these were used at three different temperatures: room temperature, warm (about 50° C.) and hot (60–80° C.), with specific temperature records kept in each case. Some egg masses were fixed, also, at various stages after having been laid, and 12 adults were fixed in the act of egg-laying (see Costello and Costello, 1939). All this material was collected and fixed during the author's stay at the Hopkins Marine Station, Pacific Grove, California, during the summers of 1936 and 1937. I am indebted to the late Director, Dr. W. K. Fisher, and his staff for many courtesies.

The fixed animals were individually embedded in hard paraffin, and sectioned serially at 8 or 10 microns. Approximately equal numbers were sectioned transversely, frontally and sagittally. The serial sections were stained by a variety of methods: Heidenhain's iron haematoxylin, Feulgen, Champy-Kull, Benda, Flemming tricolor, safranin, Delafield's haematoxylin, Mallory's triple stain, anilin blue, or crystal violet, and counterstained, in some cases, with eosin, erythrosin, light green, or orange G. Specimens fixed in room temperature Worcester's solution or in Heath's solution and stained with Heidenhain's iron haematoxylin gave preparations of superlative beauty. More than 423 sets of serial sections of the worms and egg masses were prepared by my wife and myself in 1936, 1937 and 1938. Most of these slides were studied by Helen M. Costello and the writer between 1936 and 1940. All the preparations were subsequently re-studied by Dr. Catherine Henley in 1958. This paper would not have been written without this help, and I hereby acknowledge my deep appreciation. My thanks are due, also, to Drs. Sally Hughes-Schrader and Franz Schrader for many stimulating discussions about the *Polychoerus* egg over a period of years, and for reading the manuscript; to Dr. Kenneth W. Cooper for similar conferences; and to Dr. Shinya Inoué for an all-too-brief discussion at Woods Hole during the summer of 1960.

The serial sections were examined at various magnifications (50 to 2400 diameters) and the location (slide number, row number, section number) of each significant feature noted, to serve as a record and in order that any feature might easily be found again. Photomicrographs were taken of maturing eggs, spindles, chromosomes, etc., at magnifications of 300 to 1400 diameters, using apochromatic objectives and compensating eye-pieces. Wash drawings were made by Mrs. Ernest Runyon, to whom I am greatly indebted.

Observations

The giant cleavage spindle of the egg of Polychoerus

The fully grown eggs of *Polychoerus carmelensis,* after fixation, measure 200 to 280 microns in diameter. Considerable shrinkage in certain of the fixatives is indicated by the fact that the cavities in the parenchyma in which the eggs lie are 260 to 320 microns in diameter. In one animal fixed in Champy's fluid, there were no shrinkage spaces around the eggs, and one ovum, just extruded through the body wall, measured 320 microns in diameter. There is every reason to believe that the ova are closely surrounded by the parenchyma when the animals are alive, and that the eggs shrink more than the surrounding tissues.

The resting (metaphase) first cleavage spindle of *Polychoerus* is of the amphiastral type, with the continuous fibers constituting a central spindle (Hermann,

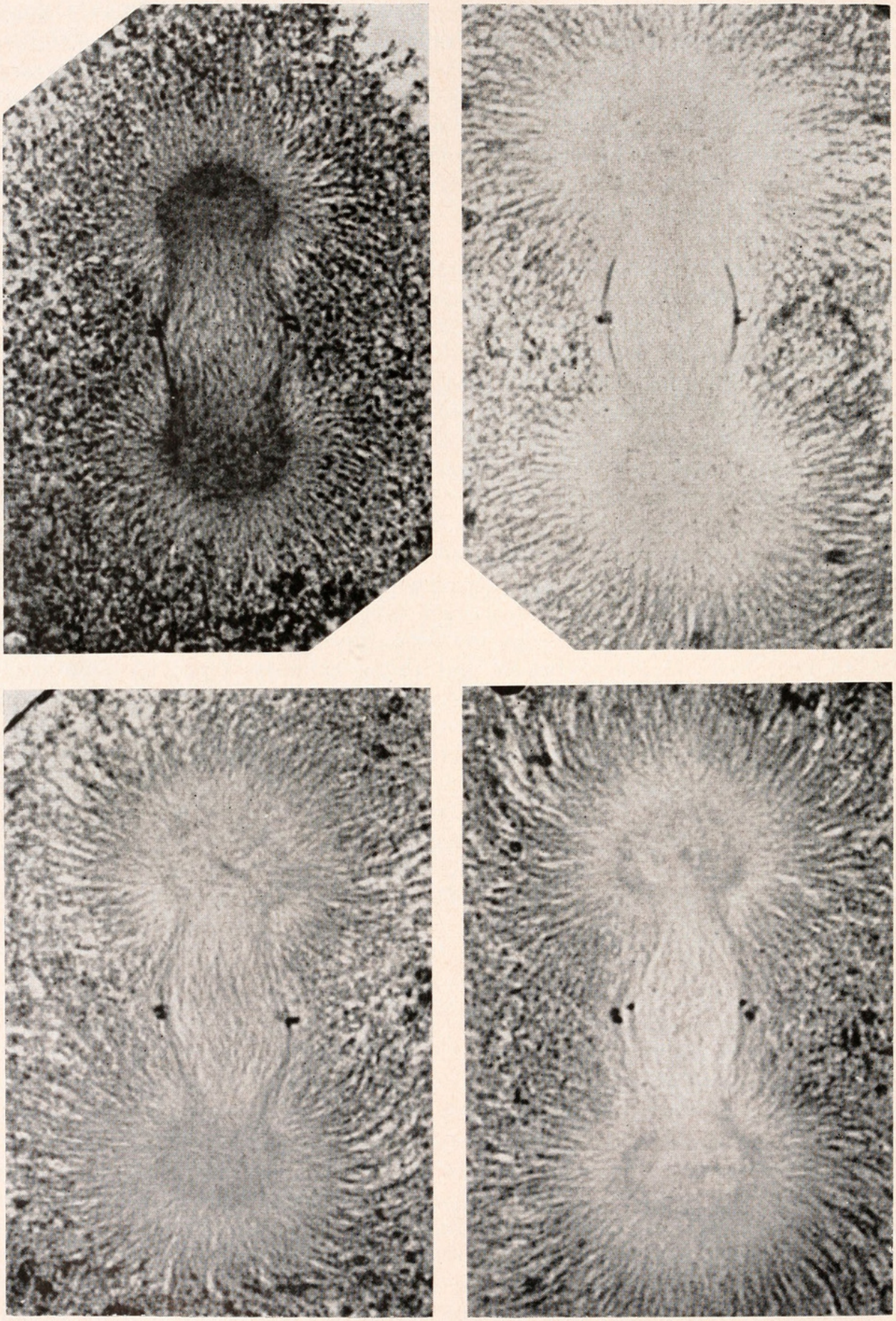

FIGURE 1. Photomicrographs of 10-micron sagittal sections through resting first cleavage metaphase of four eggs of *Polychoerus carmelensis*. Left, upper, fixed in Flemming's; right,

1891) about which the chromosomes are grouped in a ring (Fig. 1). A sheath of mantle fibers lies peripheral to the central spindle, with the chromosomal fibers attached to the kinetochores of the fringing chromosomes. The chromosomal fibers are more fibrous and less granular than the continuous fibers or the astral rays. The chromosomal fibers appear, also, to be compound, consisting of as many as 10 or 12 finer fibrils. The chromosomes are all V- or J-shaped, with median or sub-terminal attachments, and with their arms projecting radially outward from the equatorial plate (Fig. 13). The diploid number of chromosomes, repeatedly counted with ease in a large number of the resting metaphases, is 34. The chromosomes are not exceptionally large, the long arms of the J-shaped chromosomes being 5 or 6 microns in length and the arms of the V-shaped a little less. These chromosomes in the resting metaphase are never double, and have not yet visibly split at this stage.

The fixed spindle at resting metaphase measures up to 120 microns in length [2] between the centers, and a maximum of 65 microns in diameter at the equator. The spindle plus asters often exceeds 160 microns in length. The usual diameter across the equatorial plate is 45 to 50 microns. It should be noted that these dimensions are those of sectioned material; therefore, they do not give the distorted and exaggeratedly spread relationships so often seen in squashed preparations.

In many preparations (especially those fixed in Heath's and heavily stained in iron haematoxylin), a distinct centriole can be seen at each astral center, in most cases in the shape of a rod which may sometimes be straight, but is more often slightly curved, and in a few cases, somewhat twisted. In other eggs, fixed less well, the central region of the aster may show a fine granulation, but no distinct centriole. When curved, the centrioles have their free ends nearer the equatorial plate, the convex portion away from it.

The rod-shaped centrioles of the cleavage metaphase of *Polychoerus* are 4.5 to 5 microns in length by about 0.25 micron in diameter. Examination of large numbers of cleavage spindles sectioned longitudinally indicates that the centrioles at the two ends of a given spindle are never oriented parallel to each other. In fact, while they lie transversely (or, more rarely, slightly obliquely) to the long axis of the spindle, their axes never lie in the same plane but are oriented at right angles to each other and usually at right angles to the spindle axis. Under intermediate magnification, this often results in a figure that gives the superficial appearance of having a rod-shaped centriole at one pole and a small spherical centriole at the other (see Figure 2). Under high magnification (of the light microscope), the spherical centriole can be seen to be the optical section of a vertical rod, at right angles to the horizontal rod-shaped centriole at the other pole. In eggs in which the plane of section did not happen to coincide with the longitudinal axis of the spindle, the same right-angle relationship between the two centrioles of a given spindle can be demonstrated by studying the serial sections. Whatever the angle of orientation of one centriole in relation to the plane of section, the centriole at the

[2] In the ovum fixed in Champy's fluid, the spindle measured 134 microns between the centers and 40 microns in diameter at the equatorial plate. The asters were about 80 microns in diameter.

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upper, and both lower fixed in hot Heath's; all stained in Heidenhain's iron haematoxylin. The slender rod-shaped centrioles (0.25 micron in diameter) do not show clearly at this magnification. 550 ×.

other end of the same spindle was found to be approximately at right angles to the first.

Electron microscope studies of centrioles by Burgos and Fawcett (1955), by Porter (1956) and by de Harven and Bernhard (1956) indicate that centrioles are cylinders, consisting of 9 rods in a matrix, as mentioned above. In repeated cases in vertebrate cells, where two centrioles are found close together and therefore probably recently divided, the bundles of rods are oriented at 90° to each other. Here, in this resting spindle of *Polychoerus,* even though the centrioles are at opposite poles, they are also oriented at 90° to each other. Since the centrioles of most vertebrate cells are tiny, measuring about 0.3 to 0.5 micron in length by 0.15

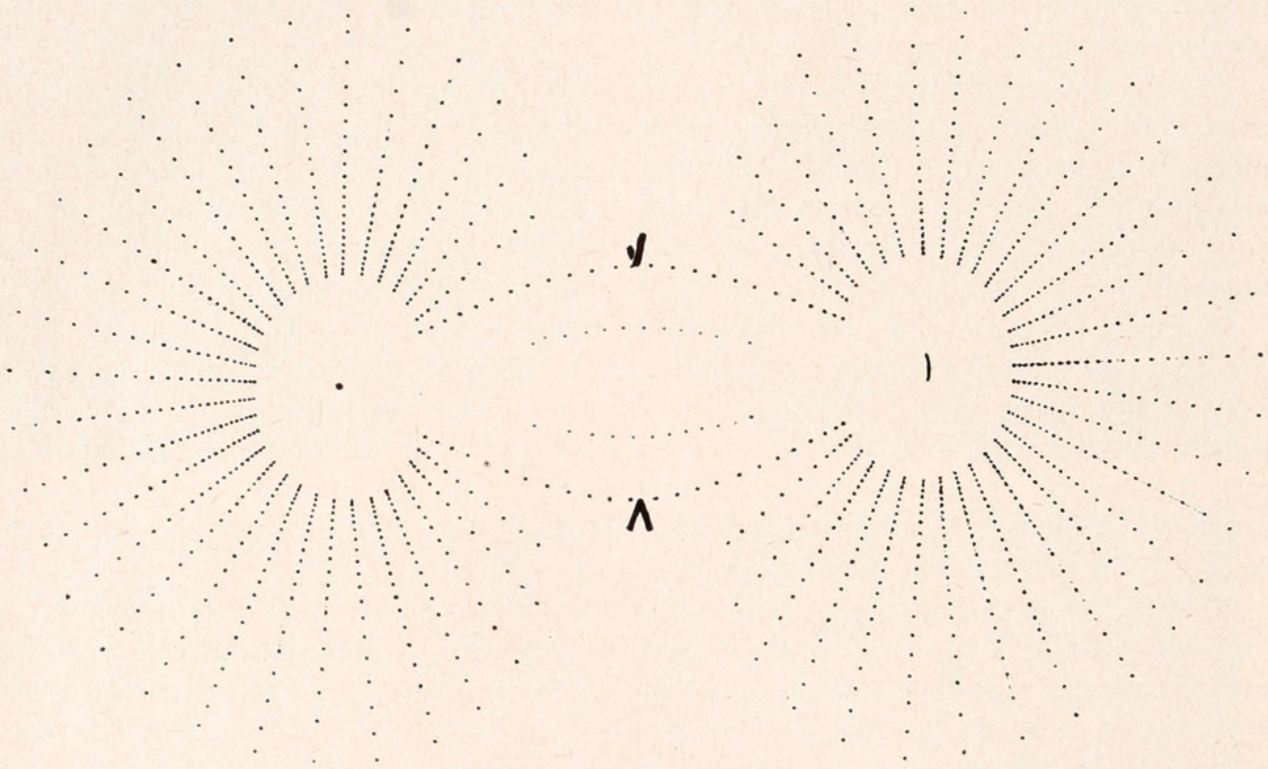


FIGURE 2. Diagram of resting first cleavage metaphase spindle of *Polychoerus,* to show centriolar orientation.

micron in diameter, there is not much chance of discovering the orientation of both of them in a given spindle by light microscopy in any appreciable number of cases. The light microscope does not give a high enough magnification to reveal their orientation. The thin sections used in electron microscopy are unlikely to include both centrioles of a spindle. However, de Robertis, Nowinski and Saez (1960) figure a thin-section electron micrograph (by Bernhard) which includes both poles of a cell in metaphase. Two centrioles are present at each pole, with the recently divided daughter centrioles at each pole at right angles to each other. The distal centriole of each pair is sectioned longitudinally, the proximal centriole transversely, in this particular case. This spindle, however, measured only 5.5 microns (calculated from the stated magnification) between the proximal centers.

Cells of the size of *Polychoerus* eggs are rare and giant spindles are practically unknown throughout the animal kingdom. So far as I have been able to ascertain from the literature, only the spindles of the cleaving egg of the whitefish approach

those of *Polychoerus* in size. In the whitefish, chromosomes extend throughout the equatorial plate, and there is no distinctive central spindle. Consequently, the details of spindle structure and chromosomal arrangement are far less clear.

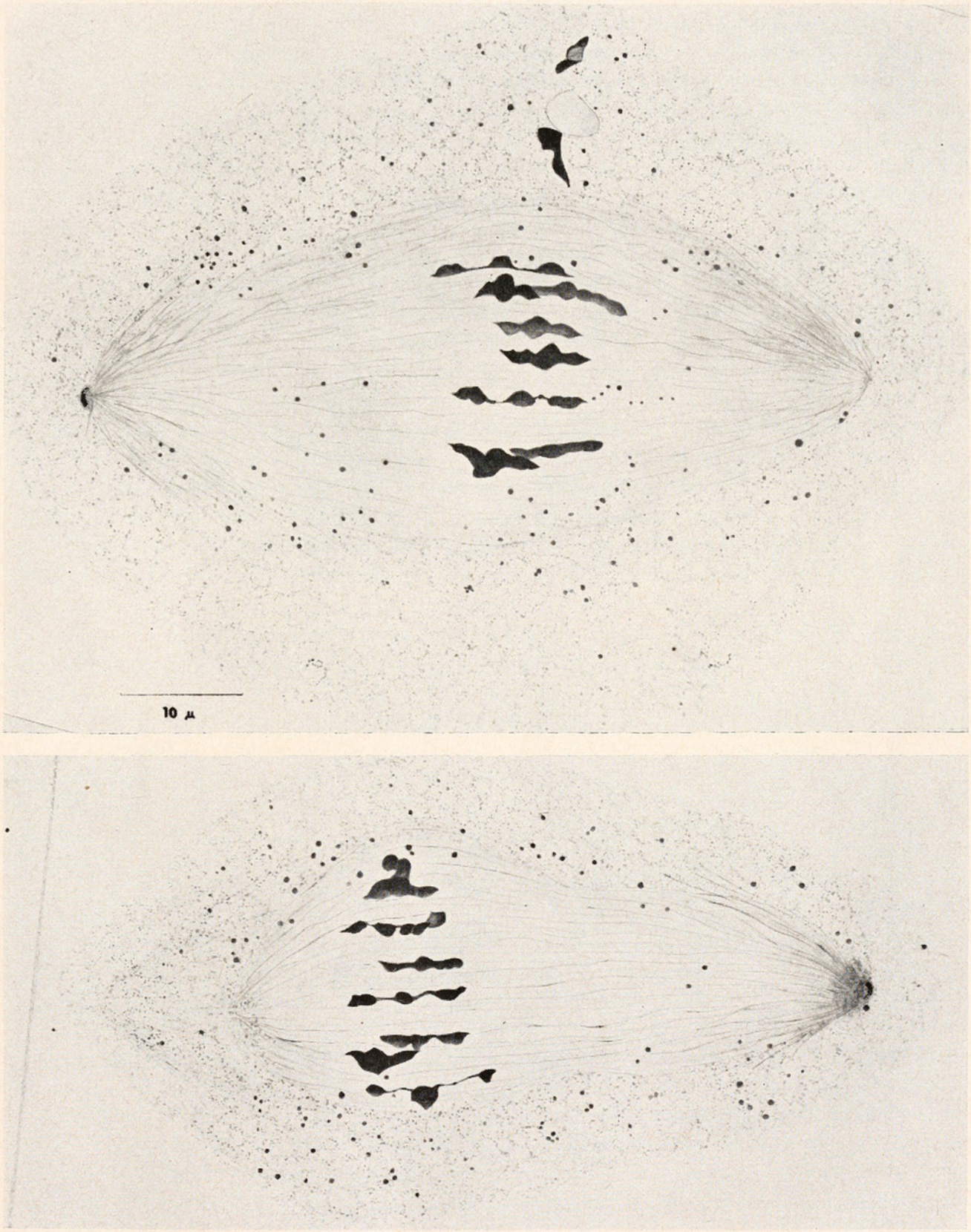


FIGURE 3. Wash drawings, of two adjacent 10-micron sections through metaphase of first maturation spindle of primary oöcyte of *Polychoerus*. Fixation in Worcester's at 60°, stained with Heidenhain's iron haematoxylin and eosin. Figures 3, 4 and 5 were drawn by Laliah Curry Runyon (Mrs. Ernest Runyon).

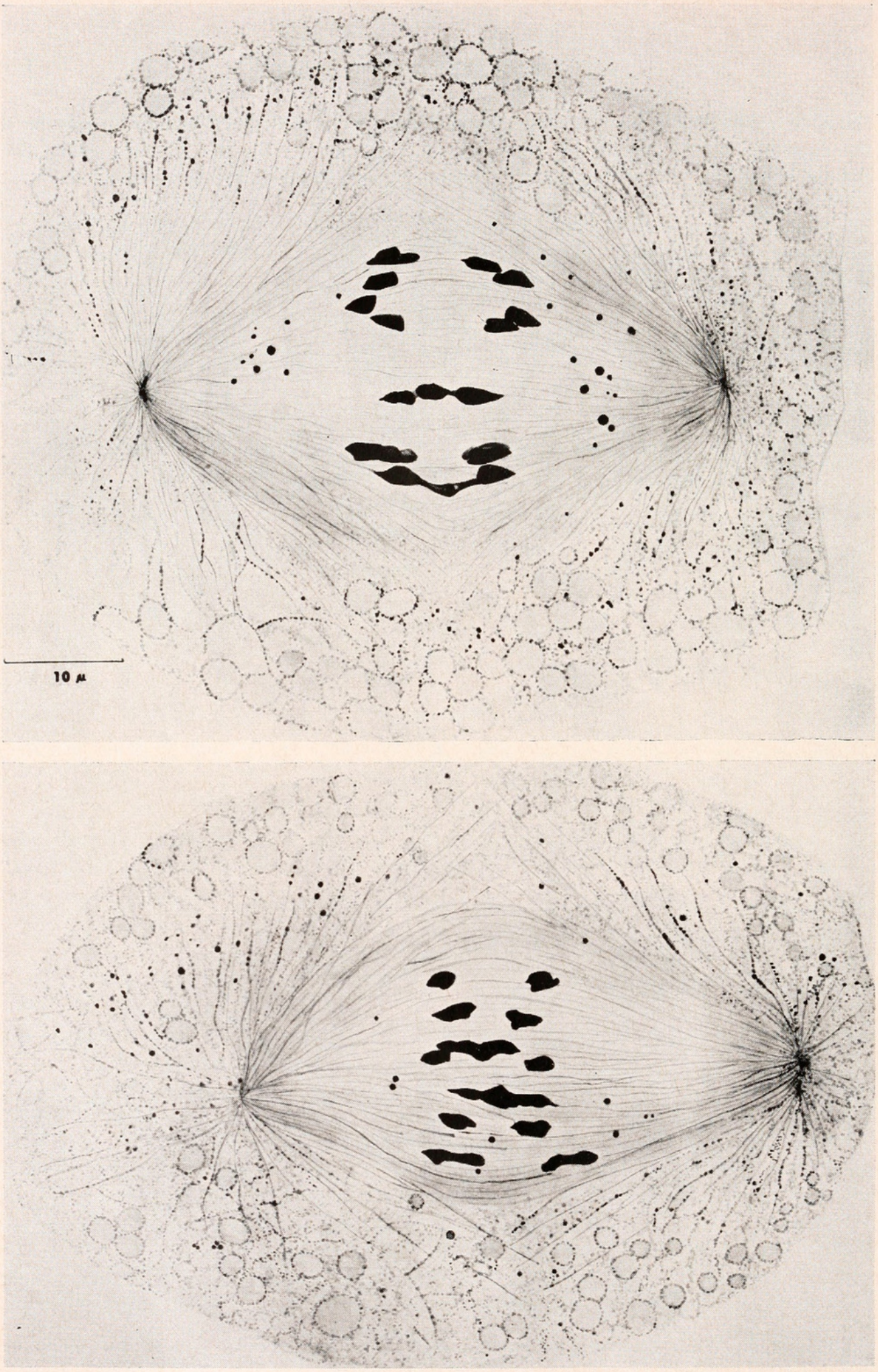


FIGURE 4. Wash drawings of two adjacent sections through first maturation anaphase of primary oöcyte of *Polychoerus*. Fixation: Heath's at room temperature; staining, Heidenhain's iron haematoxylin.

When one considers that one of the most favorable (and largest) cells used for the study of the mitotic spindle, namely, the grasshopper neuroblast, is only 30 microns in diameter, and that most mammalian cells are much smaller than this, the advantages of a form like the *Polychoerus* egg are apparent.

Giant centrioles are, however, not unknown. Cleveland (1935a, 1935b, 1935c, 1938, 1957a, 1957b) has described centrioles (in members of the flagellate genera *Pseudotrichonympha* and *Barbulanympha*) up to 80 microns in length, and in these and in other flagellates, such as *Spirotrichonympha* (Cleveland, 1958), the demonstrated relations between these and the central spindle and chromosomes are rapidly advancing our knowledge of the functions of the centrioles.

The centrioles of the first maturation spindle of the egg of Polychoerus

Since the sectioned material included many animals containing oöcytes fixed while undergoing the maturation divisions, it was imperative to examine these, also, to ascertain the structure and orientation of the centrioles, for comparison with those of the first cleavage spindle. The first maturation spindle of *Polychoerus carmelensis* measures about 75 microns between the centers at metaphase. It is 25 microns in diameter at the equator. Instead of having a central spindle consisting entirely of continuous fibers, there are at least three chromosomes located within the spindle, while the others are more peripherally arranged. This has been observed in transverse sections through the equatorial plate region. The asters of the first maturation division are much less well developed than those of the cleavage spindle, but in the better-fixed material, centrioles are clearly visible at each pole. These centrioles are relatively small, measuring about 1 micron in length by about 0.25 micron in diameter. Some variations in form of the centrioles are found at various stages during the first meiotic division. At the pre-metaphase stretch stage and also at metaphase, centrioles have been found in the shape of curved rods, which may or may not have slightly enlarged ends. These curved rods are oriented with the convex side away from the equatorial plate, the concave side (and free ends) extending toward it. An imaginary line across the free ends of the centriole at one pole is approximately at right angles to the long axis of the spindle, and in almost every case examined was found to be *at right angles to the centriole axis* at the opposite pole of the same spindle. In other spindles at approximately the same or a later metaphase stage, the centrioles were distinctly double at each end of the spindle, consisting of two short rods (0.75 to 1 micron by 0.25 micron), end-to-end or at a slight angle to each other. The apex of the angle was always directed away from the equatorial plate. Obviously these are bivalent centrioles, and they are also oriented at right angles to each other at the opposite poles of each spindle (see Figure 4). There is very little distance (0.1 to 0.2 micron) between the apices of the two rods, and it is possible that some of the centrioles which looked like single curved bodies actually consisted of two parts. They may, however, represent stages immediately preceding duplication of the centrioles, but this requires further detailed study.

There are 17 tetrads on these first meiotic spindles of *Polychoerus carmelensis*. A description of them, and of the stages of breakdown of the germinal vesicle, will be the subject of a later paper. Meanwhile, it may be mentioned that the first meiotic spindle originates as a central spindle of *extranuclear origin*, generated as

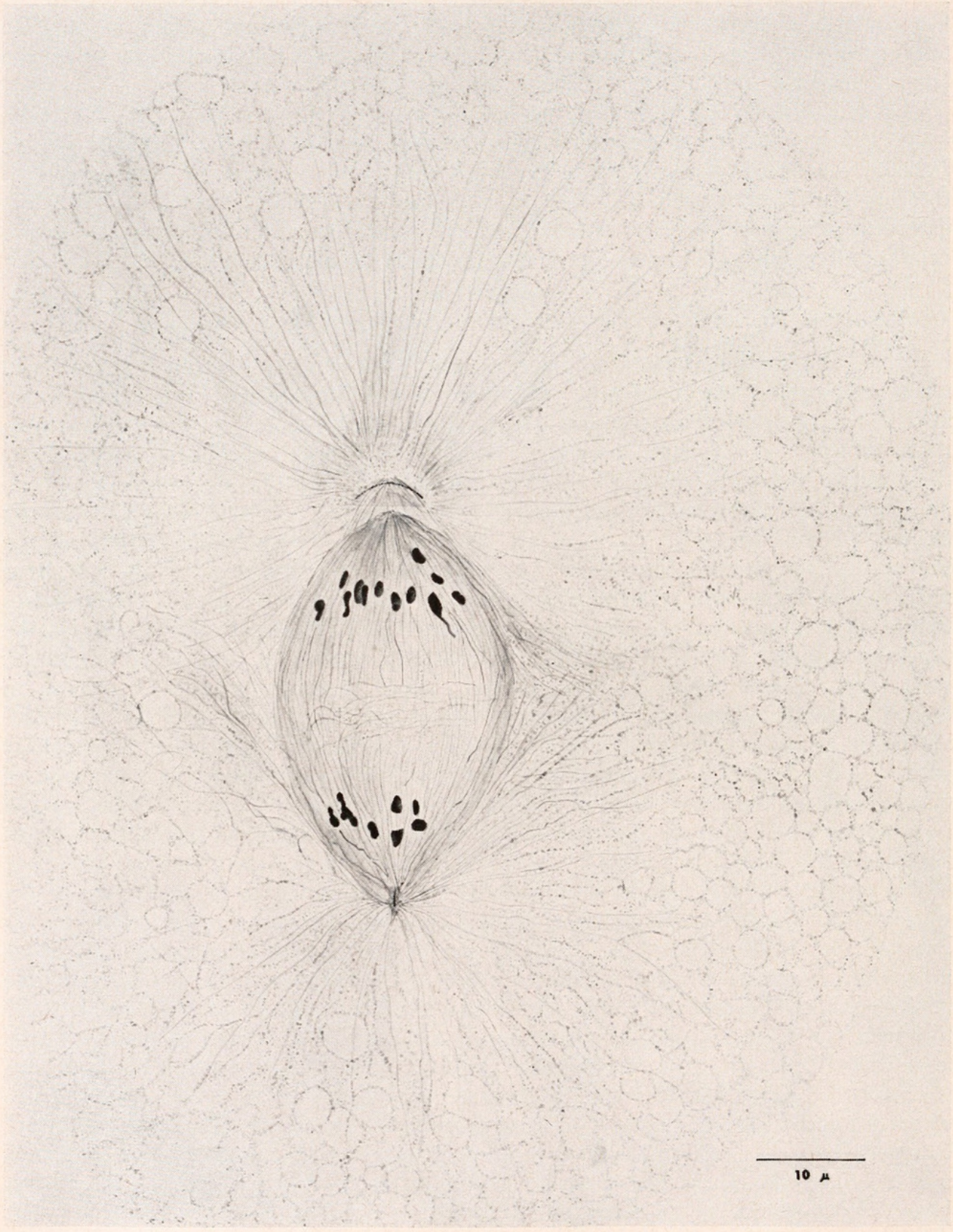


FIGURE 5. Wash drawing of 10-micron section through second maturation anaphase of a secondary oöcyte of *Polychoerus*. Fixation in Worcester's at room temperature; stained in Heidenhain's iron haematoxylin and eosin.

the two primary centrioles and asters move apart and around the oöcyte nucleus, before the wall of the germinal vesicle shows any signs of rupturing. At the same time, in numerous primary oöcytes, smaller secondary centers (with asters), between 5 and 20 in number, appear in the cytoplasm near the wall of the germinal vesicle. These secondary centers may even persist at various points in the cytoplasm, long after the first maturation spindle with its primary centers is fully established. The secondary centers are too tiny for an analysis of their shape. After the rupture of the oöcyte nucleus, the chromosomes become attached to the central spindle and undergo marked pre-metaphase stretch movements.

The centrioles of the second maturation spindle of the egg of Polychoerus

There were not many second meiotic spindles found. These are relatively shorter, broader spindles (38 microns long by 21 microns in diameter at anaphase) than those of the first meiotic division, and contain 17 dyads at metaphase. The astral regions are somewhat better developed than in the first meiotic spindle. The centrioles of these second meiotic spindles are truly remarkable. They are long, thin bodies, curved into a crescentic form. The clearest of these are 5 microns in length and so thin as to be just at the limit of microscopic visibility (*i.e.*, about 0.2 micron). The concave side (and free ends) are directed toward the equatorial plate, the convex side away from it. These resemble the centrioles of the second spermatocyte division of *Nemobius,* as described (his Fig. 39) by Baumgartner (1929), although in the gryllid material the centrioles are straight. At the two opposite ends of the same spindle, the axes of the centrioles are again at right angles to each other (see Fig. 5). There is no indication of duplication in any of these second meiotic centrioles. These appear, then, to be univalent. Presumably because of the orientation of the two centrioles, these second meiotic spindles look much more heteropolar than did the first.

Since the metaphase centrioles at the opposite poles of the first maturation spindle, of the second maturation spindle, and of the first cleavage spindle, even though differing in size, valency, etc., are oriented at right angles to each other, it would seem that this is an inherent property of either the centrioles or the protoplasm of the species.

The details of the origin of the cleavage centers for *Polychoerus carmelensis* have not yet been completely worked out. However, enough stages have been studied (including many examples of the association of the 17 maternal with the 17 paternal chromosome vesicles) to indicate that the process in *Polychoerus* is probably not unlike that in most other forms; thus far, however, a sperm nucleus has not been seen at the stage when it bears a sperm aster. Gardiner (1898) figures such a stage (his Fig. 24) for *Polychoerus caudatus.* At the period of association of the maternal and paternal chromosome vesicles, two rod-shaped centrioles, with well developed asters, are frequently found (Fig. 6). Occasionally the two centrioles and asters are sufficiently far apart as to appear to be associated with separate groups of chromosome vesicles. These centrioles resemble those of the first cleavage metaphases in size and shape. The astral rays can be seen to originate along their entire lengths.

Curiously enough, I have thus far been unable to identify with certainty any centrioles (or asters) in the first or second meiotic divisions of the spermato-

cytes, which are found in other regions in the same serial sections of the same (hermaphroditic) animals that show such beautiful egg centrioles and asters. Fusiform spindles with tetrads or dyads are clearly seen, and the material appears to be excellent for a comparison of sperm chromosomes with egg chromosomes at corresponding meiotic stages. Large, distinct black dots (after haematoxylin staining) in the spermatocytes are undoubtedly the chromatoid bodies.

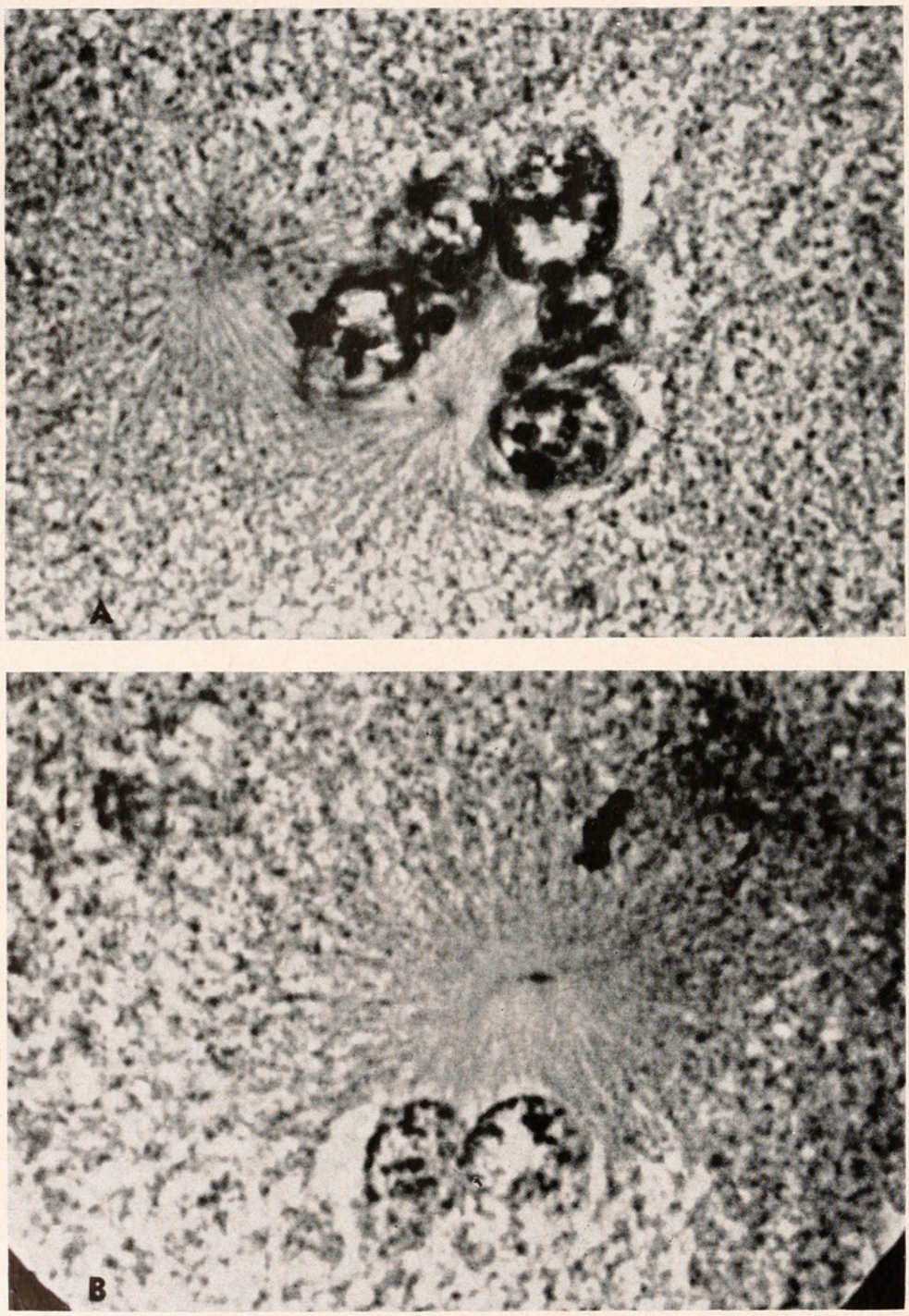


FIGURE 6, A. Beginning of cleavage diaster, and 6 of the 34 chromosome vesicles. The centriole between the vesicles is a rod, in plane of section. The other centriole is perpendicular to plane of section, and half is in the adjacent section. 910 ×.

FIGURE 6, B. Aster and one rod centriole of early cleavage diaster, associated with two chromosome vesicles, in another egg. Centriole foreshortened. Fixation: hot Heath's; stain: Heidenhain's iron haematoxylin. 910 ×.

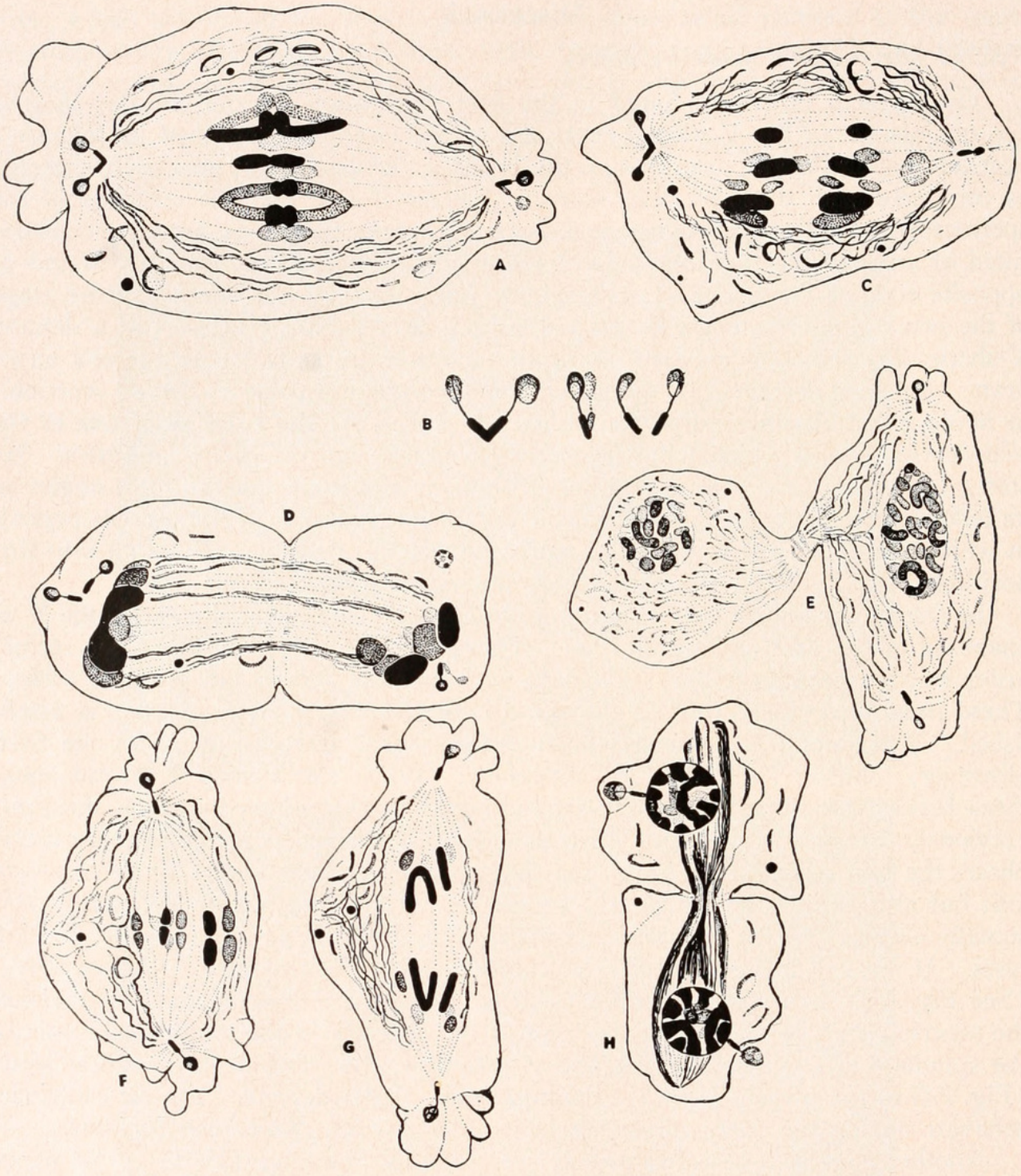
Discussion

Related observations made by others

The bivalent centrioles found in the first maturation division of *Polychoerus* are reminiscent of the V-shaped centrioles of the Gryllidae described in Johnson's excellent paper (1931), even though the V-shaped centrioles are oppositely directed as compared with those of *Polychoerus*. Johnson found that the centriole of the spermatogonium is a very short rod, scarcely longer than it is broad, frequently tilted at an angle to the long axis of the spindle. These centrioles are found at opposite ends of the nucleus *before* spindle formation. When the pachytene stage of the primary spermatocyte is reached, all the cells exhibit centrioles of a definite V-shape, directed apex inward, the tip of each limb in contact with the cell membrane. Johnson observed the migration and separation of these V-shaped centrioles in *living* material, and figured (his Figs. 83, A and B) the *rotation* of *one* of the two central bodies which had taken place over an interval of five minutes. He states (p. 124), "One central body nearly always lies in a plane at right angles to its fellow of the opposite pole, as seen in Fig. 35 [Figure 7,C of the present paper], in which the upper one is lying flat while the other is on edge. Rotation into this position is accomplished at various times during the prophase."

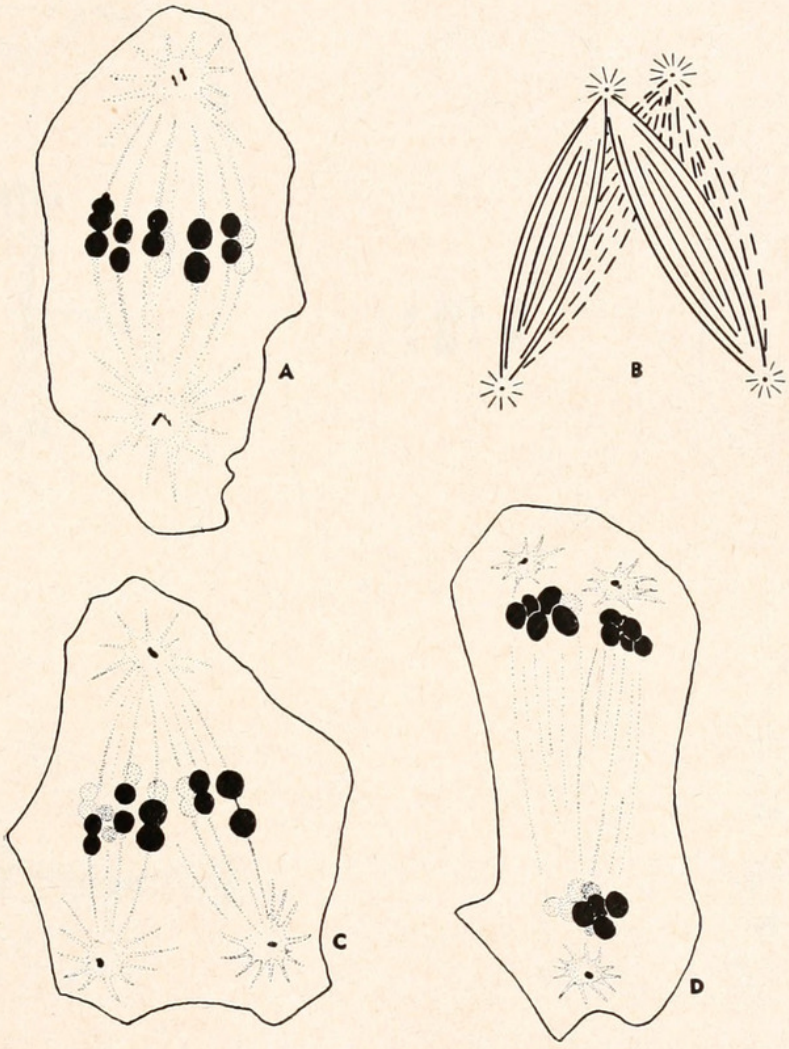
In *Oecanthus* and other Gryllidae studied, a split ocurs at the apex of the V at anaphase of the first meiotic division. This gives rise to centrioles of the second order which separate as short rods, each with its axial thread and terminal vesicle. These rods move apart 180° as the spindle for the next meiotic division is established. The second division spindle therefore forms at right angles to the first. However, Johnson (pp. 125–126) continues: "When the V-shaped central apparatus breaks into rods, the moieties move apart in that plane in which the arms previously diverged. Since the V's usually lie at right angles at the primary metaphase, the two secondary spindles will not only lie at right angles to the primary axis but also at right angles to each other. . . . That divisions of secondary cytes actually occur as I have indicated is quite evident in examination of second division cysts. If one cell is cut sagittally, its closest neighbor is usually cut transversely." (See Fig. 7,E in the present paper.) At metaphase of the second meiotic division the centriole rods of *Oecanthus* lie more or less directly in the longitudinal axis of the spindle, but sometimes, especially at anaphase, the elements are slightly tilted (Fig. 7,G in the present paper). Baumgartner (1929) described similar centriolar behavior during the spermatogenesis of another gryllid, *Nemobius fasciatus*.

Another equally interesting case is presented by Schrader (1941) in his paper on spermatogenesis in the earwig, *Anisolabis*. During the first meiotic division of the spermatocyte there are two centrioles visible at each spindle pole. He states (p. 136), "Each centriole is evidently a short rod. The axes of the two centrioles in each centrosome have no definite relation to each other and almost every possible variation has been encountered." Many of these cells divide normally (see Fig. 8,A of the present paper), while certain other cells exhibit a most interesting anomaly of spindle behavior (Figs. 8,C and D). As Schrader states (pp. 129–130), "In such cells the two centrioles at each pole, which normally remain closely associated in a single centrosome, begin to separate—usually just about the time that the metaphase is being established. . . . The precocious separation of the centrosomes occurs simultaneously at both poles of the cell involved. . . . The movement of



FIGURES 7, A through 7, H redrawn by Helen M. Costello from Johnson (1931), *Zeitschr. wiss. Zool.*, 140, with the permission of the publisher and author. All are of *Oecanthus nigricornis*. (A) First meiotic metaphase in primary spermatocyte. Centrioles at right angles to each other, with axial filaments and terminal vesicles. (B) Centrioles from first meiotic figures in front and side views. Daughter centrioles at right, after division of a V-shaped centriole. (C) First division, anaphase. The centriole at the right pole is in end view. (D) First division, telophase. Division and beginning migration of daughter centrioles. (E) Secondary spermatocytes at interphase. Adjacent daughter cells connected by spindle-bridge from the first meiotic division. Note the relation of the cell axes. The left cell is cut in transverse section. (F through H) Second meiotic division, metaphase, anaphase and telophase, respectively. Note the asymmetry of distribution of the chondrioconts in (F) and (G), and the telokinetic rotation of the nuclei and centrioles in (H). The dark dots are chromatoid bodies.

the centrosomes after separation is usually very definite. It is such that a plane passing through the original axis and the sister centrosomes at one pole is nearly always at right angles to one passing through the pair at the opposite pole. . . . The result is a quadripolar figure which, if it shows the centrosomes of one pole at the same focal level, will present the opposite pair of centrosomes optically superimposed on each other." (See Figs. 8,B and D of the present paper.)

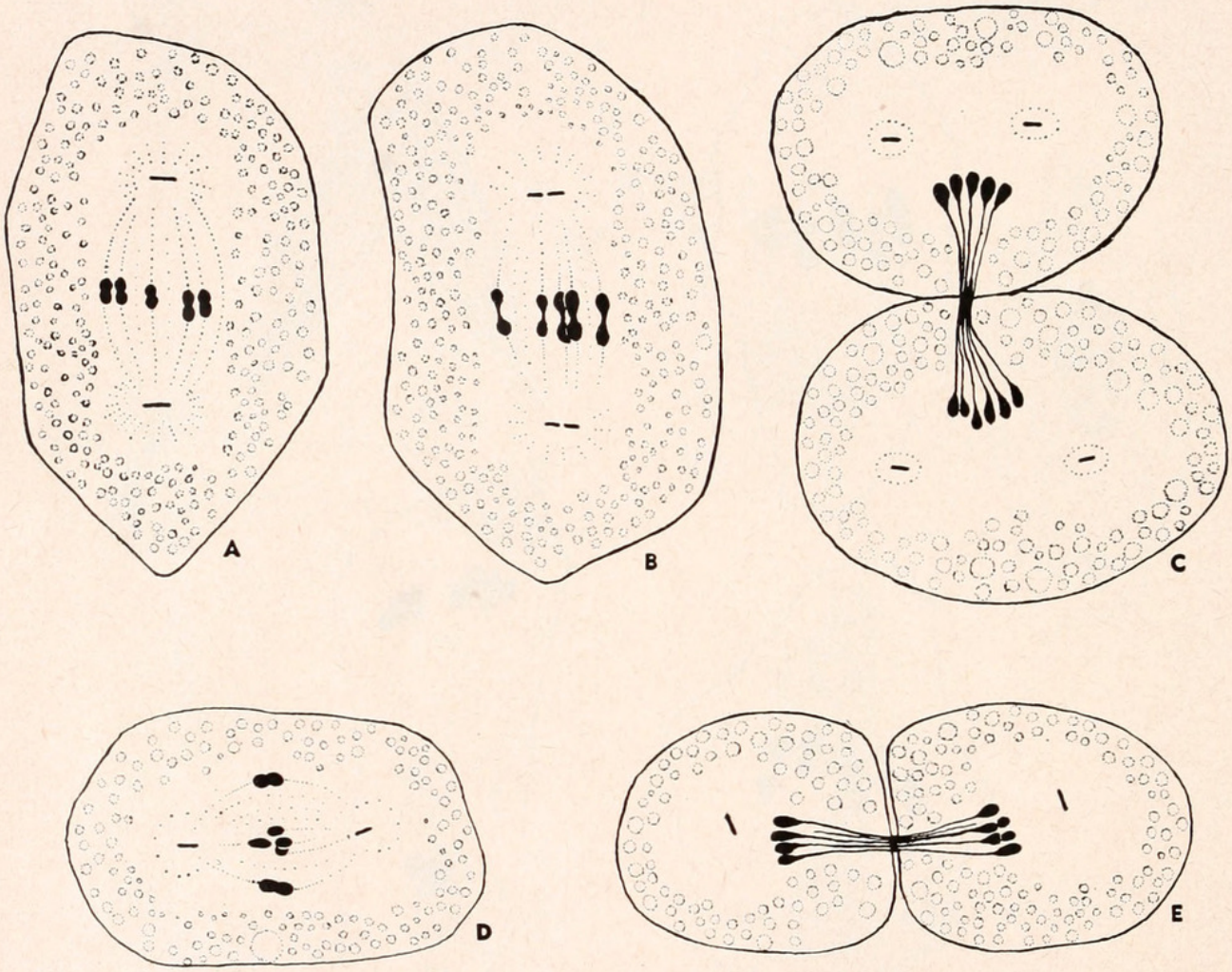


FIGURES 8, A through 8, D redrawn from Schrader (1941), *J. Morphol.*, **68**, with permission of the publishers and author. All are of *Anisolabis maritima*. (A) Normal first spermatocyte metaphase, in side view. (B) Diagram showing the four spindles that arise after precocious separation of the centrioles in two planes. (C) First meiotic metaphase with precociously divided centrosomes. Those at upper pole superimposed on each other and in a plane at right angles to those of lower pole. Only two of four spindles shown. (D) Telophase of first meiotic division, with precocious centrosomal division. The centrosomes at the lower pole superimposed on each other. Chromosomes in two groups at the lower pole.

The *Anisolabis* material of Schrader therefore shows a type of centriole behavior similar to that described by Johnson (1931) for *Oecanthus,* except that the *precocious separation* of the centrioles in the anomalous *Anisolabis* cells did not give rise (Schrader, p. 130) to the production of continuous fibers (*i.e.,* a spindle) between them.

In the spermatogenesis of the hemipteran *Gelastocoris,* as described by Payne

(1927), a different situation obtains. Payne states (p. 319), "[In the primary spermatocyte] the centriole is still single and a straight rod and lies at right angles to the long axis of the spindle [Figure 9,A in the present paper]. As the chromosomes constrict and begin to move apart the centriole divides transversely into two equal parts, which gradually separate as the chromosomes move toward the poles of the spindle [Figure 9,C in the present paper]. The second division follows



FIGURES 9, A through 9, E redrawn from Payne (1927), *J. Morphol.*, 43, with permission of publishers and author. All are of *Gelastocoris oculatus*. (A) Centriolar orientation at metaphase of the first spermatocyte division. (B) Early anaphase of the first spermatocyte division. The centrioles have divided. (C) Late anaphase of the first meiotic division. Centrioles have moved apart to become the centrioles for the second division. (D) Metaphase of second meiotic division. Centrioles have retained orientation in each daughter cell resulting from first division. (E) Telophase of the second meiotic division. Centrioles have turned and moved to one side in each daughter cell (spermatid).

without a nuclear reconstruction and the division figure is built around the two new centrioles which now lie more or less parallel to the long axis of the spindle [so that both spindles of daughter secondary spermatocytes lie in one flat plane]. As the chromosomes approach the poles in this division, the centriole moves to one side and lies here during the formation of the spermatid nucleus [Figure 9,E in the present paper]. . . ."

Thus we have two strikingly contrasting conditions of cell orientation in

Oecanthus and *Gelastocoris*, associated with differences in *centriolar orientation* and *behavior*.

Since in both these cases we are dealing with terminal divisions in spermatogenesis, the further significance is not immediately apparent.

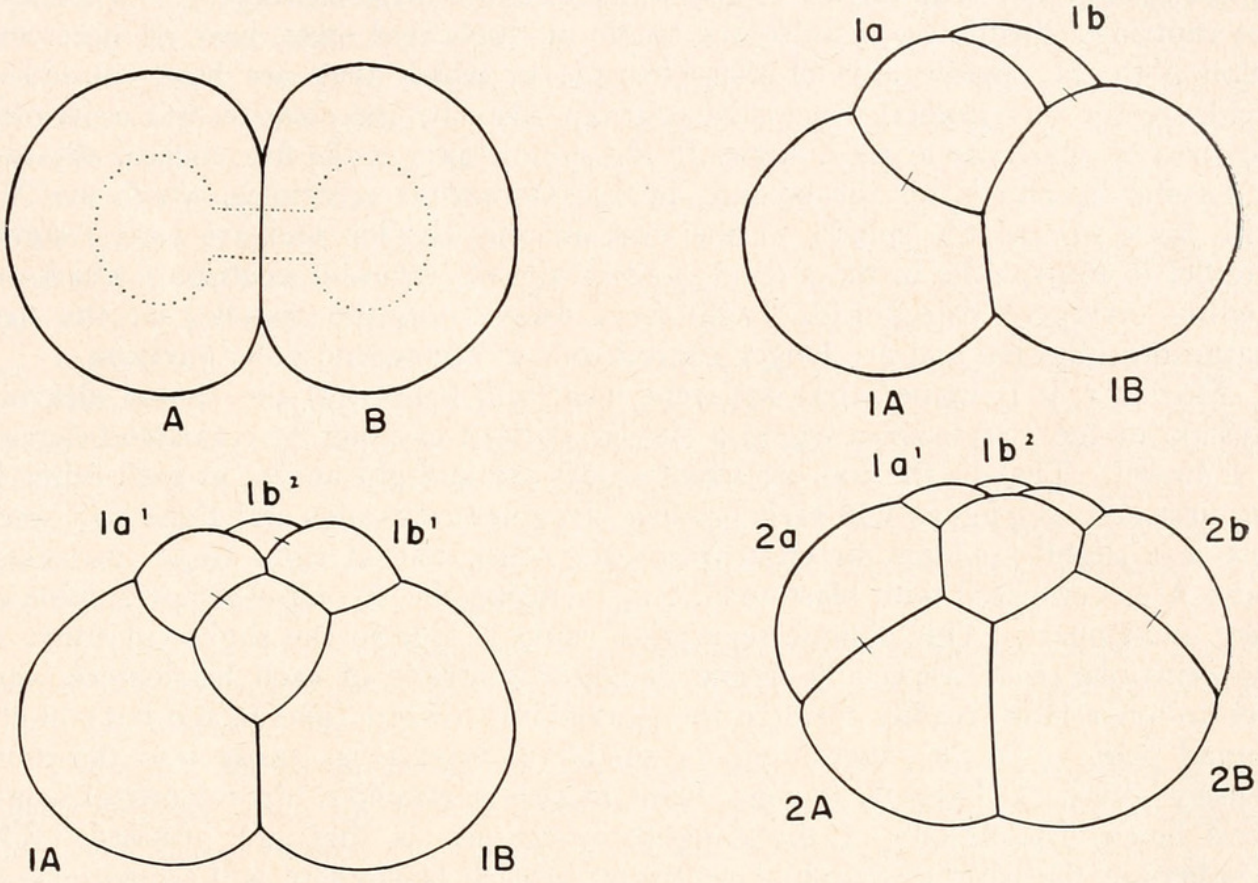


FIGURE 10. The early cleavages of *Polychoerus carmelensis* drawn from photomicrographs of living eggs. Egg membrane (chorion) and external jelly omitted. The cleavage of *Polychoerus carmelensis* differs in many details from that described by Bresslau (1909, 1933) for *Convoluta*, and by Gardiner (1895) for *Polychoerus caudatus*. (Upper left) Two-cell stage at the time of completion of the first cleavage furrow. The amphiaster is visible. (Upper right) Four-cell stage, side view. Two micromeres (1a, 1b) have been formed by a leiotropic second cleavage. (Lower left) Six-cell stage, side view, slightly tilted toward the observer. The members of the first duet of micromeres (1a, 1b) have divided by a dexiotropic cleavage, to give $1a^1$ and $1a^2$, and $1b^1$ and $1b^2$, and there has been some shifting of the blastomeres. (Lower right) Eight-cell stage, side view. The macromeres 1A and 1B have divided almost bilaterally but somewhat dexiotropically, producing 2a and 2b, and becoming 2A and 2B.

The possible significance of centriolar orientation

The *Polychoerus* material gives information not only on the orientation of centrioles during oögenesis, but also on their orientation for first cleavage. Omitting, for the moment, a discussion of the situation regarding the formation of the polar bodies, the possible significance of the relation of centriolar behavior to cleavage may be considered.

The first cleavage of the egg of *Polychoerus* is equal (or near-equal), giving rise to two cells, A and B (Fig. 10), with presumably identical cytoplasmic compo-

sition. The first cleavage plane bisects the large spindle and presumably passes through the animal pole, although this pole is not marked by any polar bodies. As I have pointed out briefly (Costello, 1960b), the polar bodies are not extruded but are intracellular, mark no axis, and eventually degenerate.

The right-angle orientation of the slightly curved centrioles to each other and to the spindle axis has obviously not influenced this first cleavage. The axes of the centrioles themselves, as also the single or duplicated state, bear no necessary relation to the spindle axis of that cleavage in which they are being observed. Evidence for this general conclusion is given, also, by the *Gelastocoris* centrioles, the axes of which are at right angles to the spindle axis in the first meiotic division and coincide with it for the second; by the *Oecanthus* centrioles, which are V's with apex toward the spindle at the first meiotic division and are rods roughly parallel to the spindle in the second; and by the *Polychoerus* centrioles which are slightly divergent paired rods (with apex away from the spindle) at the first maturation division and are longer curved rods at the second polar division.

However, it is immediately apparent what will happen at the second cleavage division of the *Polychoerus* egg if a similar pattern of inherent centriole behavior is followed. That is, the rod centrioles which are at right angles to each other in the first cleavage figure will each produce daughter centrioles and these will separate in a slightly oblique direction from each other, and at right angles in blastomere A as compared with blastomere B, generating the second cleavage spindle as they move apart. This oblique separation could be due to the slight curvature of the centriolar rods, especially if one daughter centriole of each blastomere were free to move more readily through the protoplasm toward (and to the left) of the animal pole, while the other might be unable to migrate as easily into the more densely yolk-packed vegetal region. It might also be related to the manner of origin (and hence orientation) of the daughter centrioles as they are produced. The members of the pairs of daughter centrioles in each blastomere will likewise be at right angles to each other, the upper one nearer the animal pole and at right angles to the lower one, near the middle of the vegetal hemisphere. The two oblique second cleavage spindles must likewise be assumed to be heteropolar, with the upper ends differing from the lower ends (like the heteropolar auxocyte of *Oecanthus* shown in Johnson's Fig. 5). This might be due to the type of protoplasm (animal versus vegetal) in which the ends lie. When this second cleavage is completed, we would then have, by a typical *leiotropic spiral cleavage,* a pair of micromeres, 1a and 1b, surmounting a pair of macromeres, 1A and 1B; this actually results at the second cleavage of the *Polychoerus* egg (Fig. 10, upper right).

If a similar pattern of centriole behavior is repeated at the third cleavage, with the formation of spindles determined by the axes of daughter centriole separations, the result would now be a *dexiotropic* cleavage for each cell that had arisen leiotropically. When this cleavage cycle is complete, we would have an 8-cell stage, with four micromere products ($1a^1$, $1a^2$, $1b^1$, $1b^2$), two second duet micromeres (2a, 2b) and two second generation macromeres (2A, 2B) (Fig. 10, lower right). We can thus explain the *regular alternation of leiotropic and dexiotropic* cleavages in the early development of this form, and, *presumably, in all other spirally cleaving forms.* (*Polychoerus* shows spiral cleavage by duets, practically all forms other than acoels show spiral cleavage by quartets. The mechanics of centriole behavior in the quartet type could be similar, but more complex.)

One can, therefore, set up the general hypothesis that the orientation of the centrioles with respect to each other and with respect to cell polarity, at any given division (meiotic, mitotic [or cleavage]) in which centrioles are normally present, determines the *original* position in which the daughter centrioles will separate from each other. This, in turn (provided there are not secondary re-orienting factors operating), *determines the main axis of the spindle for the next division, and hence* (barring the intervention of secondary factors), *the relative orientation of cells to each other* in the embryo, organ, or even organism. This orientation of the cells to each other should persist at least as long as the cells remain connected by the primary cell connective (spindle remnant or cell bridge), or until other forces move and re-orient them. The physico-chemical nature of the forces that orient the centrioles is as yet unknown (Schrader, 1947, 1953). It is clear that no simple explanation, in terms of electrostatic charges, nor of electromagnetic polarization, can account for rod centrioles orienting at right angles to each other.

A corollary of this hypothesis is the idea (already reasonably well established by others, as a working hypothesis) that the centrioles are the loci of origin of the orienting forces that spin out the astral rays and, at least, the continuous spindle fibers, and give rise to the distal centriole (blepharoplast) of the spermatid which spins out the axial filament of the sperm. There is likewise much evidence that the centrioles are the loci of forces orienting the mitochondria, etc. (Bowen, 1920, pp. 345-348; Johnson, 1931, p. 124).

Obviously, a theory dealing with centriolar behavior can have no direct application to cleavage patterns or cell orientations in forms where centrioles are lacking. (See also footnote 3.)

The apparent exceptions to the corollary view, in the forms where the spindle fibers originate from the kinetochores of the chromosomes (especially in anastral and acentriolar mitoses, and in forms showing diffuse chromosome fibers), may possibly be resolved by the suggestion (made years ago by Darlington, 1936; Schrader, 1936; Pollister, 1939; Pollister and Pollister, 1943; etc.) that the kinetochores contain centriolar material, or are, in effect, centrioles. This was especially beautifully demonstrated by Pollister's work on the supernumerary centrioles of certain gastropods. This matter could be resolved still more simply, if one assumes that the centrioles form the continuous spindle fibers, and the kinetochores, as slightly different counterparts of the centrioles, form the chromosomal fibers.

[3] It should be kept in mind that the role played by the centers or centrosomes in the mechanism of mitosis may vary in different organisms. Thus, Dietz (1959, *Zeitschr. Naturforsch.*, **14b**: 749–753) has shown that under experimental conditions in the insect, *Pales crocata*, a bipolar spindle may be formed even when one or both centrosomes are located in a distant part of the cell, and anaphase movements of the chromosomes may be quite normal. But how such an abnormal configuration of spindle elements affects cytokinesis is not reported by Dietz. Presumably, in *Pales*, also, irregularities in the division of the cell as a whole will result from such a displacement of the centers. Once the centrioles have done their work in spinning or orienting the fibers necessary for a given division, they may have no further function in that particular mitotic division. Hence, they could be removed and have the division continue. The kinetochores of the chromosomes might assume the entire function of fiber-formation under such circumstances, as they normally do in acentriolar mitosis. A more critical test of the effects of removal of centrioles would be to study the succeeding division (if this takes place) or to study the effect of centriole removal in a form where the centrioles clearly spin out the continuous fibers.

Further possible implications

This relation can be further checked by examination of the spindle axes in cleavage products of various other forms in which the centrioles are visibly rods of sufficient length so that their orientation can be ascertained.

If all the relations of centriolar patterns (it is obvious that there must be more than two) to cleavage patterns could be established, we could then use the more easily determined cleavage pattern as a means of ascertaining the centriolar pattern of the previous cell generation. This would enable us to predict what the centriolar pattern and behavior may be in forms in which the centrioles are too small (or too short, as cylinders) to ascertain this pattern directly. It may be possible, by merely glancing at a section of testis under the low power of the microscope, and finding that the axes of many secondary spermatocytes are at right angles to the axes of their nearest mates, to predict that the centrioles will be at right angles in the primary spermatocytes (as in *Oecanthus*). Where the spindle or cell axes lie parallel in adjacent secondary daughter cells, the *Gelastocoris* type of centriolar pattern must have obtained in the primary spermatocytes.

It is possible, of course, that there may be, in some forms, no constant relation between the daughter cells, but even in this case, the variability of arrangement may be attributable to the centriolar behavior. For example, in *Anasa tristis,* Paulmier (1899) found (p. 243) that ". . . the axes of the daughter spindles [in the two daughter secondary spermatocytes] were rarely parallel—the angles at which they lie being dependent upon the angles of the planes in which the two pairs of centrosomes [centrioles, in present terminology] move apart, these angles varying within 90°."

One further possible function of centrioles in determining movements of or within cells might be revealed through the investigation of the telokinetic movements in spermatids. In the spermatids (of the *Gelastocoris* type), when the centrioles move to one side of their respective nuclei (the same side) in both spermatids, as the interzonal connectives elongate, is there a correlated rotation of the two spermatid nuclei away from each other, one rotating clockwise, the other counterclockwise? Payne's paper does not show this stage.

The centriolar movements are presumably the first indications of the telokinetic movements, which have been described in more detail by Montgomery (1911), Bowen (1922) and Johnson (1931). Johnson (1931) states (p. 126), "Nuclei of sister spermatids may rotate into any position with respect to one another, about 55% turning at right angles to the direction of their sisters; i.e., if one is seen at the side of a spindle remnant, the sister nucleus is seen above or below the remnant. I do not believe that the direction of telokinetic movement in one cell has much effect upon the other product of the same division, but rather that the direction is controlled by the spatial relations of the cyst." But could it not be controlled by the positions of the centrioles in relation to the nuclei of the two daughter cells? Johnson's Figure 42 (Figure 7,H of the present paper) shows that the centriolar complex takes up exactly this position, so that the centriole-nuclear arrangement in one daughter spermatid is the opposite of that in the other. This is exactly opposite to the situation we should expect in *Gelastocoris.* We may tentatively suggest, therefore, that the centrioles supply the motive force that causes the interzonal fibers to continue to expand, to bring about the telokinetic rotation.

Early embryologists made many attempts to correlate the position of the first plane of cleavage or the median plane of the embryo with either the sperm entrance point or the copulation path of the migrating sperm nucleus. In various species these efforts met with some degree of success. The entrance point or sperm path may bear some constant relation, within these species, to the orientation of the sperm centriole, when it is finally in position to produce the centrioles of the sperm diaster, which become the centrioles of the first cleavage spindle. Hence, in this situation, also, the final orientation of the sperm centriole and the axis of separation of the daughter centrioles could be the significant feature in determining the first cleavage plane.

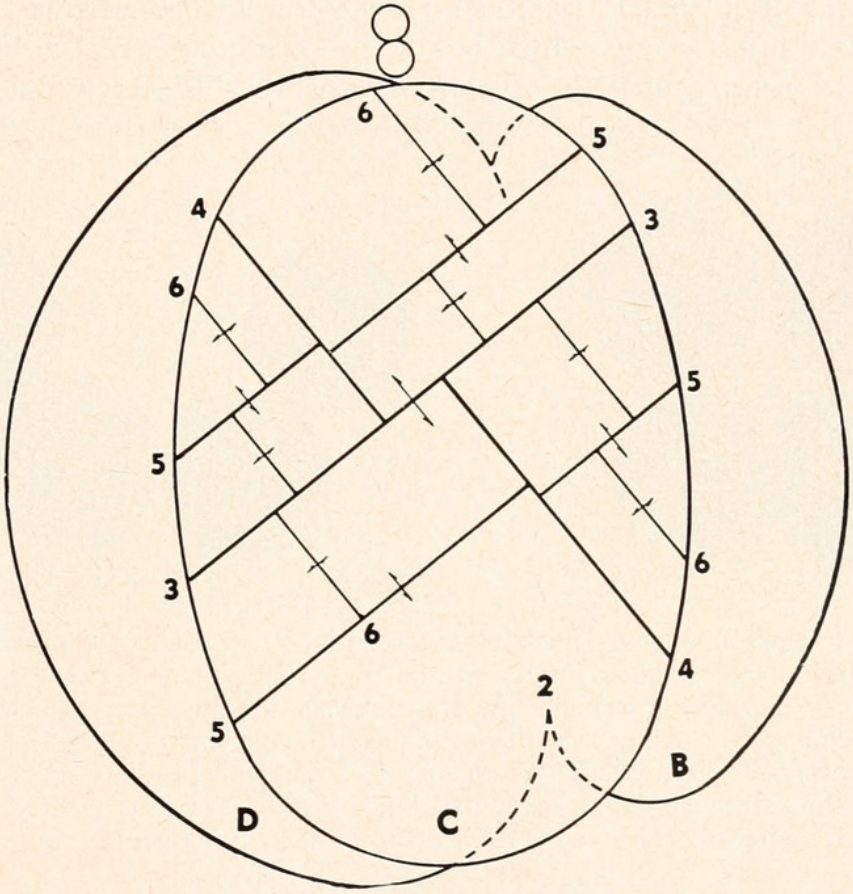


FIGURE 11. Diagram showing the positions of the successive cleavage planes from the third to the sixth (indicated by numbers), projected on the C quadrant of an unequally spirally cleaving egg (such as *Nereis*). Cleavages are alternately dexiotropic (odd-numbered) and leiotropic (even-numbered). Note that all dexiotropic cleavage planes are *at right angles* to all leiotropic planes. The A blastomere is omitted, and the second cleavage plane interrupted, for clarity. Displacements of the cells are not shown.

Patterns and transitions during spiral cleavage

The hypothesis that the orientation and behavior of centrioles may account for the pattern of spiral cleavage in forms like *Polychoerus,* where duets of micromeres are formed, can be readily extended to the quartet type of spiral cleavage, typical of *Crepidula, Nereis, Chaetopterus,* etc. In quartet type spiral cleavage, the spindle of any given cell lies approximately at right angles to those in adjacent cells in both adjoining quadrants at the 4- and 8-cell stages. The successive divisions of each cell, after the 4-cell stage, are alternately dexiotropic and leiotropic, during

the entire spiral period of cleavage. However, the first two cleavages in forms such as *Nereis* have the first two cleavage planes somewhat differently directed than the third through the sixth cleavage planes (see Figure 11). In the somewhat simpler situation of spiral cleavage by duets, as exemplified by the *Polychoerus* egg, it is only the first cleavage plane that is slightly anomalous, the others (until the transition to bilateral cleavage begins) being alternately leiotropic and dexiotropic.

There are thus two "critical" periods in spiral cleavage. The first is the period before micromere formation (examples: the first cleavage of *Polychoerus,* and the first two cleavages of *Nereis, Crepidula,* etc.). The second occurs when there is an inevitable transition from the strict alternation of dexiotropic and leiotropic cleavages to bilateral cleavages which herald the production of the bilaterally symmetrical acoel, polyclad, annelidan or molluscan larva. We know that in *Crepidula* (Conklin, 1897) the first cleavage is "prospectively" dexiotropic and the second is

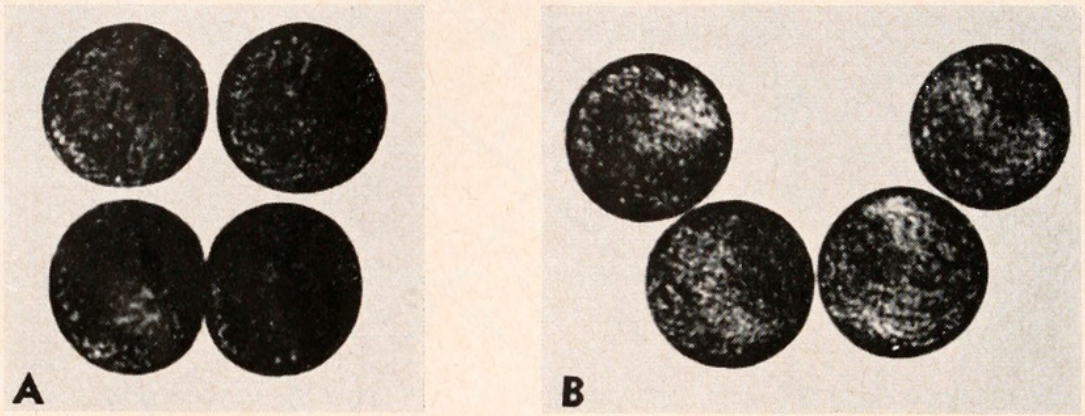


FIGURE 12. Four-cell stages of two living eggs of *Arbacia punctulata.* Magnification 340 ×. These eggs were fertilized in sea water containing Ishida (1936) hatching enzyme. They were transferred to Rulon calcium-low sea water 87 minutes later, and photographed 104 minutes after insemination. Note that the primary cell connective (spindle remnant) of the first cleavage connects the two lower blastomeres, and the two spindle remnants of the second cleavage connect the other daughter cells. All spindle axes are thus clearly indicated. (A) The initial position of the blastomeres. (B) Position after a very gentle tap on the coverglass.

"prospectively" leiotropic, as evidenced by the post-cleavage rotations of the protoplasmic areas and by the movements which lead to the formation of the cross-furrow. Perhaps someone will be able to pursue this subject, in a suitable material, and follow the behavior of the centrioles while the egg is undergoing the transition from spiral to bilateral cleavage. To my knowledge, there has been no other hypothesis suggested to attempt to account for the sequence of dexiotropic and leiotropic cleavages.

The primary cell connective

As cytokinesis is brought to completion and the two daughter cells are separated by the advancing cleavage furrow, there remains a spindle remnant of continuous and interzonal fibers, sometimes containing Zwischenkörper (Fig. 15). This spindle remnant, together with a thin layer of cortical cytoplasm and/or cell membrane, becomes the stalk or bridge connecting the daughter cells after division

is complete. In other words, the spindle remnant constitutes the major portion of the primary cell connective. This connective persists for a considerable period of time, if not permanently, in many materials. This is obvious to anyone who has ever isolated cleavage blastomeres free-hand with fine glass needles; the spindle remnant offers resistance to the needle and must be cut through. The axis of the spindle, of course, determined the point of attachment of the primary cell connective between the daughter cells.

If the eggs of *Arbacia punctulata* are treated to remove the membranes and examined at the 4-cell stage, as earlier demonstrated by Moore, (1930a, 1930b, 1945) for other echinoderm eggs, they show (Fig. 12) the primary cell connectives of the first cleavage spindle between the two middle cells, and the spindle remnants of the two second cleavage spindles at right angles to the first, between the other pairs of cells, exactly as pointed out by Moore (1930a, 1930b) for *Strongylocentrotus* and *Dendraster*. In the echinoderm, which is a radially cleaving form, these connectives are in the same plane, which is not the case in a spirally cleaving egg. The cell connectives are therefore a reflection of the pattern of orientation of the earlier spindles.

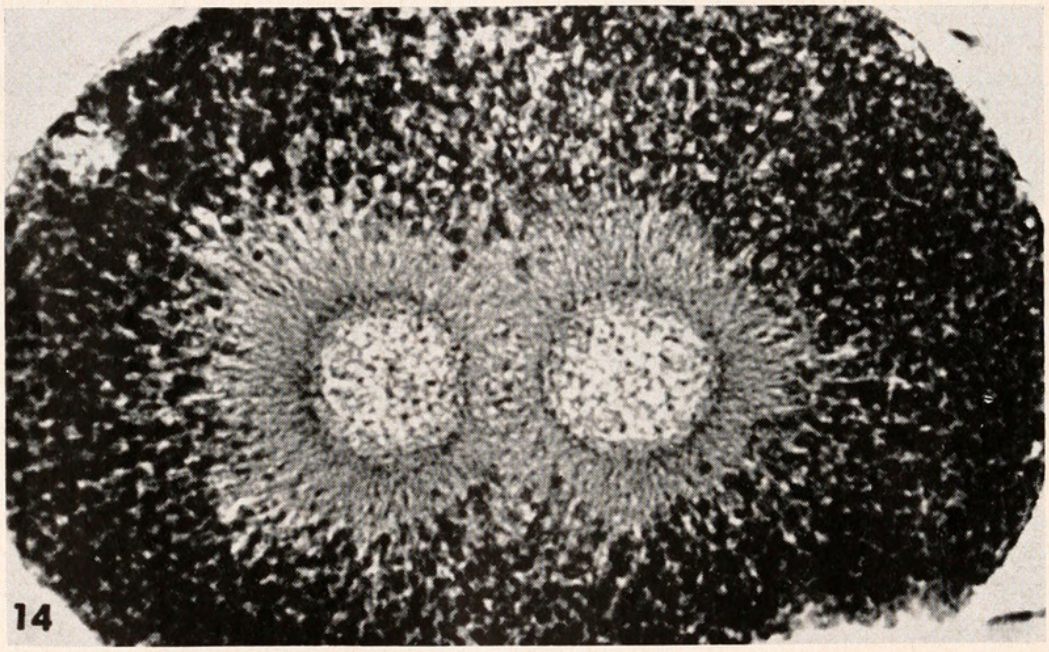
Kojima (1960) has also described the blastomere arrangements in the sea urchin egg, and has experimentally altered this relation by means of dinitrophenol. However, he does not interpret his results in terms of the centrioles, indicating only that the cause of the rearrangement is unknown. For really complex arrangements of multicellular bridges formed from spindle remnants among numerous cells during the oögenesis of *Vespa* (hornet), see the paper by Majiarski (1913).

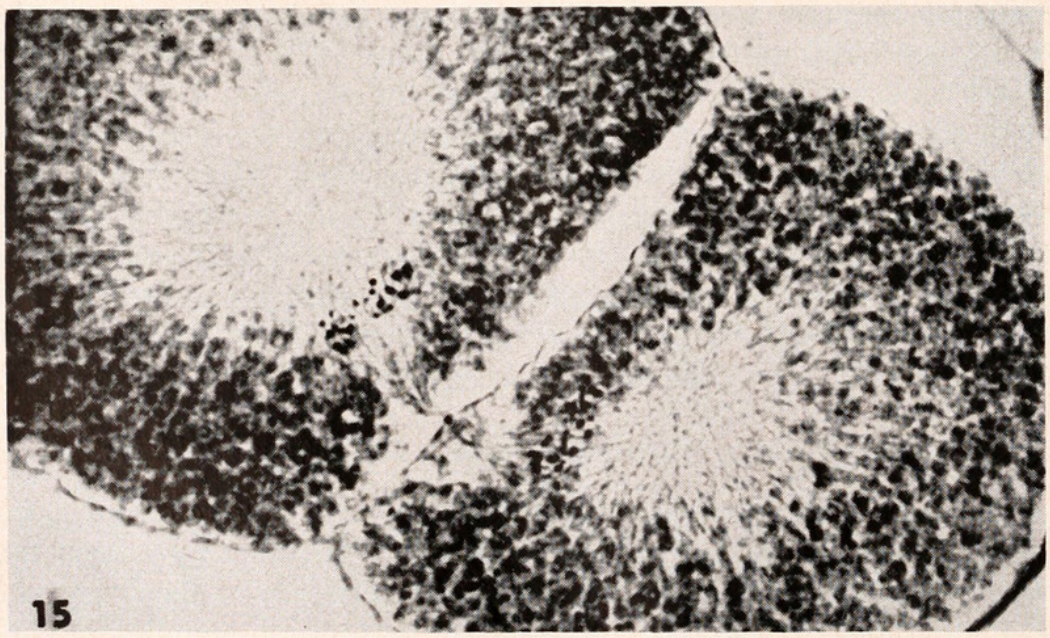
Sections through the eggs of *Polychoerus* just after the completion of the first division clearly show (Fig. 15) the nature of the primary cell connective. At the point where the furrow membrane meets the spindle remnant, Zwischenkörper may be distinctly seen. These have been described in many other cases, by early workers on cell division.

I believe that the primary cell connectives are far more important than is generally realized. In fact, the persistence of these connectives through a number of cell divisions may be a significant difference between Metazoa and colonial forms, on the one hand, and Protozoa on the other. In certain metazoan blastomeres the cell connectives are apparently totally absent. Fuliński (1916) described the complete separation of the blastomeres of the egg of *Dendrocoelum,* where the individual blastomeres lie dispersed in the external yolk. Here some other factor must have assumed the role taken by the primary cell connective in most forms. That the connectives are absent or can be relatively easily ruptured in some forms is indicated also by the experiments on the mechanical isolation of sponge cells (Wilson, 1907) and their subsequent reaggregation. Presumably, also, in many types of tissue cultures, the cells are free to wander.

Primary vs. secondary forces orienting spindles, and other problems

It is obvious that there are secondary forces which may re-orient a formed or forming spindle, and change the original relations which may have been established by centriolar orientation. The second maturation division of the ovum provides a striking demonstration of this fact. The second maturation spindle (of the secondary oöcyte), formed at the inner end of, and at right angles to, the axis of the first





FIGURES 13 through 15 are of *Polychoerus carmelensis.*

meiotic spindle (the position brought about by the separation of the inner daughter centrioles), must be rotated through 90° to become oriented perpendicular to the egg surface and give off the second polar body under the first. Similar forces may likewise be involved in orienting the first meiotic spindle perpendicular to the egg surface, at the animal pole. The nature of such polar forces is unknown, as is the nature of the forces that orient daughter centrioles with respect to each other. However, the maturation divisions of the ovum are terminal divisions—leading to no future role for the egg centrioles, in those cases in which the entering sperm centriole gives rise to the centrioles of the cleavage diaster. There may be other secondary re-orienting factors as well.

An equally important problem is whether there are differences in centriolar behavior patterns in cases where the daughter cells return to their resting interphase condition, as compared with rapidly dividing cells in which there is no nuclear reconstruction between successive cleavages. Conklin's extensive studies (1902) on centrosome and sphere during the cleavages of *Crepidula* may supply pertinent evidence here.

Division of cleavage centrioles

Because of the relative paucity of *Polychoerus* material available following the resting first cleavage metaphase stage, the stages of centriolar division have not yet been worked out. However, sufficient material was studied to suggest that the whole central apparatus of the *Polychoerus* egg may follow a pattern similar to that described by Vejdovský and Mrázek (1903) for the egg of the oligochaete, *Rhynchelmis*. In *Polychoerus* (Fig. 14), shortly after the eggs are laid, ". . . drastic changes occur at the central region of each aster, while the chromosomes are still at metaphase. The centriole disappears and the centrosomal region becomes a large sphere, which has acquired a definite boundary, not traversed by the rays, which remain outside it. Internally, the sphere is reticular, with numerous tiny granules, no one of which can be identified as a centriole. These spheres (centrospheres, astrospheres, or centrosomes), 40 μ or more in diameter, with their surrounding rays, are beautifully described as 'polar suns.'" (Costello, 1960a). In *Rhynchelmis*, there is a stage in the centrosomal cycle exactly corresponding to this "polar sun" stage. It is of transitory duration, and new daughter centrioles arise from within the reticulate mass where the old centriole had disappeared. The "disappearance" of a centriole may mean only that it has become unstable or unstainable, and has disappeared as a microscopically visible particle. Its reappearance may mean only that it has again become stainable, or of a size large enough to be microscopically detectable. This may complicate the study of the division cycle of the cleavage centrioles in *Polychoerus* by light microscopy, but it in no way negates the hypothesis outlined above.

FIGURE 13. Polar view of metaphase plate of resting first cleavage metaphase, showing all 34 chromosomes. Two were cut and one fragment of each was in the adjacent section. Fixation: Worcester's; stain, Heidenhain's iron haematoxylin. 990 ×.

FIGURE 14. Reticulate centrospheres and surrounding asters of 8-micron sagittal section of metaphase of egg just laid. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

FIGURE 15. Zwischenkörper, spindle remnant, chromosome vesicles, etc., at late telophase of first cleavage, in 8-micron section. Fixation: Flemming's; stain: Flemming's tricolor. 480 ×.

SUMMARY

1. The centrioles of the egg of *Polychoerus carmelensis,* at first meiotic metaphase, second meiotic metaphase, and resting first cleavage metaphase, are slightly curved rods which are usually oriented at right angles to each other and to the main axis of the spindle.

2. Centriole orientation and behavior in the spermatocyte divisions of Gryllidae and Hemiptera, as described by Johnson (1931) and Payne (1927), in relation to the arrangements of daughter cells, are compared with centriole orientation and predicted behavior in the egg of *Polychoerus.*

3. These considerations (on centriole orientation and behavior) constitute the basis for a new hypothesis, as follows:

a. The orientation of the centrioles at any given division determines the position in which the daughter centrioles will separate from each other.

b. The path of separation of daughter centrioles determines the position of the main axis of the spindle for the next division.

c. The axis of the spindle determines the relative positions of the daughter cells with respect to each other.

d. This arrangement of the daughter cells is maintained, for a time at least, by the primary cell connective, of which the spindle remnant is the significant portion.

e. These relations obtain in the absence of secondary intervening factors.

4. The inherent right-angle orientation of the centrioles at the two poles of the first cleavage spindle of *Polychoerus* is thus interpreted in causal relation to the alternating dexiotropic and leiotropic divisions in spiral cleavage.

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