Malorientation in Half-bivalents at Anaphase: Analysis of Autosomal Laggards in Untreated, Cold-treated, and Cold-recovering Crane Fly Spermatocytes

MARIE A. JANICKE and JAMES R. LAFOUNTAIN, JR.
Division of Cell and Molecular Biology, State University of New York at Buffalo, Buffalo, New York 14260

ABSTRACT Exposing crane fly larvae to 6°C or returning them to 22°C after exposure to 6, 2, or 0.2°C can induce any number of autosomes in their primary spermatocytes to lag near the spindle equator at anaphase. Autosomal laggards in cold-recovering cells are contained in bivalents until anaphase (Janicke, M. A., and J. R. LaFountain, 1982, *Chromosoma,* 85:619–631). We report here documentation that lagging autosomes in cold-treated and cold-recovering cells are maloriented. During meiosis I, half-bivalents usually associate with only one pole via kinetochore fibers, with sister chromatids being oriented to the same pole. In contrast, laggards had kinetochore microtubules (kMTs) extending from them toward both poles: one sister was oriented to one pole and the other had some or all of its kMTs extending toward the opposite pole. Bipolar malorientation of autosomal laggards also was observed in one untreated cell. The number of kMTs per half-bivalent was similar in lagging and non-lagging autosomes, and those kMTs were contained in long birefringent kinetochore fibers. The overall spindle structure in cold-recovering cells was similar to that observed in untreated anaphase cells. Giemsa-stained centromeric dots of sister chromatids were contiguous in non-laggards and separated in laggards at anaphase. We conclude that bipolar malorientations can exist at anaphase in chromosomes that remain paired until anaphase, that cold recovery increases the frequency of that anomaly, and that such malorientations may be one cause of anaphase lag.

During the reduction division of meiosis, homologues usually pair to form bivalents. Sister chromatids of one homologue (half-bivalent) become oriented (via a kinetochore fiber) to one spindle pole, and the sister chromatids of its partner homologue both become oriented to the opposite pole. This orientation is thought to cause the bivalent to be subjected to bipolar forces that (*a*) result in its congression to the metaphase plate, and (*b*) ensure that upon its separation into half-bivalents at anaphase onset, the coupled sister chromatids of one half-bivalent move together to one pole while the partner half-bivalent moves to the opposite pole (13, 29). In reduction-division chromosomes that do not pair or become unpaired before anaphase (univalents), sister chromatids may become oriented to opposite poles (25, 29). Univalents with such orientations may congress and then "lag" (see Materials and Methods for terminology) near the spindle equator at anaphase. Laggards may or may not eventually move poleward, and several routes exist by which lagging may lead to the production of cells with incorrect chromosome complements (7, 9, 16, 25, 27).

In crane fly primary spermatocytes (cells undergoing the reduction division of meiosis), sex chromosomes are normally unpaired during prometaphase; they congress to the metaphase plate with their sister chromatids oriented to opposite poles and lag at anaphase (4, 10). We have reported previously (16) that exposing crane fly larvae to 6°C or returning them to 22°C after exposure to 6, 2, or 0.2°C induces anaphase lagging of one or both members of any number of the three homologous pairs of autosomes in their primary spermatocytes. Anaphase lag has been induced previously in other types of reduction-division cells by other experimental treatments (14, 32), and in some cases seems to have resulted from induction of premature univalence and subsequent orientation of sisters to opposite poles (14). This clearly was not the case for the autosomes that lagged in cold-treated and in cold-recovering crane fly spermatocytes; the autosomes that lagged

© The Rockefeller University Press · 0021-9525/84/03/0859/11 $1.00

TABLE I

Quantitation of Spindle Ultrastructure in Treated and Untreated Cells

Cell No.	Treatment	Chromosome distribution	Kinetochore MTs				Equatorial MTs	
			Non-laggard autosomes		Laggard autosomes	Sex univalents	Spindle	Non-spindle
1	Untreated	∧∧∧ / ·· / ∨∨∨	[110↑↑]			[— / 64↓]	1,141	853
2	Untreated	∧∧∧ / ·· / ∨∨∨	[93↑↑]	[88↓↓]		[— / 35↓] [— / 48↓]	718	524
3	Untreated	∧∧ / ··<< / ∨∨	[83↑↑]		[44↑ / 45↓] [25↑3↓ / 36↓]			
4	62 h at 6°C without recovery	∧∧∧ / ·· / ∨∨∨	[127↑↑]			[66↑ / 71↓] [50↑ / 56↓1↑]	346	350
5	62 h at 6°C without recovery	∧∧∧ / ·· / ∨∨∨	[164↑↑]				330	454
6	62 h at 6°C without recovery	∧∧∧ / ··< / ∨∨	[116↑↑]	[97↓↓]	[39↑ / 58↓]	[51↑ / 54↓] [47↑ / 67↓]	383	441
7	62 h at 6°C without recovery	∧∧ / ··<< / ∨∨			[67↑ / 69↓4↑] [57↑ / 43↓]		547	530
8	63 h at 6°C, then 69 min at 22°C	∧∧∧ / ·· / ∨∨∨	[86↑↑]					
9	63 h at 6°C, then 77 min at 22°C	∧∧∧ / ·· / ∨∨∨				[53↑ / 70↓]	832	1,290
10	63 h at 6°C, then 77 min at 22°C	∧∧∧ / ··< / ∨∨	[103↑↑]		[76↑ / 56↓6↑]	[— / 45↓] [— / 59↓]	547	530
11	25.5 h at 0.2°C, then 81 min at 22°C	∧∧∧ / ··< / ∨∨			[47↑ / 48↓]	[49↑ / 51↓] [— / 34↓]	693	1,142
12	21.5 h at 2°C, then 66 min at 22°C	∧∧∧ / ·· / ∨∨∨					505	1,618
13	21.5 h at 2°C, then 48 min at 22°C	∧∧∧ / ·· / ∨∨∨	[82↑↑]			[72↑ / 54↓] [76↑ / 63↓]	1,142	641
14	22 h at 2°C, then 58 min at 22°C	∧∧∧ / ··< / ∨∨	[93↑↑]		[32↑11↓ / 49↓]	[— / 43↓] [— / 50↓]	797	1,245

15	21.5 h at 2°C, then 58 min at 22°C	ΛΛΛ ··< VV		[46↑ / 47↓]			
16	21.5 h at 2°C, then 48 min at 22°C	ΛΛΛ ··< VV		[45↑ / 39↑10↓]		1,223	794
17	48 h at 2°C then 55 min at 22°C	ΛΛ ··<< VV	[116↑↑]	[40↑ / 39↓2↑]			
18	21.5 h at 2°C, then 58 min at 22°C	ΛΛ ··<< VV		[37↑1↓ / 45↓]			
19	48 h at 2°C, then 55 min at 22°C	ΛΛ ··<<< V	[93↑↑]	[55↑1↓ / 60↓] [45↑ / 54↓]			
20	22 h at 2°C, then 58 min at 22°C	Λ ··<<<< V	[114↑↑]	[48↑ / 58↓] [43↑ / 45↓] [49↑ / 50↓] [40↑ / 46↓]	[— / 58↓]	913	789
21	48 h at 2°C, then 55 min at 22°C	··<<<<<<		[57↑ / 51↓] [55 / 63↓]			

The identification number of each cell is followed by the treatment applied to the larva from which the cell was obtained. The next column indicates diagrammatically the number of non-lagging autosomal half-bivalents at the upper pole (Λ), non-laggards at the lower pole (V), lagging sex chromosomes (·), and lagging autosomes (<). For each non-lagging autosome analyzed, the combined number of kMTs of both sister kinetochores is given, followed by two arrows pointing toward the same pole, to indicate that sister kinetochores were adjacent and that all their kMTs extended toward the same pole. For lagging autosomes and sex chromosomes, sister kinetochores were separate from one another; the kMT complement of the kinetochore closest to the upper pole is given above the complement of its sister, with the two values being included together in brackets. The number of kMTs extending from each kinetochore toward the upper pole is indicated by an arrow pointing up, and the number extending toward the lower pole is indicated by an arrow pointing down. In many cases, kMT counts were made for only one kinetochore of a sex chromosome, and the kinetochores that were not analyzed are denoted by dashes. Counts of non-kinetochore spindle MTs and non-spindle MTs were made from single sections of cells through an equatorial plane containing both sex chromosomes; such planes contain few if any kMTs.

in those cells were half-bivalents that were contained until anaphase in bivalents, the latter often appearing unusually tilted at metaphase (16).

The purpose of the present work was to investigate the basis of the lagging that we induced using cold and cold recovery. We report here light and electron microscopic evidence that the half-bivalents that lagged in cold-treated and cold-recovering cells had bipolar malorientations (that is, kinetochore fibers extended from them toward both poles instead of toward only one pole at anaphase). We believe that the treatments used here induce lagging by inducing failure of half-bivalents contained in bivalents to achieve proper orientation by anaphase onset.

MATERIALS AND METHODS

Terminology: We use the words lagging and laggards to refer to any chromosome remaining near the spindle equator at a time in anaphase when most chromosomes normally have reached the poles. The term is applied regardless of the reason the chromosome is found at the equator and regardless of whether or not it eventually moves to a pole.

The kinetochore is the structure that is located at the centromere (primary constriction) of a chromosome and mediates a connection between the chromosome and the spindle (31). Spindle microtubules (MTs)[1] that terminate in a kinetochore are referred to as kinetochore microtubules (kMTs), while those that do not are referred to as non-kinetochore microtubules (nkMTs) (11). A kinetochore fiber appears in thin sections as a bundle of spindle MTs (many of them kMTs) extending from the kinetochore of a chromosome toward a pole.

A pair of sister chromatids is said to be amphitelically oriented if its sister kinetochores are associated via kinetochore fibers with opposite poles. Syntelic orientation refers to orientation of sister kinetochores to the same pole (4). An individual kinetochore is merotelically oriented if it has kMTs extending from it toward both spindle poles (Östergen, cf. reference 2).

Cold Treatment: Crane flies (*Nephrotoma suturalis*) were maintained in the laboratory and fourth-instar larvae containing spermatocytes in desired stages were selected and subjected to cold treatments as described previously (16). Ultrastructural analysis was done on anaphase primary spermatocytes obtained from larvae exposed to treatments previously shown to induce autosome lagging in high percentages of cells. Those treatments consisted of either exposing larvae to 6°C for ~60 h or exposing larvae to 6, 2, or 0.2°C for a day or more and then returning them to 22°C for ~1 h. Kinetochore fiber birefringence and the positions of Giemsa-stained centromeric dots were examined in cells from larvae recovering from exposures of a day or more to 2°C. Results from these analyses were compared with results obtained from untreated cells.

Ultrastructural Analysis: To prepare crane fly spermatocytes from untreated or cold-recovering larvae for electron microscopy, testes were dissected from the larvae in Tricine insect buffer (5) and ruptured, and their contents were spread as a monolayer over a thin film of agar (0.5%) containing 2% glutaraldehyde in 0.05 M cacodylate buffer, the film having been applied previously to a carbon-coated coverslip. The moment the cells were spread was taken to be the end of the cold-recovery period in the case of cold-recovering cells. The fixed cells were secured to the coverslip by overlaying them with a second thin film of molten agar and gelatin (0.75% agar and 0.75% gelatin in distilled water) and then immersed in a solution of 2% glutaraldehyde in 0.05 M cacodylate buffer for 1 h. Fixation of cells dissected from larvae in the cold room was accomplished in a similar manner, except that the coverslips with their agar-glutaraldehyde films were pre-chilled, and after dissection the monolayers of fixed cells were kept in closed dishes containing glutaraldehyde for ~1 h; the closed dishes then were transferred to 22°C and the monolayers were immediately overlaid with agar/gelatin films. Post-fixation, dehydration, infiltration, and embedding in epoxy resin were done as described by LaFountain (21). The wafers of cells embedded in plastic were removed from the glass coverslips in liquid nitrogen. Cells in desired stages of division were selected using phase-contrast microscopy, and sections were cut on a Porter-Blum ultramicrotome MT-II. For most cells, thin sections (~0.1 μm) were cut, but a few cells were cut into relatively thick (~0.25 μm) sections. Serial sections were collected on Formvar-coated slot grids and stained for 20 min in uranyl acetate and for 10 min in lead citrate. The grids were then carbon coated and examined on a Hitachi-500 electron microscope operated at 75 kV.

Ultrastructural analysis was confined to primary spermatocytes in early to mid-anaphase. In these cells, the sex chromosomes and any autosomal laggards present were at or near the spindle equator. For all cells analyzed, the positions of lagging and non-lagging autosomes were recorded before sectioning. Analysis was restricted to cells in which the number of chromosomes at each pole and the number of laggards could be clearly determined. Lagging chromosome fragments and bridges such as those described by others (12) were rare in both untreated and treated cells and were not included in the analysis.

Four anaphase cells that were obtained from larvae at recovery times of ~1 h after exposures of a day or more to 2°C and that exhibited autosome lagging were serially sectioned parallel to the spindle axis. Serial transverse sections were collected from 21 cells: 3 cells from untreated larvae, 4 from larvae exposed to 6°C without recovery, 3 from larvae recovering from 6°C, 1 from a larva recovering from 0.2°C, and 10 from larvae recovering from 2°C (Table I). Of those cells, 13, including one untreated cell and at least one cell subjected to each of the treatments, contained autosomal laggards. Cells analyzed after recovery from 2°C had zero, one, two, three, four, or six of their six autosomal half-bivalents lagging.

In order to track MTs in serial transverse sections, the profile of each MT in the area of interest (e.g., surrounding a kinetochore) was coded in a printed enlargement of an electron micrograph and followed in successive sections. For analysis of the number and distribution of MT profiles in an entire transverse section of a cell, overlapping micrographs were taken of all areas of the cell in that section, and printed enlargements of those micrographs were assembled into a montage. Student's t test was used at $\alpha = 0.05$ for comparison of average counts of MTs.

Giemsa Staining of Centromeric Dots: The method of Sumner (33) was modified in order to stain centromeric dots (see also reference 26) on crane fly chromosomes, enabling rapid assessment of centromere position in populations of spermatocytes. Air-dried smear preparations of testicular contents (16) were immersed in Carnoy's fixative (3:1 ethanol/acetic acid), air-dried, aged for several days, immersed in Carnoy's, and air-dried again before being treated for 30 min with 0.2 N HCl at room temperature. After a rinse in distilled water, the smear preparations were transferred to a freshly made aqueous solution of 5% barium hydroxide octahydrate [$Ba(OH)_2 \cdot 8H_2O$] for 60 min. They were then rinsed three times in distilled water, incubated for 1 h in 2 × SSC (2 × SSC = 0.03 M trisodium citrate in 0.3 M sodium chloride) at 60°C, rinsed, and stained in 5 or 7.5% Giemsa in M/15 Sorensen's phosphate buffer, pH 7.2. Staining was monitored by viewing the preparations under the microscope after a brief rinse in distilled water. When centromeric dots were sufficiently differentiated (generally after 15 min to 2 h of staining), the preparations were allowed to air-dry and were mounted on glass slides using Permount (Fischer Scientific Co., Pittsburgh, PA). The clarity with which the centromeric dots were stained was quite variable, even among cells on the same slide. In some cases, testes were hypotonically treated in distilled water or 0.17% NaCl before preparation of the smear, but no consistent improvement in staining was obvious after this treatment. Cells were observed using a 100×/

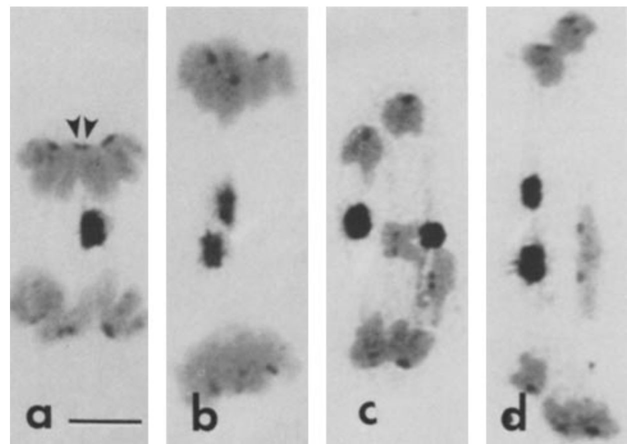

FIGURE 1 Light micrographs of anaphase cells that were subjected to a Giemsa-staining technique for centromeric heterochromatin. The lagging sex chromosomes stain intensely throughout. Centromeric dots, indicated by two arrowheads in *a*, are closely apposed at the centromeric region of each non-lagging autosomal half-bivalent. In lagging autosomes, however, the dots are separated (*c*–*d*). The cells in *a* and *b* were obtained from untreated larvae; *c* and *d* were from a larva exposed to 2°C for 22 h then returned to 22°C for 76 min. Bar, 5 μm. × 1,900.

[1] *Abbreviations used in this paper:* kMT, kinetochore microtubule; MT, microtubule; nkMT, non-kinetochore microtubule.

154 153 152
151 150 149

FIGURE 2 Selected micrographs of serial transverse thin sections through the kinetochore fiber and kinetochores of a non-lagging autosomal half-vibalent in cell 17 (Table I). The distribution of kMTs, which are circled, is similar to that observed in untreated cells. Sister kinetochores (sections 151–153) are adjacent, and kMTs extend from them toward the same pole (in the direction of section 154). As in non-lagging autosomes in untreated cells, no MTs begin at the kinetochores and extend toward the equator. Bar, 0.5 μm. × 60,000.

1.25 NA oil-immersion objective and recorded on Kodak Panatomic-X film (Eastman Kodak Co., Rochester, NY).

Polarization Microscopy: For analysis of kinetochore fiber birefringence, living-cell preparations were made in halocarbon oil (23), and cells to be observed with polarization optics were located using Nomarski differential interference contrast microscopy. The polarization microscope was set up with a relatively strain-free achromatic (40×/0.85 NA oil immersion) objective, a λ/30 rotatable compensator, a mercury arc lamp (HBO-200), a narrow-band green (546-nm) interference filter, and a heat-absorbing filter. Photographic records were made on Kodak Tri-X film.

RESULTS

Non-lagging Autosomes

In treated and in untreated cells, non-lagging autosomes were shaped as a V at anaphase, with their arms trailing toward the spindle equator. At the poleward apex of that V, the sister chromatids were joined near their centromeres. Giemsa-stained centromeric dots of sister chromatids of non-lagging half-bivalents were contiguous and located at the apex

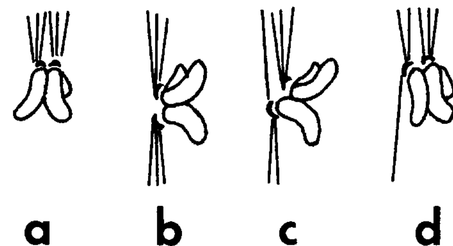

FIGURE 3 Diagram of types of orientation in anaphase half-bivalents described in this report: (*a*) syntely, (*b*) amphitely, (*c*) amphitely with merotely, (*d*) syntely with merotely.

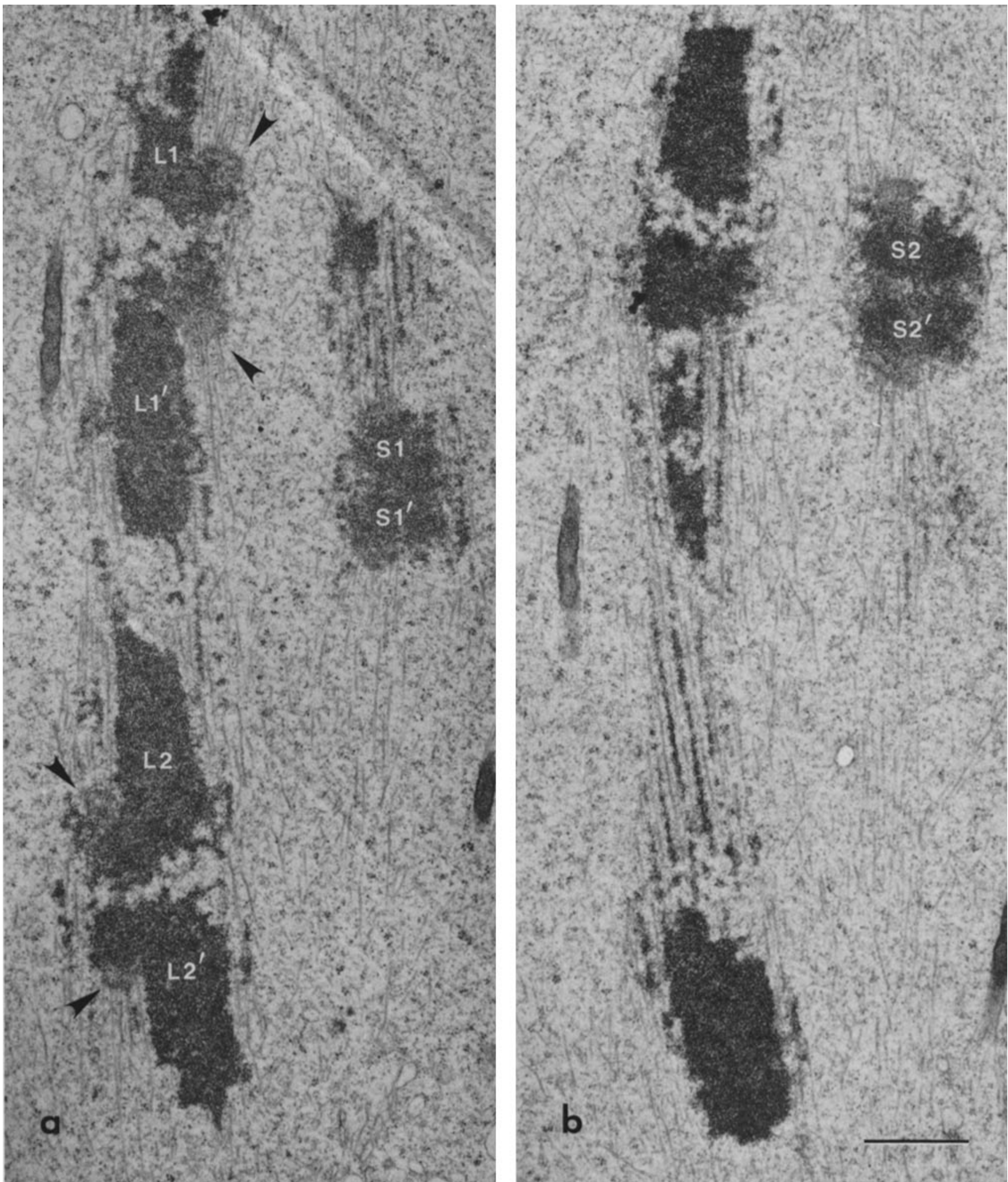

FIGURE 4 Longitudinal thin sections through the lagging autosomes and sex chromosomes of a cell containing two lagging autosomes. The cell was obtained from a larva exposed to 2°C for 21.5 h then returned to 22°C for 66 min. The pole-to-pole axis of the spindle is approximated by the vertical axis of the micrograph. In *a*, the sister kinetochores of both lagging autosomes are visible. The kinetochores (arrowheads) of sister chromatids of one laggard (L1 and L1′) face opposite poles and have kMTs extending from them toward opposite poles. Similar amphitelic orientation of kinetochores (arrowheads) is evident in the sister chromatids (L2 and L2′) of the other autosomal laggard. Sister kinetochores of one sex univalent (with its sister chromatids labeled S1 and S1′) also are visible and have kMTs extending from them toward opposite poles. Some of the kMTs extending from the kinetochore of S1 appear to run through grooves in the periphery of the other sex univalent, located above it (see also reference 10). In *b*, which is four sections from *a*, MTs are in chromosome grooves at the peripheries of L2 and L1′. Kinetochores of the second sex univalent (the chromatids of which are labeled S2 and S2′) are included in this section. Also depicted are MTs extending through grooves at the periphery of S1-S1′. Bar, 1 μm. ×, 18,000.

of the half-bivalent (Fig. 1). Each dot was ~0.5 μm in diameter. In electron micrographs, kinetochores could be distinguished from chromatin on the basis of their lower electron density. Each sister kinetochore appeared as a curved disk, ~0.4 μm in diameter and 0.2 μm thick, with the concave face of the disk adjacent to the chromosome and the convex face of the disk directed away from the chromosome. Sister kinetochores of non-lagging autosomes were located closely apposed to one another and faced the same pole (22).

For all cells examined (untreated or subjected to any of the temperature treatments used), regardless of whether the cells contained no autosomal laggards or as many as four of them, serial transverse section analysis of non-lagging autosomes (Fig. 2) revealed perfect syntelic orientation without merotely (see *Terminology* in Materials and Methods and Fig. 3*a*). Three autosomal half-bivalents in two untreated cells without autosomal laggards averaged 97 ± 12 (SD) kMTs per half-bivalent (Table I). That number was not statistically different from the 83 kMTs that were attached to a non-lagging autosome in an untreated cell with autosomal laggards or from the 98 ± 13 (SD) kMTs attached to non-lagging autosomes in cold-recovering cells. Non-lagging autosomes in cells fixed after 62 h at 6°C with no recovery generally had at least as many (if not more) kMTs as autosomes in untreated cells (Table I).

Lagging Autosomes

In contrast to non-laggards, the arms of lagging autosomes were usually spread apart at anaphase, extending toward opposite spindle poles. Sister chromatids were held together near their centromeres, but Giemsa-stained centromeric dots were separated from one another along the pole-to-pole axis of the spindle (Fig. 1). Electron microscopy of lagging autosomes revealed that the two sister kinetochores of a lagging autosome were generally separated by ~1–2 μm along the pole-to-pole axis during early to mid-anaphase. Sister kinetochores of autosomal laggards faced opposite poles (Figs. 4 and 5), with some of them being tilted slightly toward the sides of the spindle (Fig. 5).

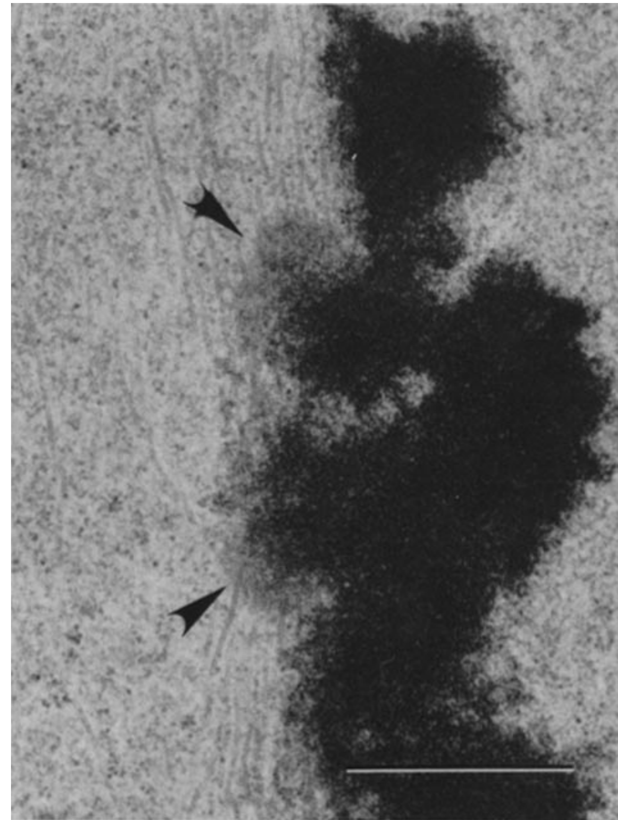

FIGURE 5 Electron micrograph of a thick (0.25-μm) longitudinal section through a lagging autosomal half-bivalent in a cell obtained from a larva that had been exposed to 2°C for 22 h then returned to 22°C for 58 min before fixation. The vertical axis of the micrograph approximates the pole-to-pole axis of the spindle. The two sister kinetochores (arrowheads) are separated from one another and face essentially toward opposite poles but have portions that jut out laterally from the chromosome. The half-bivalent clearly is amphitelic and also appears to display some merotely, although the latter is difficult to identify with certainty in a thick longitudinal section. Bar, 1 μm. × 30,000.

For each autosomal laggard, a bundle of kMTs extended along the length of each of the outspread arms and inserted into a kinetochore (Figs. 4–7). Other MTs extended along the length of the laggard and did not terminate in a kinetochore. Many of those MTs were contained in grooves that extended along the length of the laggards and penetrated a variable depth into the chromatin (Figs. 4*b*, 6, and 7), each groove being ~0.10–0.15 μm wide. Reports by Fuge (10, 12) and by Forer and Brinkley (8) on crane fly spermatocytes include descriptions of similar chromosomal grooves containing MTs in lagging sex chromosomes, trailing autosomal arms, and X-ray-induced fragments. We also observed such grooves in sex chromosomes at anaphase (Fig. 4*a*).

All autosomal laggards were found to have bipolar malorientation. Autosomal laggards exhibiting amphitely without merotely (Fig. 3*b*) were observed in cells fixed at 6°C, in a cell recovering from exposure to 0.2°C, in cells recovering from exposure to 2°C, and in one autosomal laggard in an untreated cell (Table I, cells 3, 6, 7, 11, 15, 19–21). For one of the sister kinetochores of each of the other autosomal laggards, another arrangement was seen. Successive sections going through the kinetochore fiber and toward the equator revealed MT profiles that could be tracked into the kinetochore material for a few sections and then could be seen no more, as was also the case for kMTs attached to correctly oriented kinetochores. What was unusual was that a few sections after the first set of profiles had ended, a few MT profiles appeared in the kinetochore region and extended toward the equator. The MTs extending from the equatorial side of the kinetochore were not in register with the MTs that extended from its poleward side. In many cases, both sets of MTs were perpendicular to the plane of section. One possibility is that the MTs seen extending from the kinetochore toward the equator were continuations of MTs that penetrated the kinetochore from its poleward side, became obscured and bent as they passed through the kinetochore, and became visible again as they emerged on the other side. This possibility cannot be ruled out. However, in the absence of any evidence for such continuity, we have interpreted this arrangement as merotely: after the kMTs extending to one pole had ended, a few additional kMTs began that extended toward the opposite pole. Those additional MTs were located near the periphery of the kinetochore and extended along the side of the laggard, often within chromosomal grooves (Figs. 6 and 7). Merotely was observed in one of the sister kinetochores of one or more autosomal laggards in a cell exposed to 6°C, a cell recovering from exposure to 6°C, cells recovering from exposure to 2°C,

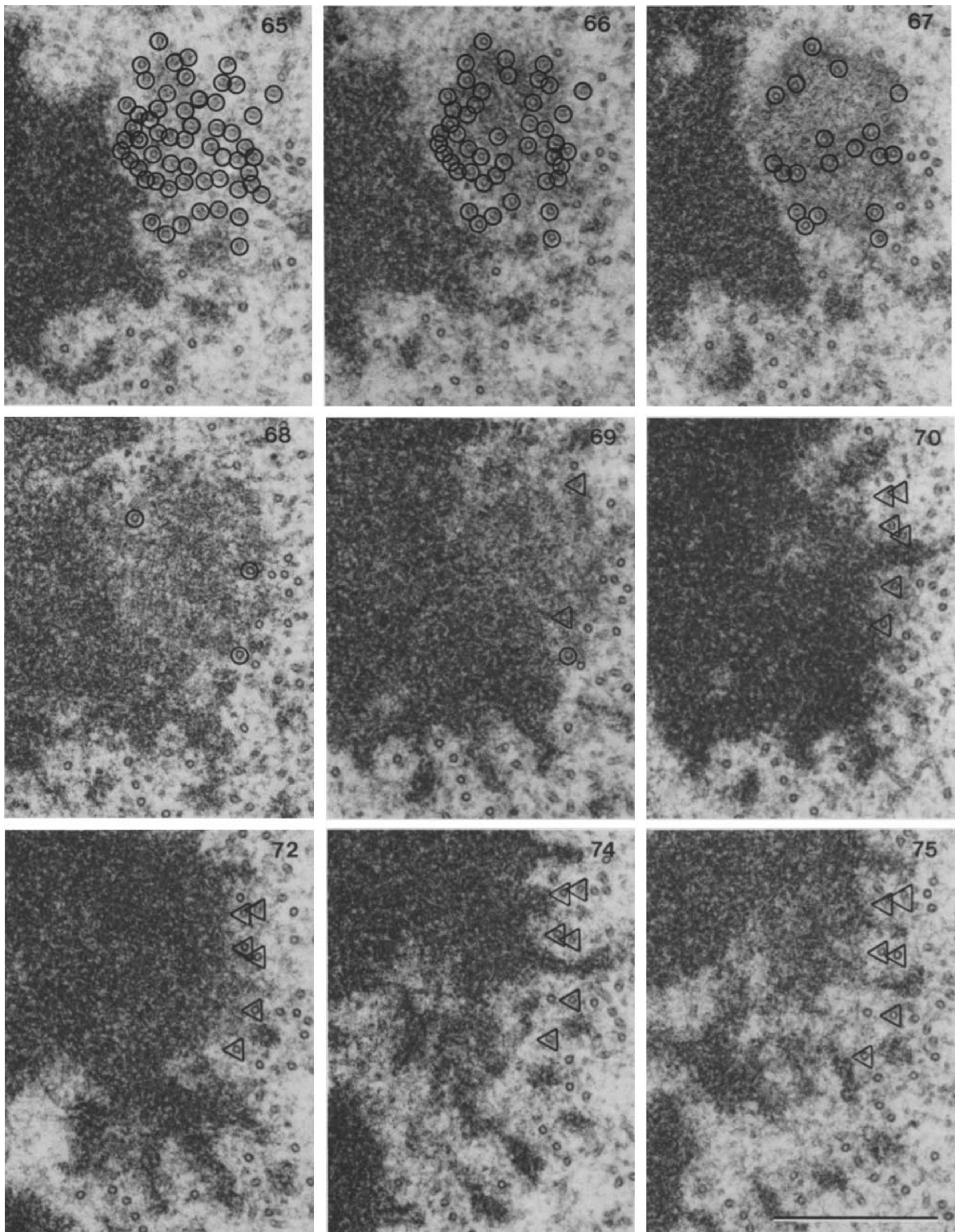

FIGURE 6 Selected micrographs of serial transverse thin sections through the lagging autosome in cell 10 (Table I). The circled kMTs terminate in section 66, 67, 68, or 69 in a single sister kinetochore. The MTs in triangles in section 69 and subsequent sections are kMTs that begin in the kinetochore on its equatorial face and extend toward the pole opposite from the one toward which the circled kMTs extend. The presence of these kMTs defines this kinetochore as being merotelic. Chromosomal grooves containing MTs that extend along the side of the laggard are seen here in transverse section. (The series continued in Fig. 7.) Bar, 0.5 μm. × 60,000.

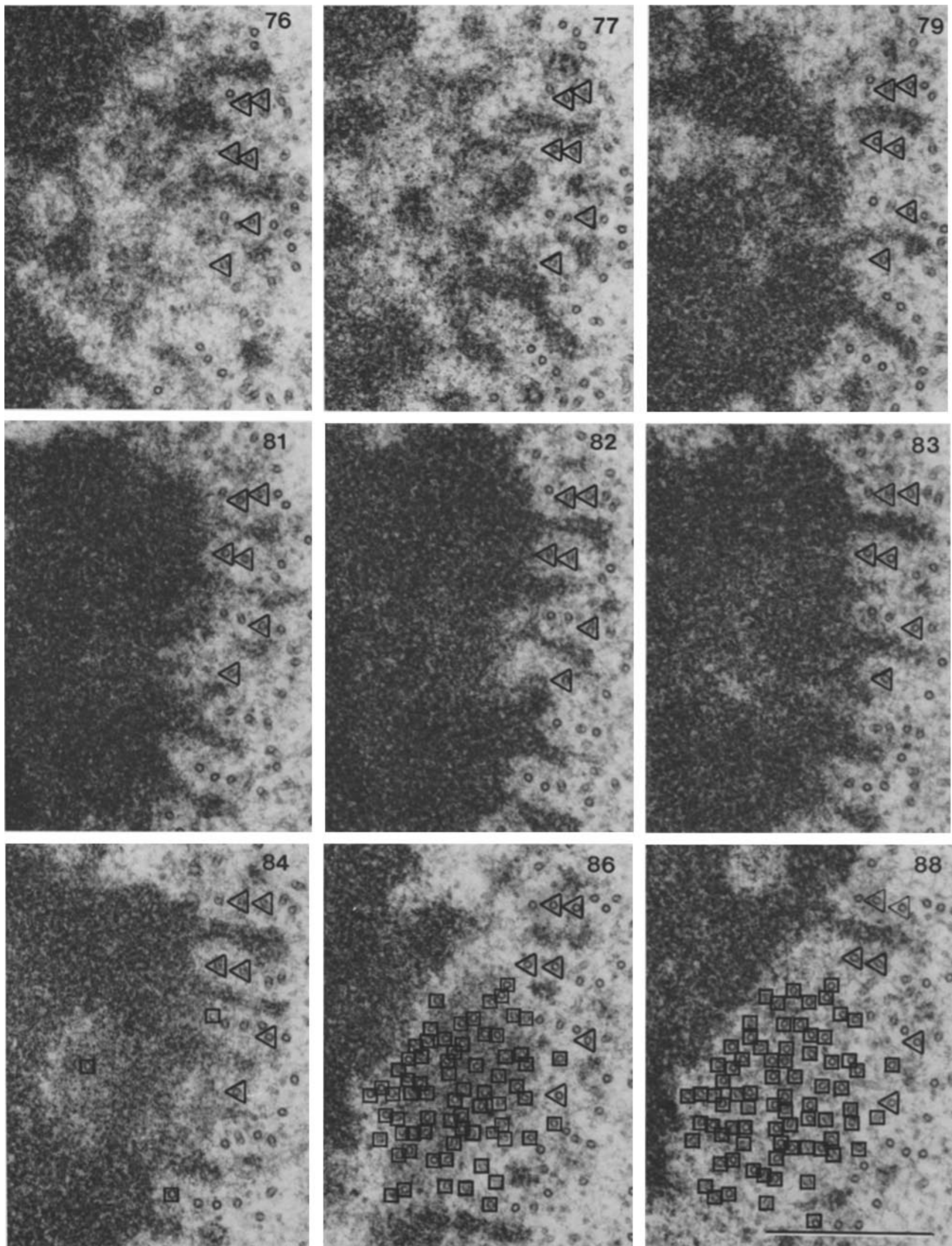

FIGURE 7 Sections 84–86 show the other sister kinetochore of the lagging half-bivalent in Fig. 6. All of the kMTs (which have been enclosed in squares) of this kinetochore extend toward the pole opposite from that to which the majority of kMTs extend from the kinetochore in Fig. 6. Overall, then, this laggard has amphitelic orientation with one of its kinetochores exhibiting merotely. Bar, 0.5 μm. × 60,000.

and one autosomal laggard in an untreated cell (Table I, cells 3, 7, 10, 14, 16, 17–19). Ultrastructural reports of merotely have been made previously for prometaphase plant and animal cells undergoing equational divisions (1, 17, 24) and for prometaphase univalents (34).

For all but one of the laggards exhibiting merotely, the majority of kMTs at the merotelic kinetochore extended toward the pole opposite from the one toward which its sister kinetochore was oriented. We classify such laggards as being amphitelic with merotely (Fig. 3 *c*). The one autosomal laggard analyzed that displayed neither pure amphitely nor amphitely with merotely was found in an early-anaphase cell recovering from exposure to 2°C (Table I, cell 16). Its kinetochores were facing the same pole, as was the case in syntelically oriented half-bivalents, and were not separated as much as were the sister kinetochores of other laggards. One of its sister kinetochores had 45 kMTs directed toward the pole the kinetochores faced, while the other had only 39 directed toward that pole and 10 directed toward the opposite pole. We classify this laggard as being syntelic with merotely (Fig. 3 *d*).

We did not find a statistical difference between the average number of kMTs per lagging half-bivalent after any treatment and the average number of kMTs per non-lagging half-bivalent in untreated cells (Table I). Polarization microscopy of cells from cold-recovering larvae revealed that kMTs of laggards were contained in long kinetochore fibers (Fig. 8), as were kMTs of non-lagging chromosomes.

Other Aspects of Spindle Ultrastructure

Analysis of serial transverse sections through the sex chromosome kinetochore fibers of untreated, 6°C-treated, and cold-recovering cells (including those that contained autosomal laggards and those that did not) showed that all sex chromosomes were amphitelically oriented and all possessed comparable numbers of kMTs (Table I). One sister kinetochore in a sex chromosome of a cell fixed after 63 h at 6°C exhibited merotely (Table I, cell 4), but in all other cases, pure amphitely was observed in sex chromosomes. The number of kMTs per sister kinetochore of each sex chromosome in treated cells did not show a statistical difference from the number in untreated cells.

The number and distribution of MTs other than kMTs was examined in transverse sections of the spindle at an equatorial plane that included both sex chromosomes. The only kMTs that may have been included in such sections were the few kMTs that extended from merotelic kinetochores toward the equator. These sections contained numerous nkMTs and numerous MTs in the cytoplasm peripheral to the spindle. Many of the latter were associated with the mitochondria that ensheath the spindle in crane fly spermatocytes (20). The cells we examined after 62 h at 6°C without recovery had significantly fewer spindle MTs at the equator than did the untreated cells we examined (Table I). Most of the nkMTs present at the equator in cells fixed at 6°C were found around the peripheries of the lagging sex chromosomes and of any autosomal laggards present. In cells recovering from exposure to 6, 2, or 0.2°C, the numbers of MTs at the equator at anaphase were not statistically different from those numbers in untreated cells.

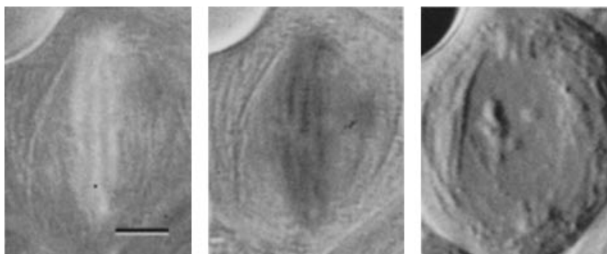

FIGURE 8 Micrographs (polarized light, positive and negative contrast, and Nomarski differential interference contrast) of a living cell obtained from a larva that had been exposed to 2°C for 25 h and returned to 22°C 79–85 min before these micrographs were taken. The cell contains a single lagging autosomal half-bivalent to the left of the two lagging sex univalents. The five non-lagging half-bivalents already have reached the poles. Birefringent kinetochore fibers extend from the lagging autosomal half-bivalent toward each of the two spindle poles. Bar, 5 μm. × 1,400.

DISCUSSION

Naturally occurring autosomal laggards in crane fly primary spermatocytes and the autosomal laggards induced by exposing larvae to 6°C or by returning larvae to 22°C after exposure to 6, 2, or 0.2°C exhibited bipolar malorientation: instead of having syntelic orientation, as autosomes normally do in crane fly primary spermatocytes, they exhibited amphitely and/or merotely (see *Terminology* in Materials and Methods and Fig. 3). To our knowledge, this is the first ultrastructural documentation of malorientation in anaphase chromosomes. The only previous ultrastructural demonstrations of unorthodox orientations at anaphase (e.g., amphitely during reduction-division anaphase) were made in cell types in which those orientations are normal (10, 19).

Bipolar malorientation has been invoked as an explanation for the congression to the metaphase plate and anaphase lagging of univalents in reductionally dividing plant and animal cells (3, 4, 14, 34). Indeed, amphitely has been shown at the ultrastructural level at metaphase in univalents of a wheat hybrid in which variable numbers of chromosomes were unpaired during prometaphase and lagged at anaphase (34). Half-bivalents reattaching to the anaphase spindle of crane fly spermatocytes after experimental detachment at anaphase also can establish bipolar malorientation, as evidenced by analysis of kinetochore fiber birefringence (9).

A common assumption has been that, in contrast to the situation for univalents, bipolar malorientations in paired chromosomes are always corrected before anaphase. This is based on results from plant and animal reduction-division chromosomes that remain paired before anaphase, which show ultrastructural evidence for bipolar malorientations at prometaphase but not at anaphase (6, 24). Indeed, there is strong evidence that when unipolar malorientations occur in paired chromosomes (that is, malorientations in which all kinetochore fibers extend from the chromosome pair to the same pole during prometaphase), those orientations are abandoned before anaphase (4, 29).

The only evidence contrary to the assumption that bipolar malorientations in paired chromosomes are always abandoned by anaphase has been based on light microscopic assessment of chromosome configurations in: (*a*) translocation trivalents in crane fly primary spermatocytes (4), (*b*) bivalents having unusually long intercentromeric distances as a result of structural hybridity in plant and animal hybrids (18, 30), and (*c*) two grasshopper bivalents, one micromanipulated into that configuration, and a second that was not manipulated but was observed in a micromanipulated cell

(28). Our ultrastructural data confirm that bipolar malorientation can exist at anaphase in half-bivalents that were contained in bivalents until anaphase.

Malorientation of the lagging autosomes in untreated, cold-treated, and cold-recovering crane fly primary spermatocytes was probably the cause of their lagging. All non-lagging chromosomes in reduction-division cells exhibited syntelic orientation, and all the lagging autosomes analyzed exhibited amphitely and/or merotely. In untreated and cold-recovering cells, the number of kMTs per lagging autosome was similar to the number per non-lagging autosome, and the overall spindle ultrastructure at anaphase seemed to be similar in cells with and without autosomal laggards. Also, the kinetochore fibers of laggards extended long distances toward the poles, as do the kinetochore fibers of non-lagging autosomes. The sister chromatids of the autosomal laggards had not disconnected as a result of forces acting upon them toward opposite poles. However, sister kinetochores were separated from one another in laggards, and that could have resulted from transmission of antagonistic forces by the kinetochore fibers of the lagging half-bivalents.

There are two reports in the literature of large-scale induction of unusual orientation behavior in bivalents during prometaphase, but neither of those reports provided evidence for the persistence of bivalent malorientations until anaphase (14, 15). We believe the methods used here are the first reported for large-scale induction of errors in the mechanism(s) that normally ensures that proper orientation is achieved by anaphase in half-bivalents that remain paired until anaphase. Perhaps our understanding of those mechanisms will be improved through comparison of the orientation process in untreated cells and in cells subjected to malorientation-inducing treatments.

We would like to thank Ed Cahill, Alan Siegel, Leah Steinberg, and Pat Winkler for skilled technical assistance. We also are grateful to Jim Drake and Dennis McCarthy for help with the Giemsa staining of centromeric dots.

This work was supported by National Institutes of Health grant GM-27288.

Received for publication 29 June 1983, and in revised form 10 November 1983.

REFERENCES

1. Bajer, A., and J. Molé-Bajer. 1969. Formation of spindle fibers, kinetochore orientation, and behavior of the nuclear envelope during mitosis in endosperm. Fine structural and in vitro studies. *Chromosoma (Berl.).* 27:448–484.
2. Bajer, A., and J. Molé-Bajer. 1972. Spindle dynamics and chromosome movements. *Int. Rev. Cytol. Suppl.* 3:1–271.
3. Balog, C. 1979. Studies on triploid *Allium triquetrum*. II. Metaphase I univalents and their influence on anaphase I distribution. *Chromosoma (Berl.).* 73:181–205.
4. Bauer, H., R. Dietz, and C. Röbbelen. 1961. Die Spermatocytenteilungen der Tipuliden. II. Das Bewegungsverhalten der Chromosomen in Translokationheterozygoten von *Tipula oleracea. Chromosoma (Berl.).* 12:116–189.
5. Begg, D. A., and G. W. Ellis. 1979. Micromanipulation studies of chromosome movement. II. Birefringent chromosomal fibers and the mechanical attachment of chromosomes to the spindle. *J. Cell Biol.* 82:542–554.
6. Church, K., and H. P. Lin. 1982. Meiosis in *Drosophila melanogaster*. II. The prometaphase I kinetochore microtubule bundle and kinetochore orientation in males. *J. Cell Biol.* 93:365–373.
7. Dietz, R. 1969. Bau und Funktion des Spindelapparats. *Naturwissenschaften.* 56:237–248.
8. Forer, A., and B. R. Brinkley. 1977. Microtubule distribution in the anaphase spindle of primary spermatocytes of a crane fly (*Nephrotoma suturalis*). *Can. J. Genet. Cytol.* 19:503–519.
9. Forer, A., and C. Koch. 1973. Influence of autosome movements and of sex chromosome movements on sex chromosome segregation in crane-fly spermatocytes. *Chromosoma (Berl.).* 40:417–442.
10. Fuge, H. 1972. Morphological studies on the structure of univalent sex chromosomes during anaphase movement in spermatocytes of the crane fly *Pales ferruginea. Chromosoma (Berl.).* 39:403–417.
11. Fuge, H. 1974. The arrangement of microtubules and the attachment of chromosomes to the spindle during anaphase in Tipulid spermatocytes. *Chromosoma (Berl.).* 45:245–260.
12. Fuge, H. 1975. Anaphase transport of akinetochoric fragments in Tipulid spermatocytes. Electron microscopic observations of fragment-spindle interactions. *Chromosoma (Berl.).* 52:149–158.
13. Hays, T. S., D. Wise, and E. D. Salmon. 1982. Traction force on a kinetochore at metaphase acts as a linear function of kinetochore fiber length. *J. Cell Biol.* 93:374–382.
14. Henderson, S. A. 1962. Temeprature and chiasma formation in *Schistocerca gregaria*. II. Cytological effects at 40°C and the mechanism of heat-induced univalence. *Chromosoma (Berl.).* 13:437–463.
15. Henderson, S. A., R. B. Nicklas, and C. A. Koch. 1970. Temperature-induced orientation instability during meiosis: an experimental analysis. *J. Cell Sci.* 6:323–350.
16. Janicke, M. A., and J. R. LaFountain, Jr. 1982. Chromosome segregation in crane-fly spermatocytes: cold treatment and cold recovery induce anaphase lag. *Chromosoma (Berl.).* 85:619–631.
17. Jensen, C. G. 1982. Dynamics of spindle microtubule organization: kinetochore fiber microtubules of plant endosperm. *J. Cell Biol.* 92:540–558.
18. Klingstedt, H. 1939. Taxonomic and cytological studies on grasshopper hybrids. I. Morphology and spermatogenesis of *Chorthippus bicolor* Charp. × *Ch. biguttulus* L. *J. Genet.* 47:389–422.
19. Kubai, D. F., and D. Wise. 1981. Nonrandom chromosome segregation in *Neocurtilla (Gryllotalpa) hexadactyla*: an ultrastructural study. *J. Cell Biol.* 88:281–293.
20. LaFountain, J. R., Jr. 1972. An association between microtubules and aligned mitochondria in *Nephrotoma* spermatocytes. *Exp. Cell Res.* 71:325–328.
21. LaFountain, J. R., Jr. 1974. Birefringence and fine structure of spindle in spermatocytes of *Nephrotoma suturalis* at metaphase of the first meiotic division. *J. Ultrastruct. Res.* 46:268–278.
22. LaFountain, J. R., Jr. 1976. Analysis of birefringence and ultrastructure of spindles in primary spermatocytes of *Nephrotoma suturalis* during anaphase. *J. Ultrastruct. Res.* 54:333–346.
23. LaFountain, J. R., Jr. 1982. Chromosome movement during meiotic prophase in crane-fly spermatocytes. I. Observations on living cells and the effects of cyanide and cold treatment. *Cell Motil.* 2:183–195.
24. Luykx, P. 1965. Kinetochore-to-pole connections during prometaphase of the meiotic divisions in *Urechis* eggs. *Exp. Cell Res.* 39:658–668.
25. Luykx, P. 1970. Cellular mechanisms of chromosome distribution. *Int. Rev. Cytol. Suppl.* 2:1–162.
26. Marks, G. E. 1975. The Giemsa-staining centromeres of *Nigella damascena. J. Cell Sci.* 18:19–25.
27. Myers, W. M., and H. D. Hill. 1940. Studies on chromosomal association and behavior and occurrence of aneuploidy in autotetraploid grass species, orchard grass, tall oat grass and crested wheatgrass. *Bot. Gaz.* 102:139–172.
28. Nicklas, R. B. 1967. Chromosome micromanipulation. II. Induced reorientation and the experimental control of segregation in meiosis. *Chromosoma (Berl.).* 21:17–50.
29. 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.
30. Richardson, M. M. 1936. Structural hybridity in *Lilium martagon* album × *L. Hansonii. J. Genet.* 32:411–450.
31. Rieder, C. L. 1982. The formation, structure, and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.
32. Sugawara, S., and K. Mikamo. 1980. An experimental approach to the analysis of mechanisms of meiotic nondisjunction and anaphase lagging in primary oocytes. *Cytogenet. Cell Genet.* 28:251–264.
33. Sumner, A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* 75:304–306.
34. Wagenaar, E. B., and D. F. Bray. 1973. The ultrastructure of kinetochores of unpaired chromosomes in a wheat hybrid. *Can J. Genet. Cytol.* 15:801–806.