Nonrandom Chromosome Segregation in *Neocurtilla (Gryllotalpa) hexadactyla*: An Ultrastructural Study

DONNA F. KUBAI and DWAYNE WISE

Department of Zoology, Duke University, Durham, North Carolina 27706. Dr. Wise's present address is the Department of Biological Sciences, Mississippi State University, Mississippi State, Mississippi 39762.

ABSTRACT During meiosis I in males of the mole cricket *Neocurtilla (Gryllotalpa) hexadactyla*, the univalent X_1 chromosome and the heteromorphic X_2Y chromosome pair segregate nonrandomly; the X_1 and X_2 chromosomes move to the same pole in anaphase. By means of ultrastructural analysis of serial sections of cells in several stages of meiosis I, metaphase of meiosis II, and mitosis, we found that the kinetochore region of two of the three nonrandomly segregating chromosomes differ from autosomal kinetochores only during meiosis I. The distinction is most pronounced at metaphase I when massive aggregates of electron-dense substance mark the kinetochores of X_1 and Y chromosomes. The lateral position of the kinetochores of X_1 and Y chromosomes and the association of these chromosomes with microtubules running toward both poles are also characteristic of meiosis I and further distinguish X_1 and Y from the autosomes. Nonrandomly segregating chromosomes are typically positioned within the spindle so that the kinetochoric sides of the X_2Y pair and the X_1 chromosome are both turned toward the same interpolar spindle axis. This spatial relationship may be a result of a linkage of X_1 and Y chromosomes lying in opposite half spindles via a small bundle of microtubules that runs between their unusual kinetochores. Thus, nonrandom segregation in *Neocurtilla hexadactyla* involves a unique modification at the kinetochores of particular chromosomes, which presumably affects the manner in which these chromosomes are integrated within the spindle.

The model for chromosome behavior during meiosis in all higher organisms is based upon Carothers' (3) careful analysis of the behavior of heteromorphic chromosome pairs and a univalent sex chromosome in grasshopper spermatocytes. Each heteromorph was found to assort completely randomly with respect to every other heteromorphic pair and with respect to the single X chromosome. These results are in accord with the chromosomal theory of inheritance as first stated by Sutton (13); pairs of homologous chromosomes are arranged on the spindle at meiosis I without regard to one another and, therefore, assort independently, just as do unlinked genes in crosses.

Ironically, observations that contradict the generality of this model were reported by Payne (10, 11) at about the same time as the work of Carothers. In spermatocytes of the Northern mole cricket, a relative of the grasshopper, Payne recognized a heteromorphic chromosome pair formed by association of two chromosomes of markedly different sizes and found that the members of this pair segregate nonrandomly with respect to a univalent X chromosome; the larger member of the heteromorph always moves to the same spindle pole as the X chromosome at anaphase I. This nonrandom behavior was observed in every cell, despite the fact that Payne could discover no physical connection between the chromosomes involved. The accuracy of Payne's observations has been confined by White (15) who studied fixed cells and by Camenzind and Nicklas (2) who observed living spermatocytes.

The truly remarkable nature of nonrandom segregation in the mole cricket was revealed by the study of living cells (2). Micromanipulation of the chromosomes involved supported the opinion of earlier workers that there is no physical connection between those chromosomes. Moreover, when the inappropriate orientation (X chromosome and larger member of the heteromorph oriented to opposite poles) was induced with the micromanipulation needle, the appropriate orientation was

THE JOURNAL OF CELL BIOLOGY · VOLUME 88 FEBRUARY 1981 281-293

actively restored; the sex chromosome usually reoriented spontaneously so as to ensure its passage to the same spindle pole as the large member of the heteromorph.

Preferential distribution of unconnected chromosomes is not unique to the Northern mole cricket; it has been reported for a number of unrelated organisms (for review see references 7 and 8[1]). However, fundamental differences between the various examples suggest that no single mechanism governs all cases of nonrandom chromosome behavior. In crane flies, for example, physically separate chromosomes that assume similar orientation on the spindle regularly move toward opposite spindle poles. It is, therefore, necessary to explain how poleward movement of one chromosome can induce oppositely directed movement of another chromosome (5). For the mole cricket, on the other hand, the fact that chromosomes orient on the spindle in nonrandom manner requires that we discover how orientation of the X chromosome can be influenced by the orientation of the heteromorph (2).

Camenzind and Nicklas (2) suggested that the structural organization of the spindle could dictate nonrandom orientation of particular chromosomes if there exist specific differences between the spindle fibers of non-randomly segregating chromosomes and those of the remaining chromosomes of the complement. Our study was undertaken to discover if there are ultrastructural indications of such differences. We have found that an electron-dense material is associated with the kinetochores of the X chromosome and of the smaller chromosome of the heteromorphic pair and that microtubules are arrayed about these particular kinetochores in an unusual fashion. We discuss the manner in which these structural peculiarities might be related to nonrandom chromosome behavior.

MATERIALS AND METHODS

Living specimens of *Neocurtilla* (*Gryllotalpa*) *hexadactyla* Perty (4) were collected near Grassy Pond in the Sandhills Wildlife Management Area in the vicinity of Southern Pines, N. C.

All of the preparative procedures used in this study have been described previously. To select cells in the desired stages of spermatogenesis, living cultures of testicular tissue were prepared for light microscopic observations as described by Nicklas et al. (9). Cells were observed on an inverted phase contrast microscope and photographed by time-lapse cinemicrography. During observation and recording, fixation of selected cells was initiated by microinjection of fixative into the vicinity of the target cell and was completed by immersion of the coverslip bearing the attached target cell in a large volume of fixative. This routine of "microfixation" followed by "macrofixation" and subsequent steps of preparation for electron microscopy were essentially those of Nicklas et al. (9). However, the composition of fixatives, buffers, etc. and the schedule of fixation differed. Our conditions were as follows: (*a*) Microfixation: 6% glutaraldehyde in PIPES-NaCl buffer (100 mM PIPES [Sigma Chemical Co., St. Louis, Mo.], 0.3% NaCl, pH 6.8–6.9) for 4–5 min. (*b*) Macrofixation: 3% glutaraldehyde in PIPES-NaCl buffer for 30 min. (*c*) Rinse: PIPES-NaCl buffer; two changes of buffer at 10-min intervals followed by overnight storage in buffer at 4°C. (*d*) Second Fixation: 2% osmium tetroxide in PIPES-NaCl buffer for 0.75–1 hr. (*e*) Rinse: distilled water; two changes at 10-min intervals. (*f*) Uranyl acetate treatment: 0.5% uranyl acetate in veronal-acetate buffer for 0.75–1 h (12).

Fixatives were prepared immediately before use. As an assurance of the quality of the glutaraldehyde used, fixatives were prepared from a 70% stock solution stored in sealed ampoules (Ladd Research Industries, Inc., Burlington, Vt.); only those lots having an absorption ratio $A_{230\ nm}/A_{280\ nm}$ <0.2 were used (1).

Serial sections through entire cells were examined in a Siemens 101 electron microscope, and micrographs of each section including nucleus or spindle were prepared using Kodak 70-mm roll film (Kodalith LR 2572). The overall analysis was based upon the study of serial negatives prepared at original magnifications ranging from × 2,300 to × 3,900 and viewed using the × 14 or × 31 additional magnification provided by an aerial roll film viewer (Hoppmann Corp., Springfield, Va.). For illustrations in this publication, new micrographs of the sections of interest were taken at magnifications ranging from × 2,500 to × 6,500.

Reconstructions were prepared to examine certain spatial relationships in the serially sectioned cells. In some cases, three-dimensional reconstructions were required. For these, the micrograph negative of a section was projected at × 14 or × 31 magnification onto a transparent cellulose acetate sheet attached to the viewing screen of the roll film viewer, and all structures of interest (e.g., chromosome outlines including kinetochore position, microtubule profiles, and centrioles) were traced onto the acetate. One tracing of each section was made. The reconstruction was then made by superposing individual acetate sheets in order, using chromosome outlines as a guide to correct registration (registration of individual microtubule profiles was not an objective except as noted in the legend of Fig. 8). The correct spacing between tracings was maintained by interposing plexiglas sheets of appropriate thickness (section thickness × final magnification) between them. In other cases, accurate three-dimensional information was not necessary, as, for example, when attempting to identify particular chromosomes on the basis of their shape or when examining the overall disposition of chromosomes in spindle or nucleus. For such purposes, a simplified reconstruction method was used to record information from a large number of sections on one or a few acetate sheets. After the structures in one section were traced on an acetate sheet, the image of the succeeding section was projected onto the viewing screen, and the chromosome outlines previously traced on the acetate were brought into register with the chromosome profiles appearing in the projected image. For this second section and for all following sections, only significant changes or new structures (e.g., increased chromosome size, the appearance of new chromosomes, the appearance of kinetochores, kinetochore-associated microtubules and centrioles, changes in outline of the nuclear envelope) were added to the acetate tracing. The use of different colored inks for different structures, particularly for separate chromosomes, eliminated confusion caused by overlapping of structures in the tracing. In this way, a single tracing incorporating a two-dimensional composite of information from many sections was produced. We refer to these as "two-dimensional reconstructions".

RESULTS

Our phase contrast microscopic observations of meiosis in living spermatocytes of *N. hexadactyla* are in accord with previous descriptions derived from fixed materials (10, 11, 15) or living cells (2). For a summary of chromosome behavior during meiosis, see Fig. 1. In this account, X_1 refers to the univalent sex chromosome, whereas Y_2 and Y designate, respectively, the larger and smaller chromosomes composing the heteromorphic pair, as suggested by White (15).

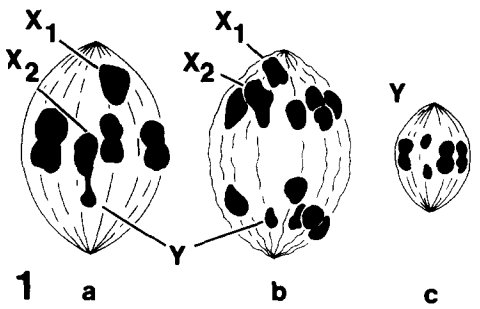


FIGURE 1 Meiosis in *Neocurtilla hexadactyla*. (*a*) At metaphase of the first meiotic division, the chromosomes destined to segregate nonrandomly are easily recognized; the univalent chromosome (X_1) usually does not lie at the spindle equator, and the X_2Y chromosome pair (X_2;Y) is distinguished from autosomal pairs on the basis of its assymmetrical shape. Note that the X_2Y pair is somewhat shifted off the equator so that Y is closer to a pole than is X_2. Characteristically, chromosome X_1 is found in the half-spindle toward which X_2 is oriented. This orientation presages the nonrandom chromosomes distribution of the ensuing anaphase (*b*) when the physically unlinked chromosomes X_1 and X_2 invariably pass to the same pole while chromosome Y passes to the opposite pole. As a result of the first meiotic division, two types of secondary spermatocytes are produced; one carries the Y chromosome but neither X chromosome (*c*); the other (not illustrated) includes both X_1 and X_2 but no Y. (Redrawn from reference 10.)

[1] In reference 7, see pages 167 ff. and 243 ff. In reference 8, see page 276 ff.

The First Meiotic Division

The features that distinguish nonrandomly segregating chromosomes in *N. hexadactyla* are most evident during the later stages of the first meiotic division. Therefore, the description of metaphase and anaphase cells precedes that of the earlier prometaphase and prophase cells.

KINETOCHORE STRUCTURE AND SPINDLE ORGANIZATION IN METAPHASE CELLS: Four metaphase cells were examined in serial sections; they are designated cells A–D. In each, massive aggregates of extremely electron-dense material are located on the X_1 and Y chromosomes, as illustrated in cell A (Figs. 2, 3, and 4). These aggregates are of indeterminate structure but are characterized by an elongate profile and by the irregular, labyrinthine relationship among the patches of electron-dense material composing them. The electron-dense material marks the X_1 and Y kinetochore regions — only here are microtubules associated with these chromosomes.

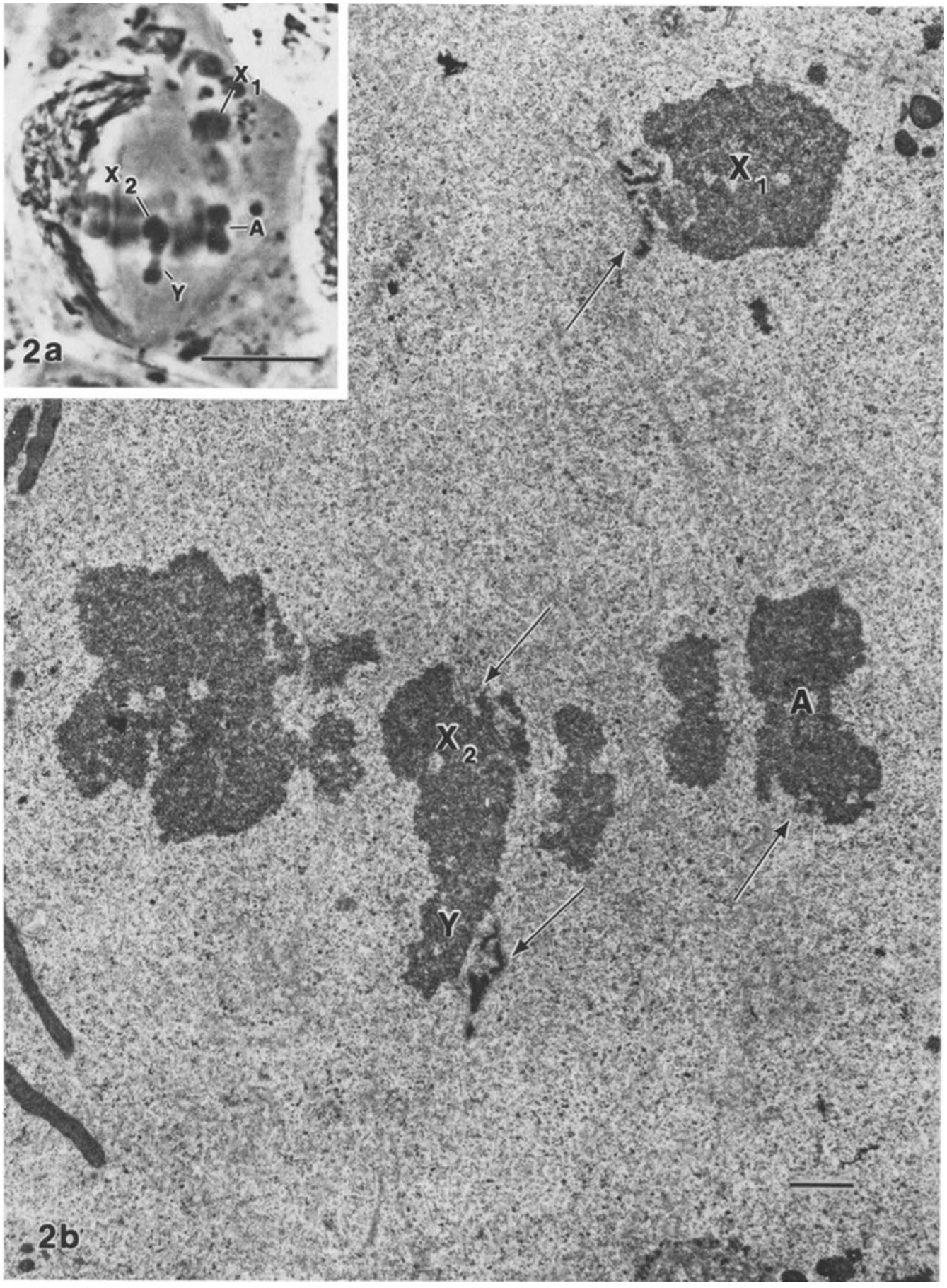
The remaining kinetochores in metaphase cells, those of the autosomes (Fig. 5) and of the X_2 chromosome, are discrete fibrillar masses less dense than the chromosomes; they are found at the poleward extreme of the chromosome, sometimes in a cuplike depression. Those that lie near the unusual X_1 or Y kinetochore regions may be marked by a single electron-opaque patch, as is the case for one autosomal kinetochore and the X_2 kinetochore in cell A and for two autosomal kinetochores in cell B (Fig. 6). In general, though, autosomal and X_2 kinetochores are free of such electron-dense material; none appears in cells C and D (because a number of sections of cell D were lost, the kinetochores of only nine of its 10 autosomal pairs were examined). During other division stages (see descriptions of meiosis II and mitosis), X_1 and Y kinetochores are discrete fibrillar structures indistinguishable from the autosomal and X_2 kinetochores just described. Therefore, we attempted to recognize such structure within the X_1 and Y kinetochore regions during metaphase of meiosis I. We conclude that if X_1 and Y kinetochores per se are similar to autosomal and X_2 kinetochores, this is obscured by the aggregate of electron-dense material in the kinetochore region.

The X_1 and Y chromosomes at metaphase I differ further from autosomes and the X_2 chromosomes in the position of the kinetochore on the chromosome and in their associations with microtubules. The autosomes and the X_2 chromosome display exactly the sort of kinetochore-microtubule-pole relationship which is characteristic of organisms with localized kinetochores (6) (Figs. 2, 5, and 6); kinetochores on oriented metaphase chromosomes are situated so that they face directly toward one centriolar pole and microtubules that emanate from the kinetochore run directly toward that pole. In contrast, the kinetochore regions of X_1 and Y chromosomes (Figs. 2, 3, and 4) are not pointed directly toward only one pole. Instead, the elongate electron-dense kinetochore region is lateral to the body of the chromosome; it is, thus, aligned parallel to the interpolar axis and cannot be considered to face only one of the two poles. Furthermore, the microtubules that impinge on the electron-dense aggregate are directed not toward the nearer spindle pole exclusively, but microtubule profiles are arrayed about both ends of the aggregate as well as in the interstices between the electron-dense patches (Figs. 3 and 4; see also Fig. 8). Three-dimensional reconstructions prepared so as to include all microtubule profiles between the chromosome and the near pole demonstrate that a microtubule bundle proceeds from the kinetochore region poleward. Presumably, this bundle is involved in the chromosome's orientation to that pole. It is possible that some or all of the microtubules that compose that bundle actually pass through the electron-dense aggregate and emerge at its opposite end. This would account for the microtubules found at the two ends of the aggregate and in among the electron-dense patches (Figs. 3 and 4). Alternatively, separate bundles of microtubules may associate with the opposite ends of the kinetochore region. We could not evaluate these alternatives because the density of the electron-opaque material in the kinetochore region makes it impossible to recognize any microtubules which may run through it.

At metaphase, nonrandomly segregating chromosomes are positioned in the spindle in a particular manner. Most obvious, of course, is the characteristic orientation of the physically unlinked X_1 and X_2 chromosomes to one pole, whereas the Y chromosome (which is paired with the X_2 chromosome) is oriented to the opposite pole. Also, as illustrated in earlier publications (e.g., references 2 and 10), the X_2Y pair is shifted off the equator so that the Y chromosome is closer to a pole than is X_2 (Figs. 1 and 2). More subtle spatial relationships are also discernible. Three-dimensional reconstructions of all four metaphase cells demonstrate that the X_2Y chromosome pair is centered within the spindle volume so that it lies almost exactly on a line drawn between the centrioles at opposite spindle poles. Moreover, in three of the four cells (cells A, B, and D), the relationship between the central X_2Y pair and the more peripheral univalent X_1 chromosome is as illustrated in Figs. 2 and 7 *a* and *b*. In Fig. 2, because both X_2 and Y chromosomes are included in the same section, it is plain that the kinetochoric sides of these chromosomes are aligned more or less along the same spindle axis. In cells B and D, this relationship is not so immediately apparent because X_1 and Y appear in different section planes. However, the reconstructions show that the chromosomes are situated exactly as in cell A. Cell C is an exception in this regard; the chromosomes in question are 90° out of the arrangement that would bring their kinetochoric sides onto the same axis (Fig. 7 *c* and *d*).

We looked for structural features that might account for a definite spatial relationship between X_1 and Y chromosomes. For both cells A and B, the reconstructions suggest that a few microtubules link the kinetochore regions in question. The clearest example is presented by cell A, in which microtubule profiles running in a very different direction from the majority of spindle microtubules trace a direct line between X_1 and Y kinetochore regions (Fig. 8). In cell B, also, a small bundle of microtubules extends between these kinetochore regions. This was not the case in cell C where the kinetochoric sides of X_1 and Y chromosomes are not on the same axis. Similar examination of cell D was impossible because of section losses.

INAPPROPRIATE CHROMOSOME ORIENTATION: We examined a single cell showing a well-defined metaphase plate but with the nonrandomly segregating chromosomes in inappropriate orientation, i.e., with X_1 oriented to the same pole as the Y chromosome (Fig. 9 *a* and *b*). Again, the kinetochores of chromosomes X_1 and Y are marked by patchy electron-dense aggregates and these face a common axis, as in the typical metaphase cells already described. However, the X_1 kinetochore region is distinctly different in this cell; it is not elongated along the interpolar axis and all of the associated microtubules run directly toward the nearest pole (Fig. 9 *c*). The only feature of the X_2Y chromosome pair worthy of note is its somewhat eccentric placement in the spindle so that it does not lie on the central interpolar axis as it does in more typical metaphase


X1
X2
A
Y
2a
X1
X2
A
Y
2b

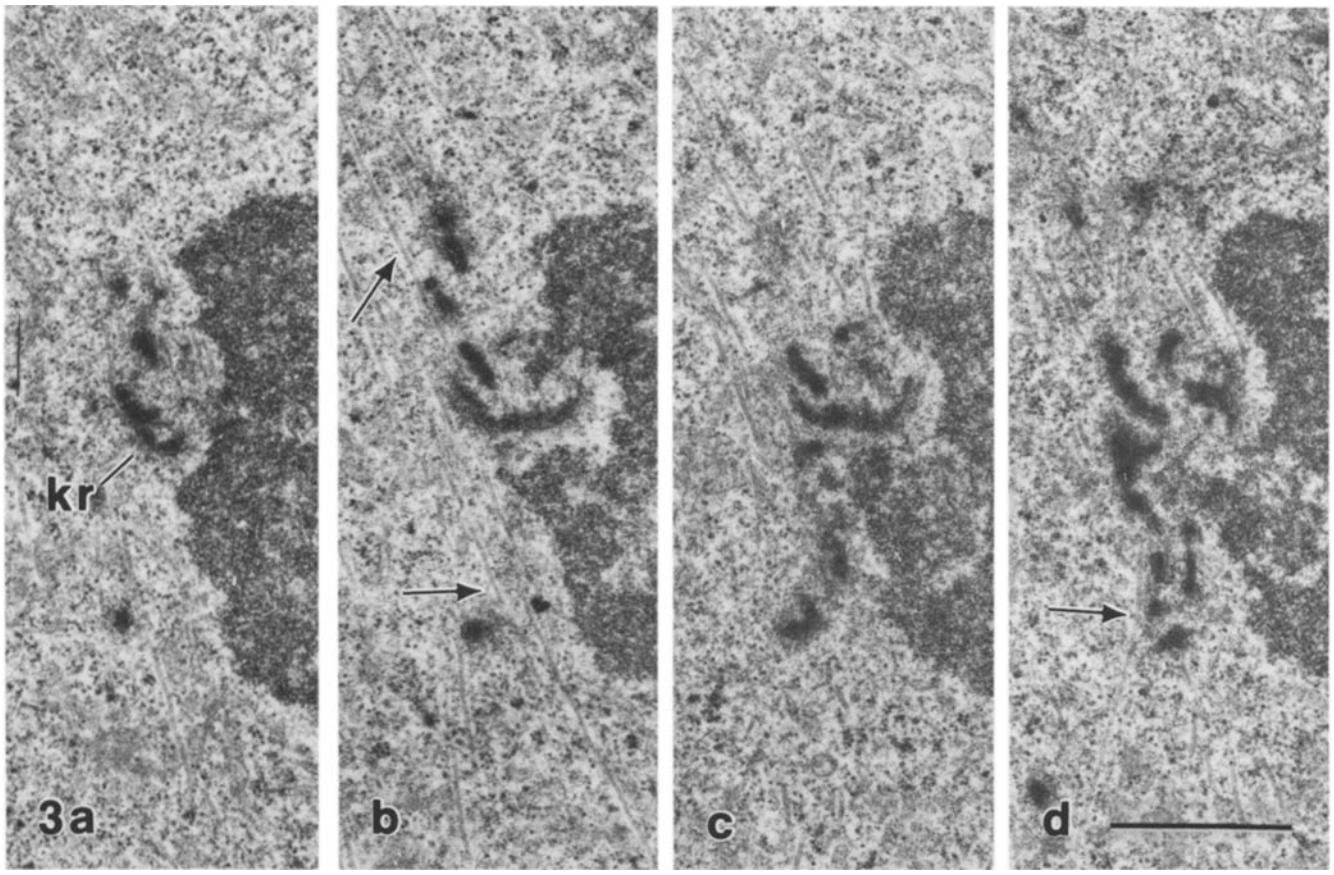


FIGURE 3 Kinetochore region of the X_1 chromosome in metaphase of meiosis I, cell A. The chromosome is shown in the same orientation as in Fig. 2; as determined from serial sections, the nearer pole is above and to the left of the area included in these micrographs. The patches of electron-dense material found in the kinetochore region (*kr*) are arranged in an irregular mass; they appear in 14 serial sections and, thus, constitute a sizeable elongate aggregate (sections *a*, 4; *b*, 6; *c*, 7; and *d*, 9 of the series are shown). Microtubules (arrows) are found at the end of the kinetochore region closer to a pole as well as at its opposite end and in among the electron-dense patches. Bar, 1 μm. × 24,000.

cells. This is evident in a three-dimensional reconstruction and in the phase micrograph included in Fig. 9*a*.

ANAPHASE: One early anaphase cell was studied in detail. At the time of fixation, the X_2 and Y chromosomes had already disjoined and moved a significant distance poleward, but only two of the 10 autosome pairs were clearly disjunct. The patchy electron-dense substructure of X_1 and Y kinetochore regions, the array of microtubules at both ends of those electron-dense aggregates, the position of X_2 and Y chromosomes on the central spindle axis, and turning of the kinetochoric sides of the X_1 and Y chromosomes toward a common axis are just as they are in typical metaphase cells. The spindle region between X_1 and Y kinetochore regions contains no microtubules with the directionality which would be expected if microtubules formed a link between these chromosomes. All autosomal kinetochores are free of electron-dense material.

The only cell fixed at a later stage in anaphase was, unfortunately, not very well preserved (poor visibility of microtubules, chromatin fusion). Nevertheless, it is worth noting that single small electron-dense patches appear on the kinetochores of several chromosomes but that no massive aggregations of such material occur; thus, X_1 and Y chromosomes are not distinctive in this cell.

PROMETAPHASE: In the three prometaphase cells examined (designated cells E, F, and G), the X_1 and Y chromosomes are not recognizable from inspection of single sections because the kinetochore regions of all chromosomes have a similar appearance. These resemble the peculiar X_1 and Y kinetochore regions

FIGURE 2 Metaphase of the first meiotic division, cell A. (*a*) In this phase micrograph taken immediately after microfixation, the X_1 univalent (X_1), the heteromorphic bivalent formed by association of a large (X_2) and a small (*Y*) chromosome, and autosomes (*A*) are distinguishable. As is typical for this stage of meiosis, X_1 has already approached one pole and the X_2 chromosome is oriented to the same pole. Note that the X_2Y pair is shifted off the spindle equator so that the Y chromosome lies closer to a pole than does X_2. Bar, 10 μm. × 2,100. (*b*) This section includes the kinetochore regions (arrows) of the four chromosomes identified in *a*. The kinetochore regions of X_1 and Y chromosomes appear as elongate electron-dense masses lateral to the body of the chromosomes. Note that the kinetochoric sides of these two chromosomes face toward a common axis—a line from pole to pole lying between the X_2Y pair and the X_1 chromosome. The kinetochores of the X_2 chromosome and of an autosome are difficult to discern because of their moderate electron density and the low magnification of this micrograph. See Fig. 5 for a micrograph of the autosomal kinetochore at higher magnification. Bar, 1 μm. × 11,200.

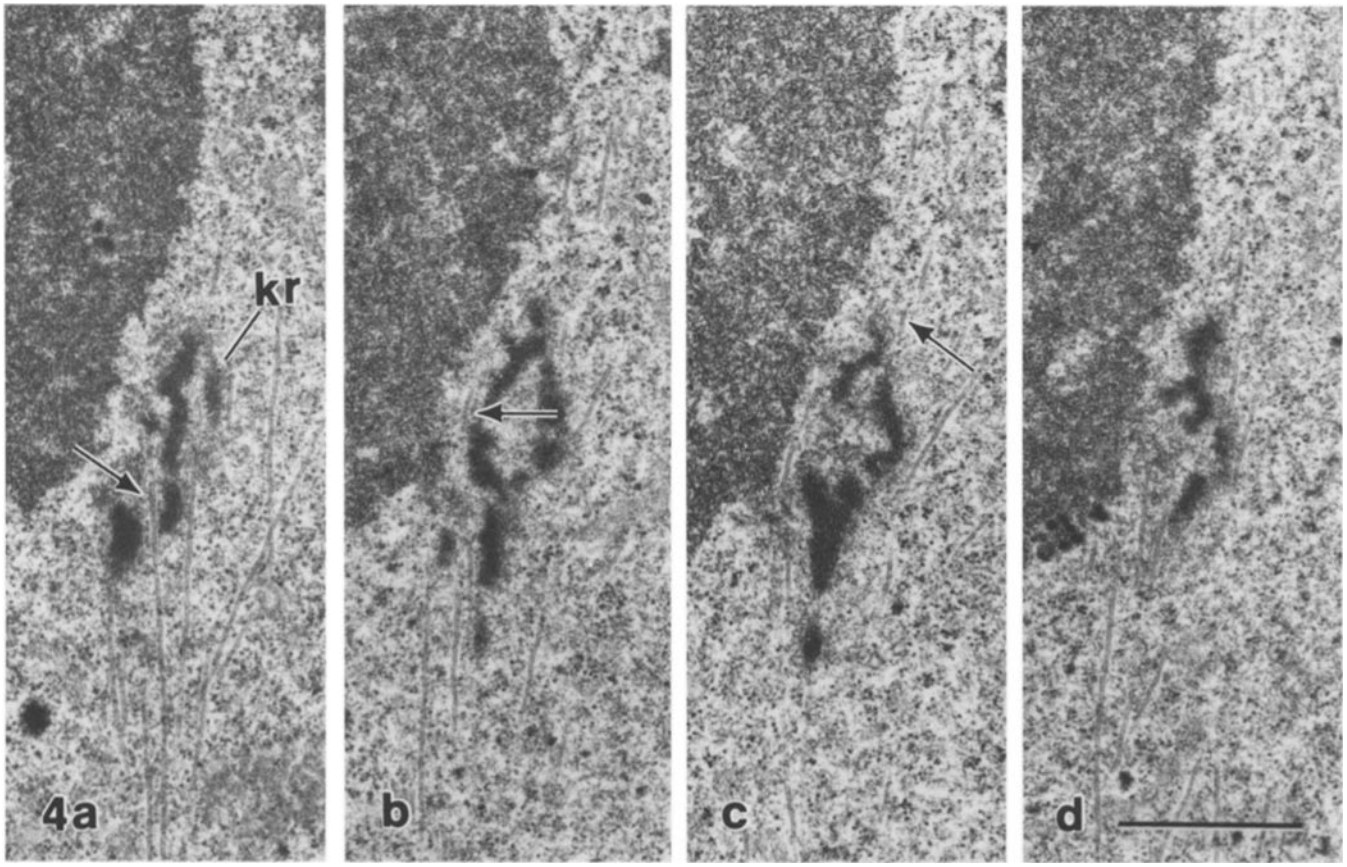


FIGURE 4 Kinetochore region of the Y chromosome in metaphase of meiosis I, cell A. The chromosome is oriented as in Fig. 2; the nearer pole is directly below the area included in these micrographs. The Y kinetochore region (*kr*) is similar to the X_1 kinetochore region shown in Fig. 3 in its electron density, complicated elongate structure, and its association with microtubules (arrows). The kinetochore is massive and continues in a series of 12 sections (sections *a*, 5; *b*, 7; *c*, 8; and *d*, 10 are shown). Bar, 1 μm. × 24,000.

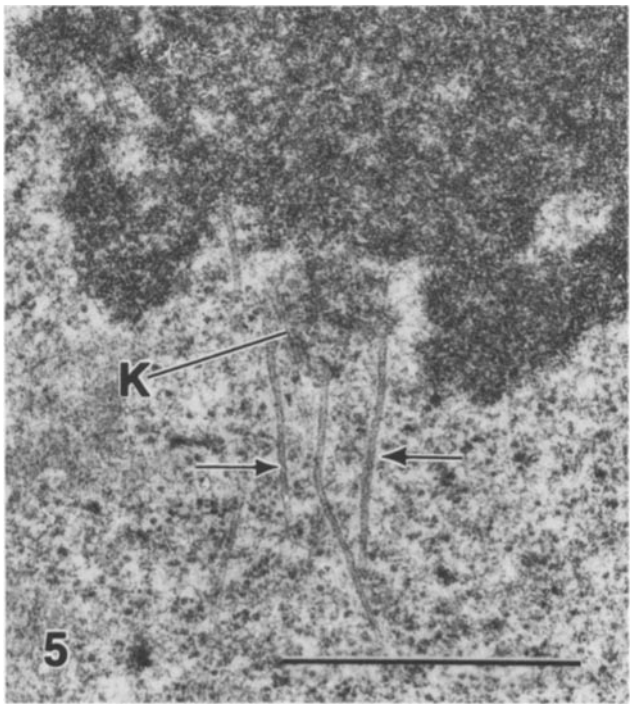


FIGURE 5 An autosomal kinetochore at metaphase of meiosis I, cell A. This is another section of the autosomal kinetochore indicated in Fig. 2. The kinetochore (*k*) is a moderately electron-dense, fibrillar mass embedded in a cuplike depression of the chromosome. Microtubules (arrows) run from the kinetochore toward the nearer pole. Bar, 1 μm. × 38,900.

in metaphase cells insofar as they have a mottled or patchy character, but they are far less electron-opaque (Figs. 10 and 11). Because cell G is in a relatively advanced stage of prometaphase (all chromosomes in approximately equatorial position, six of 11 chromosome pairs bipolarly oriented) and well-defined bundles of microtubules are associated with the majority of kinetochores, it is clear that the observed kinetochore structure is not a feature restricted to earliest prometaphase or to kinetochores which are not associated with microtubules.

Two-dimensional reconstructions permit identification of the univalent X_1 chromosome and the X_2Y chromosome pair (Fig. 12) in all three cells. By this means, we can see that the X_1 and Y kinetochore regions are more massive than those of other chromosomes and that they are elongate and lateral to the body of the chromosome just as in metaphase. Also, as in metaphase, microtubules are associated with both ends of the kinetochore regions; this was evident for both X_1 (Fig. 10) and Y chromosomes in cell E and for the X_1 chromosome in cell F.

Because prometaphase is the stage during which chromosomes assume appropriate orientation on the spindle, association between X_1 and Y chromosomes during this stage might dictate their nonrandom behavior at later stages. In this context, we note that the X_1 univalent and the X_2Y pair are not obviously connected in any of the three cells but that the kinetochoric sides of X_1 and Y chromosomes are turned toward each other in two of the three cells (Fig. 12).

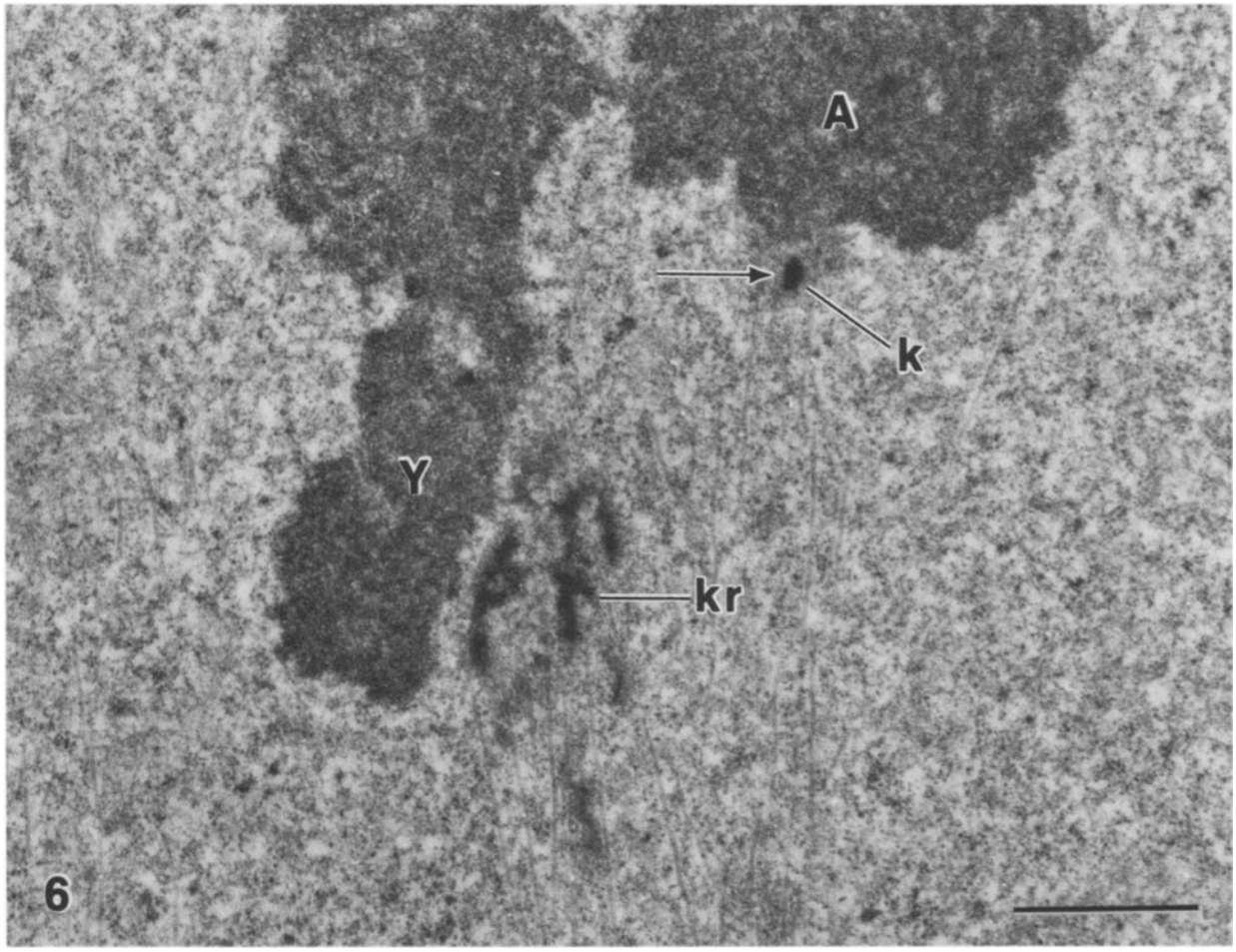


FIGURE 6 Kinetochore of an autosome and the Y kinetochore region in metaphase of meiosis I, cell B. A patch of electron-dense material (arrow) marks the kinetochore of an autosome (*A*) immediately adjacent to the massive electron-dense kinetochore region (*kr*) of the Y chromosomes (*Y*). The autosome-associated electron-dense material is confined to the single patch illustrated and appears in only three consecutive sections. Bar, 1 μm. × 23,800.

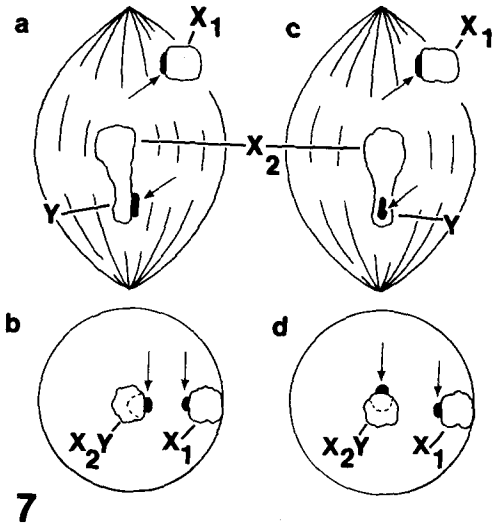


FIGURE 7 The relationship between nonrandomly segregating chromosomes as determined from three-dimensional reconstructions of the spindle. (*a*) In metaphase cells A, B, and D, the central X_2Y pair (X_2;*Y*) and the more peripheral X_1 chromosome (X_1) lie in the spindle with their kinetochore regions (arrows) aligned along a common spindle axis; if the spindle were viewed from one pole, as in *b*, X_1 and Y kinetochore would appear to face each other across one radius of the spindle. (*c*) In metaphase cell C, the X_1 and Y chromosomes have a different relationship; if the X_2Y pair is viewed so that the Y kinetochore region is on the side of the chromosome facing the reader, a nonkinetochoric side of the chromosome lies on a common axis with the X_1 kinetochore region. When viewed from the pole as in *d*, the X_1 kinetochore region appears to face a side of the X_2Y pair ~90° away from the Y kinetochore region.

PROPHASE: We examined one cell in diakinesis, the final prophase stage before nuclear envelope breakdown signals the onset of prometaphase. Kinetochores are not recognizable on the loosely condensed chromosomes in this nucleus (Fig. 13). In two-dimensional reconstruction, individual chromosomes are easily recognized. Because 12 chromosomes (10 autosome pairs, the X_2Y pair, and chromosome X_1) appear as discrete entities in the reconstruction, there is evidently no close association between X_1 and the X_2Y pair such as might promote their coordinated orientation in the ensuing prometaphase and nonrandom segregation at anaphase.

The Second Meiotic Division

The nonrandom behavior of X_1, X_2, and Y chromosomes in the first meiotic division assures that cells in the second meiotic division carry either the X_1 or the Y chromosome, never both. Therefore, if the differentiation of the kinetochores of these particular chromosomes persists in the second division, we can

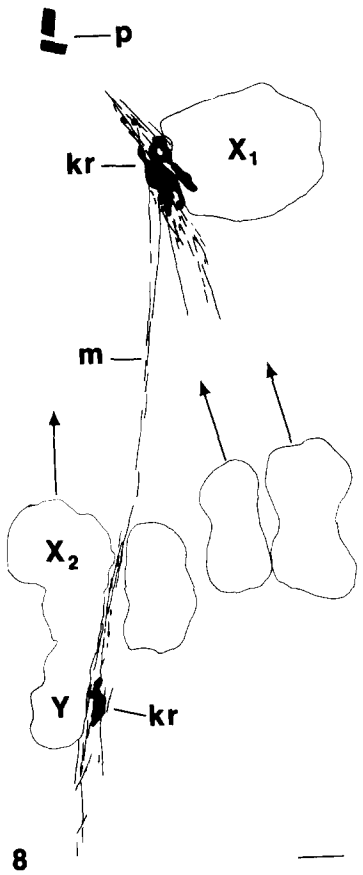


FIGURE 8 Reconstruction of a small portion of the spindle in metaphase of meiosis I, cell A. In this composite drawing, made by retracing the superposed tracings of six sections, only a few of the microtubules (*m*) in the spindle regions between the X_1 chromosome (X_1) and the X_2Y pair (X_2;*Y*) are included; the great majority of microtubules have been eliminated to avoid confusion. The microtubules shown were chosen because they have an orientation clearly different from that of the majority of spindle microtubules and because they follow a path between X_1 and Y kinetochore regions (*kr*). The predominant directionality of other spindle microtubules is indicated by the arrows, which represent autosomal kinetochore fibers. Despite care taken to discover any complete microtubules that might span the distance between X_1 and Y kinetochore regions, no clearly continuous microtubules were found (original reconstruction was made at × 96,000 magnification; all clearly identifiable microtubule profiles were included; registration of succeeding tracings was based not only upon the superposition of chromosome outlines but also upon alignment of microtubule profiles so as to generate the greatest degree of microtubule continuity through contiguous sections). The microtubules illustrated, however, suggest that at least some microtubules associated with the ends of X_1 and Y kinetochore regions interconnect those chromosomes. Other microtubules are clearly not involved in such linkage, in particular those numerous microtubules at the nonpolar end of the X_1 kinetochore region which lie at angles precluding their inclusion in a microtubule bundle running between the two chromosomes. *p*, Pole. Bar, 1 μm.

expect to find distinctive kinetochore regions on only one chromosome per cell.

The metaphase II cell we examined is a Y-bearing cell, as judged by the appreciably smaller size of one chromosome in the complement (Fig. 14; cf. Fig. 1). At this stage, the kinetochores of the putative Y chromosome are not distinctive. Though electron-dense material appears at the kinetochores of both chromatids of the Y, this material is restricted to a single small patch. Furthermore, the location of the kinetochores on this chromosome and the association between kinetochore and microtubules is conventional. Thus, the kinetochore regions of the Y chromosome are not structurally peculiar as they were at metaphase of the first meiotic division. Most of the autosomal kinetochores are also associated with a patch of electron-dense material. The kinetochores of six of the eight autosomes examined are so marked, four of them at each of their two kinetochores, whereas two others have a patch at only one kinetochore.

Mitosis

Kinetochore structure was examined in spermatogonial mitosis to discover whether X_1 and Y chromosomes manifest peculiar kinetochore structure even when their behavior is not exceptional. For one spermatogonial metaphase and one spermatogonial anaphase cell, all kinetochores were examined in serial sections. All resemble the undifferentiated autosomal kinetochores described for the first meiotic metaphase and anaphase; no electron-dense patches appear on any of them. Thus, X_1 and Y kinetochores are not distinctive during mitosis.

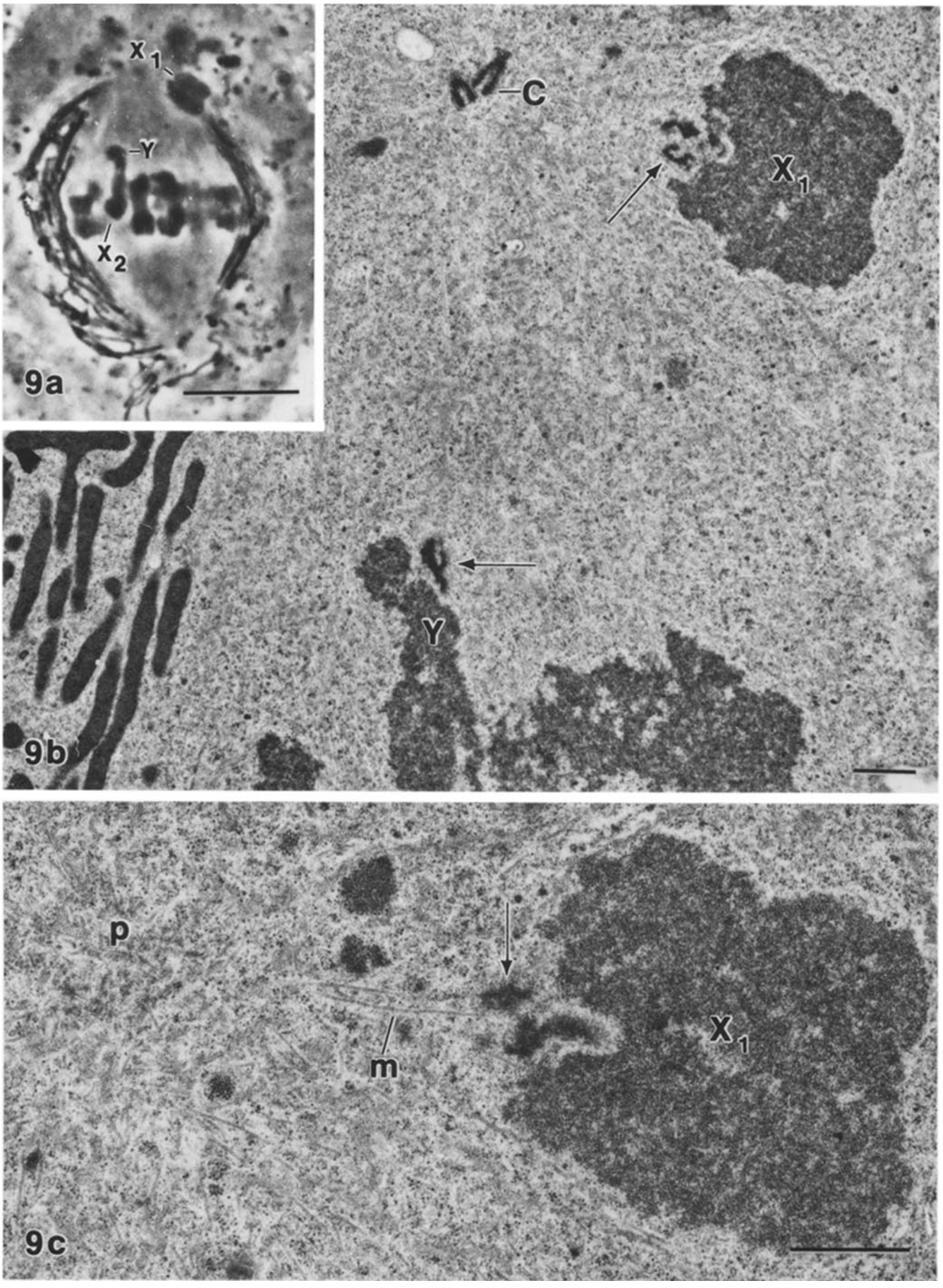
DISCUSSION

Any hypothesis formulated to explain nonrandom segregation in *N. hexadactyla* must take into account the information derived from experimental study of living cells (2) together with the following structural facts established by our study.

First, distinctive kinetochores regions are found on only two of the nonrandomly segregating chromosomes, the unlinked X_1 and Y chromosomes, which orient to opposite poles.

Second, the kinetochore regions of X_1 and Y chromosomes become progressively more distinctive during meiosis I. In mitosis and meiosis II, X_1 and Y kinetochores are as small as the kinetochores of all other chromosomes and they interact with microtubules in the same way as do other kinetochores. In prometaphase of meiosis I, changes in X_1 and Y kinetochore regions are already evident. They are appreciably larger than

FIGURE 9 Inappropriate chromosome orientation in meiosis I. (*a*) In this phase micrograph taken immediately after microfixation, the inappropriate orientation of the X_1 chromosome (X_1) and the X_2Y pair (X_2;*Y*) is apparent. Presumably X_1 would have reoriented to restore appropriate orientation (2). The position of the X_2Y pair, with Y closer to a pole, is as in typical metaphase cells, whereas its somewhat eccentric position is not. Bar, 10 μm. × 2,100. (*b*) Laterally positioned electron-dense kinetochore regions (arrows) are found on the inappropriately oriented X_1 and Y chromosomes. The X_1 kinetochore region, however, is not elongate as it always is when the chromosome is appropriately oriented. The electron density of centrioles (*c*) is a characteristic of all cells, meiotic or mitotic. Bar, 1 μm. × 10,900. (*c*) Another section of the kinetochore region shown in *b*. It is clear that microtubules (*m*) run between the kinetochore region and the near pole (*p*). In the serial sections, all of the microtubules associate with this kinetochore region have the same directionality as those illustrated. Bar, 1 μm. × 21,800.


X1
Y
X2
9a
C
X1
Y
9b
p
m
X1
9c

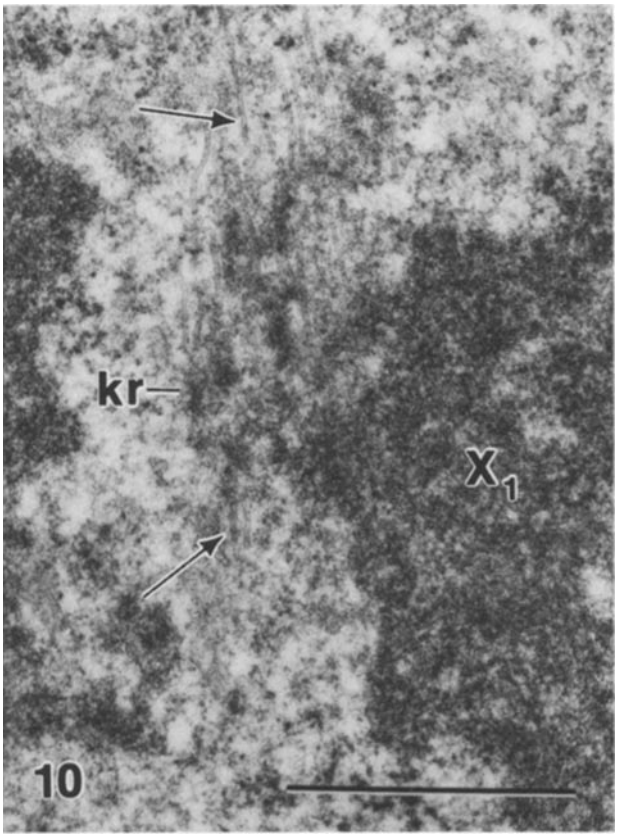


FIGURE 10 The X_1 kinetochore region in prometaphase of meiosis I, cell E. The presence of only one kinetochore region (*kr*) on this chromosome identifies it as the X_1 univalent (X_1) (see also Fig. 12). The kinetochore region is similar to X_1 kinetochore regions in metaphase cells in its inhomogeneous, patchy substructure, elongate profile, position lateral to the body of the chromosome and association with microtubules (arrows) at opposite ends. Yet, it differs from metaphase X_1 kinetochore regions in being no more electron-dense than the chromosome. Bar, 1 μm. × 38,000.

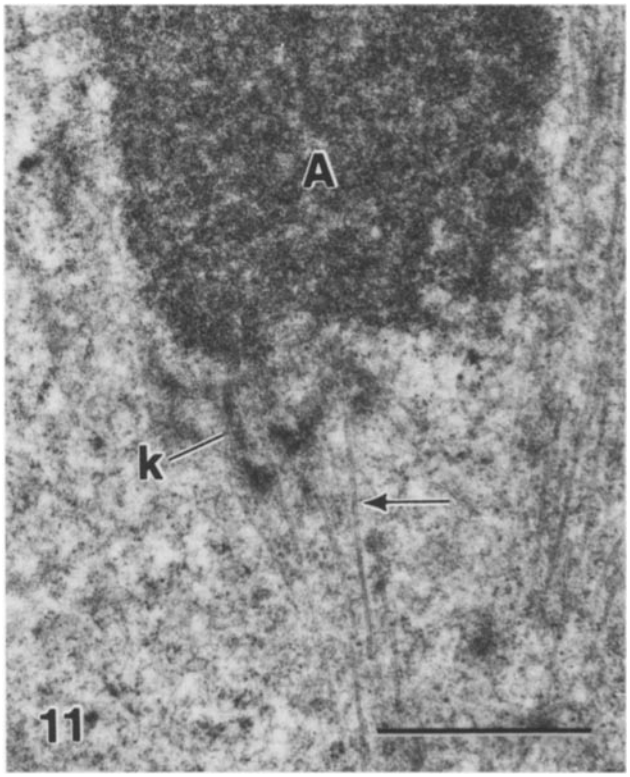


FIGURE 11 The kinetochore of an autosome (*A*) in prometaphase of meiosis I, cell G. This autosomal kinetochore (*k*), like all autosomal kinetochores in the prometaphase cells examined, is similar to the prometaphase kinetochore regions of X_1 and Y chromosomes (cf. Fig. 10) in its inhomogeneous, patchy appearance. Nevertheless, the position of the kinetochore at a chromosome end, the presence of a second kinetochore at the opposite end of the bivalent, and the microtubules (arrow), which clearly terminate at the kinetochore, all permit an unambiguous distinction between autosomal and X_1 or Y kinetochore regions. Bar, 1 μm. × 28,500.

other kinetochores, they have an atypical location lateral to the body of the chromosome, and they associate with microtubules in a distinctive manner. Later, in metaphase I and early anaphase I, these features are especially clear; and, additionally, the X_1 and Y kinetochore regions have become very conspicuous by virtue of their extreme electron density and convoluted structure.

Third, the X_1 univalent and the X_2Y pair are not physically associated during the early stages of meiosis I. There is significant distance between these chromosomes in the diakinesis and prometaphase cells we examined.

Fourth, an unusual spatial relationship between the X_1 chromosome and the X_2Y pair is evident in later stages of meiosis I. There is an obvious tendency for the kinetochoric sides of the X_1 and Y chromosomes to be turned toward a common axis. Of six cells in later stages of meiosis I (metaphase cells A-D, the atypical metaphase cell, and the early anaphase cell), only one did not conform to this pattern (metaphase cell C). Moreover, the X_2Y chromosome pair is centered within the spindle in metaphase cells showing appropriate orientation (cells A-D) as well as in the early anaphase cell, where appropriate distribution was in progress at the time of fixation, but is shifted off the central axis in the one case where inappropriate orientation was observed (the atypical metaphase cell).

It was thought that pairing between the X_1 univalent and

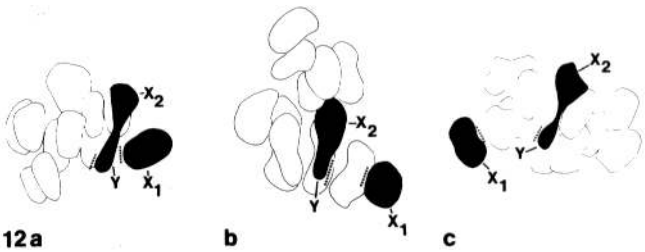


FIGURE 12 Chromosomes in prometaphase of meiosis I, cells E, F, and G. For the sake of clarity, only chromosome outlines and the kinetochore regions (dotted lines) of X_1 and Y chromosomes are included in these drawings, which were traced from two-dimensional reconstructions of complete spindle regions. In each cell, the X_2Y (X_2;Y) pair is unambiguously identifiable on the basis of its drumsticklike shape. The X_1 chromosome (X_1) is identified as the only chromosome in the cell bearing but one elongate, lateral kinetochore region, as expected for this univalent. The X_1 and Y kinetochore regions are both elongate and lateral to the body of the chromosome, exactly as in metaphase cells. *a*, Cell E; *b*, cell F; *c*, cell G.

the X_2Y chromosome pair in early meiotic stages might provide a relatively simple device for their nonrandom orientation (15, 16[2]); if present, the pairing could constrain X_1 and Y kineto-

[2] In reference 15, see page 292; in reference 16, see page 656; and in reference 8, see page 258 ff.

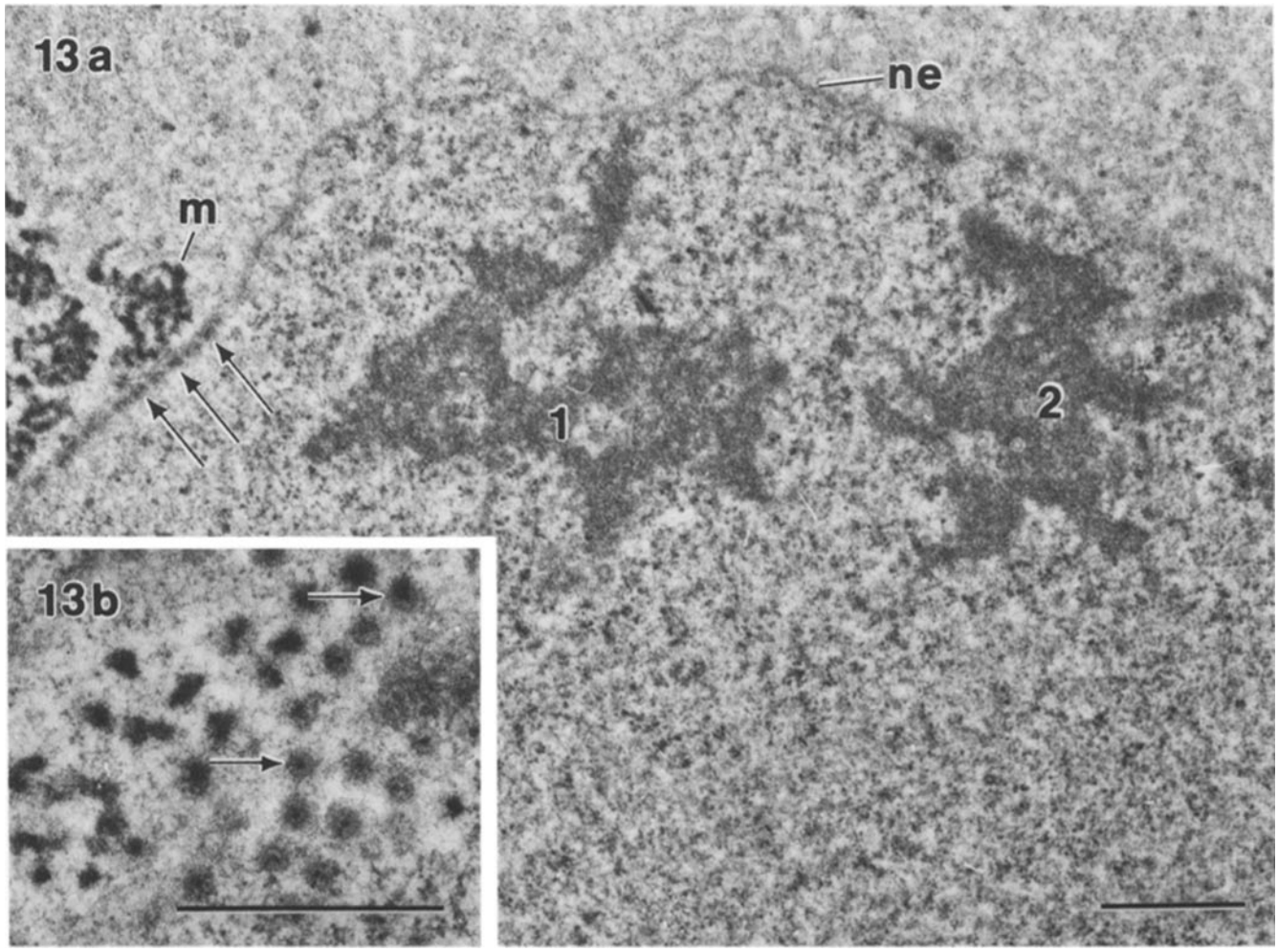


FIGURE 13 Phosphase of meiosis I. (*a*) In diakinesis, the chromosomes (*1* and *2*) are loosely condensed and each is in contact with regions of the nuclear envelope (*ne*) free of nuclear pores. Nuclear pores (arrows) are concentrated in the remaining chromosome-free areas of the envelope and sizeable flocculent electron-dense masses (*m*) appear on the cytoplasmic side of the envelope in these areas (14.). Bar, 1 μm. × 19,400. (*b*) In an earlier prophase cell (chromosomes more loosely organized and nucleolus less dispersed than in diakinesis), the association of electron-opaque material with individual pores is clear (arrows). The presence of aggregates of similar electron-dense substance in the cytoplasm of metaphase cells (not illustrated) indicates the persistence of the material in later stages of meiosis. Bar, 1 μm. × 36,700.

chores to face oppositely so that the sort of explanations offered for bipolar orientation of ordinary meiotic bivalents (8^2) would be applicable. The experiments of Camenzind and Nicklas (2) demonstrated that reorientation leading to appropriate segregation proceeds without pairing, but the possibility that initial orientation requires pairing remained open. Now, with the knowledge that the X_1 univalent and the X_2Y pair are not associated either before (diakinesis) or during (prometaphase) the time chromosomes are establishing their spindle connections, we can dismiss the idea that pairing is ever concerned with the preferential distribution of these chromosomes.

We must instead consider that nonrandom segregation in *N. hexadactyla* is a function of extraordinary properties of the X_1 and Y kinetochores and their associated microtubules. The inference that nonrandom segregation is somehow dependent on the unique nature of X_1 and Y kinetochores is justified by the contrast between various divisions. X_1 and Y kinetochore regions are grossly different from other kinetochores only during the first meiotic division in males, precisely when preferential orientation and distribution occur, but they are not at all distinctive in the second meiotic division or in mitosis when all chromosomes behave similarly. Although diverse kinetochore types are known from a variety of organisms (6), this mole cricket is the first example in which massive aggregates of electron-dense material distinguish particular kinetochores; thus, although kinetochores like those of the autosomes and the X_2 chromosome are known, the distinctive X_1 and Y kinetochore regions as observed during the first meiotic division in males are unlike any previously described.

The differentiation of X_1 and Y kinetochore regions during meiosis I involves increases in size and electron density which presumably occur because those kinetochores associate with an electron-dense substance. The electron-dense material responsible for the conspicuous appearance of X_1 and Y chromosomes at metaphase I and anaphase I is obviously not an integral part of the chromosome nor is it absolutely specific for these particular kinetochores, as is indicated by the presence of solitary patches of electron dense material on autosomal or X_2 kinetochores lying near the massive electron-dense aggregates on X_1 and Y chromosomes (metaphase cells A and B) and by the

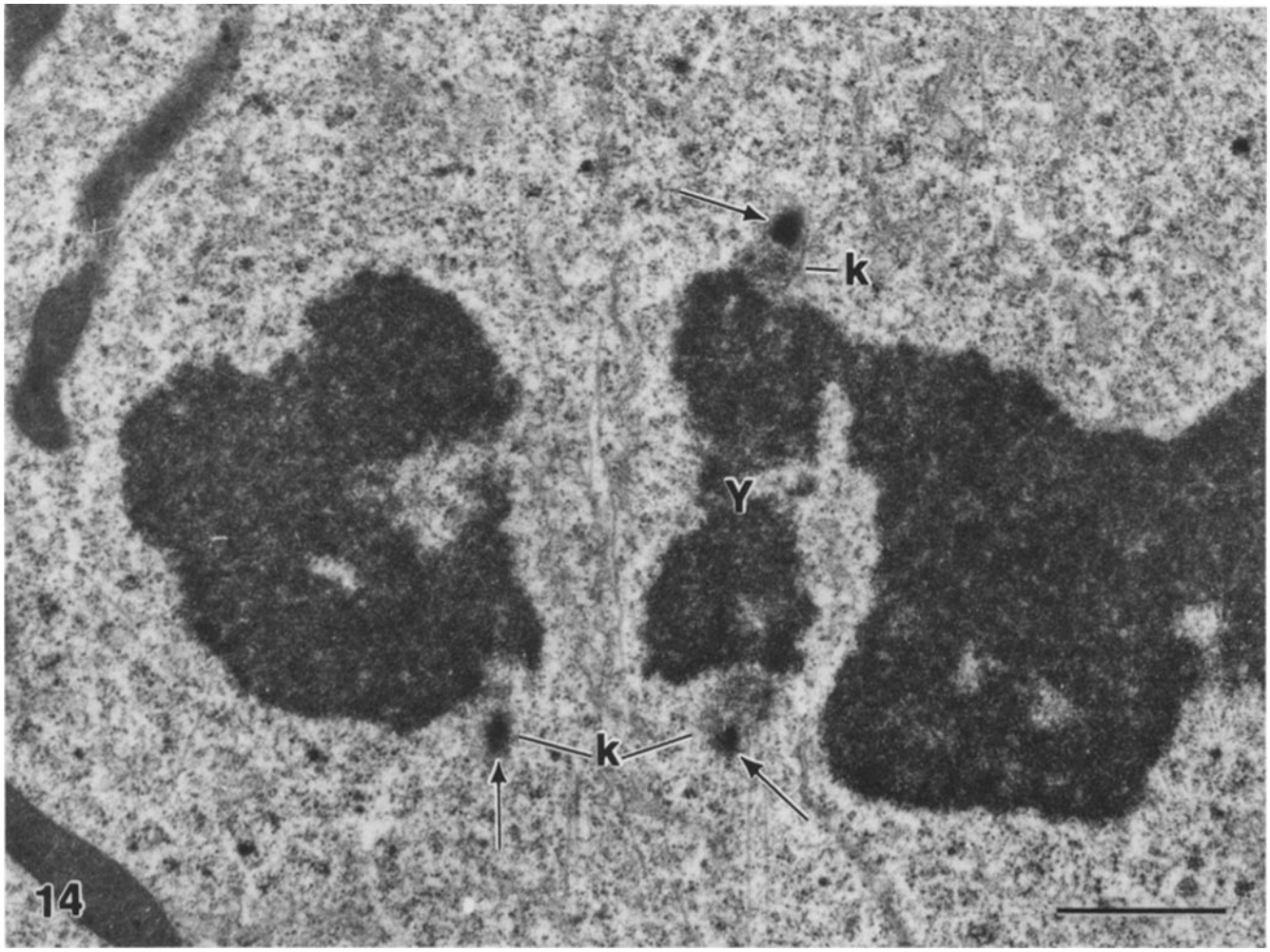


FIGURE 14 Metaphase of meiosis II. Most of the kinetochores (*k*) in this cell are marked by a patch of electron-dense material (arrows). The small size of one of the chromosomes suggests that this is the Y chromosomes (*Y*). Bar, 1 μm. × 23,100.

occurrence of such patches on numerous chromosomes when massive electron dense aggregates are not present (the late anaphase I cell and the metaphase II cell). Nonetheless, X_1 and Y chromosomes are the only ones ever associated with substantial quantities of electron-dense material, and the electron-dense material appears on both X_1 and Y chromosomes even when they are inappropriately oriented. We can do no more than speculate as to what role, if any, this substance may play in chromosome behavior. An obvious and interesting possibility is that the electron-dense material confers unusual properties on the X_1 and Y chromosomal fibers and that stable spindle structure (hence, stable orientation of X_1 and the X_2Y pair) is possible only when one atypical chromosomal fiber is included in each half spindle (cf. reference 2). Alternatively, the electron-dense material may not directly alter the properties of spindle fibers; instead its presence may be a secondary effect which derives from presently unrecognized special properties of the X_1 and Y chromosomes. In any case, the specificity of association of this material with particular kinetochores does at least signal that those chromosomes have unique properties which produce an affinity between kinetochore and electron-dense material and probably determine the observed pattern of nonrandom segregation.

The different behavior of autosomes and the nonrandomly segregating chromosomes must depend most directly on the very different chromosome-microtubule interactions observed. The kinetochore microtubules of autosomes and the X_2 chromosome run only toward the near pole. It is obvious that these ordinary chromosomes are integrated in the spindle syntelically; that is, both sister kinetochores on the two chromatids composing a chromosome connect to the same pole via a single chromosomal fiber. In contrast, appropriate orientation of the unlinked X_1 and Y chromosomes is at least correlated with an unusual association of microtubules with not only the end of the kinetochore region closest to a pole but also with the opposite end. This peculiar relationship is the rule for appropriately oriented X_1 and Y chromosomes in metaphase cells and is already evident in prometaphase (X_1 and Y chromosomes in cell E; and X_1 chromosome in cell F). Conceivably, the array of microtubles in the vicinity of the extraordinary X_1 and Y kinetochore regions indicates amphitelic orientation (sister kinetochores connected to opposite poles via microtubules running toward opposite poles). Because meiotic univalents are often amphitelically oriented (for review see reference 8[3]), this would not be unexpected for the univalent X_1, and in this light it is the Y chromosome, which is anomalous. However, we cannot conclude that either X_1 or Y is in fact amphitelically oriented because we could not establish that separate bundles of microtubules emanate from each end of the kinetochore, with each bundle connected to a different sister kinet-

[3] See page 272.

ochore.

Whereas the microtubule bundles associated with X_1 and Y chromosomes are unmistakably different from the ordinary kinetochore microtubule bundles in the same spindle, it is not clear that the integration of such atypical fibers within the spindle would necessarily constrain the X_1 and Y chromosomes to segregate to opposite poles. The position of X_1 and Y relative to each other may be influenced by the microtubules that we found to run directly between X_1 and Y kinetochore regions lying in opposite half spindles (metaphase cells A and B). If these microtubules constitute a mechanically significant link between X_1 and Y chromosomes, they could certainly cause these chromosomes to lie with their kinetochoric sides facing a common axis, as is typically observed. Whether such linkage also promotes the orientation of X_1 and Y chromosomes to opposite poles is a matter for conjecture. Perhaps, for example, the elongation of microtubules, or sliding between interdigitated microtubules, which extend between X_1 and Y kinetochores increases the distance between those kinetochores so that they must come to lie in opposite half spindles. Thus, it is at least conceivable that long-range interactions between X_1 and Y chromosomes via microtubules provides a structural basis for nonrandom segregation.

In summary, we have suggested that nonrandom segregation in *N. hexadactyla* could, in principle, depend upon *a*) half-spindle effects—only one chromosome bearing a modified kinetochore can be accommodated in half spindle and upon *b*) "interzonal" effects—the linkage of modified kinetochores via microtubules that cross the equator influences the relative position of these kinetochores. These are not mutually exclusive possibilities; for example, initial orientation of modified kinetochores to opposite poles may be promoted by half-spindle effects, whereas stabilization of that orientation is dependent upon interzonal effects. This idea is consistent with experimental data which demonstrate that the X_1 chromosome does not achieve stable orientation when the X_2Y pair is inappropriately oriented or has been removed from the cell (2).

We are grateful to R. B. Nicklas for enthusiastic support and critical discussions. Thomas Hays, Robert Kraft, and Leslie Murray contributed in various ways; we thank them especially for their labor in digging for the mole crickets. Henry Wilbur and his associates provided the first animals used in this study and subsequently endured our intrusions in their study areas for further collecting; we appreciate their patience. We thank Michael Reedy and John Boynton who generously shared their electron microscope facilities with us and Sally Schroh-enloher Anderson who provided the drawings for Figs. 1 and 7.

This study was supported in part by research grants from the Division of Physiology, Cellular and Molecular Biology of the National Science Foundation (PCM 79-11481) and the Institute of General Medical Sciences, National Institutes of Health (GM-13745).

Received for publication 4 August 1980, and in revised form 10 October 1980

REFERENCES

1. Anderson, P. J. 1967. Purification and quantitation of glutaraldehyde and its effect on several enzyme activities in skeletal muscle. *J. Histochem. Cytochem.* 15:652–661.
2. Camenzind, R., and R. B. Nicklas. 1968. The non-random chromosome segregation in spermatocytes of *Gryllotalpa hexadactyla*. A micromanipulation analysis. *Chromosoma (Berl.)*. 24:324–335.
3. Carothers, E. E. 1917.The segregation and recombination of homologous chromosomes as found in two genera of Acrididae (Orthoptera). *J. Morphol.* 28:445–521.
4. Chopard, L. 1968. Gryllides. *In* Orthopterorum Catalogus. M. Beier, editor. Dr. W. Junk BV, Publishers, The Netherlands. 12:215–500.
5. Dietz, R. 1969. Bau and Funktion des Spindelapparats. *Naturwissenschaften.* 56:237–248.
6. Fuge, H. 1977. Ultrastructure of the mitotic spindle. *Int. Rev. Cytol. Suppl.* 6:1–58.
7. John, B., and K. R. Lewis. 1965. The meiotic system. *In* Protoplasmatologia. Springer-Verlag KG, Vienna, Austria. VIf1:1–335.
8. Nicklas, R. B. 1971. Mitosis. *In* Advances in Cell Biology. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton-Century-Crofts, New York. 2:225–297.
9. Nicklas, R. B., B. R. Brinkley, D. A. Pepper, D. F. Kubai, and G. K. Richards. 1979. Electron microscopy of spermatocytes previously studied in life: methods and some observations on micromanipulated chromosomes. *J. Cell Sci.* 35:87–104.
10. Payne, F. 1912. The chromosomes of *Gryllotalpa borealis* Burm. *Archiv Zellforsch.* 9:141–148.
11. Payne F. 1916. A study of the germ cells of *Gryllotalpa borealis* and *Gryllotalpa vulgaris*. *J. Morphol.* 28:287–327.
12. Ryter, A., and E. Kellenberger. 1958. Etude au microscope électronique de plasmas contenant de l'acide désoxyribonucleique. I. Les nucléoides des bactéries en croissance active. *Z. Naturforsch. Teil B Anorg. Chem. Org. Chem. Biochem. Biophys. Biol.* 13:597–605.
13. Sutton, W. S. 1903. The chromosomes in heredity. *Biol. Bull. (Woods Hole).* 4:231–251.
14. Virkki, N., and M. Kimura. 1978. Distribution of nuclear pores and perinuclear dense substance in spermatocytes I of some oedionychina flea-beetles. *Biosystems.* 10:213–225.
15. White, M. J. D. 1951. Cytogenetics of orthopteroid insects. *Adv. Genet.* 4:267–330.
16. White, M. J. D. 1973. Evolution of Sex. *In* Animal Cytology and Evolution. Third edition. Cambridge University Press, Cambridge, England. 573–681