LOCAL REDUCTION OF SPINDLE FIBER BIREFRINGENCE
IN LIVING NEPHROTOMA SUTURALIS (LOEW)
SPERMATOCYTES INDUCED BY ULTRAVIOLET
MICROBEAM IRRADIATION

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ABSTRACT
Irradiation of the mitotic spindle in living Nephrotoma suturalis (Loew) spermatocytes with an ultraviolet microbeam of controlled dose produced a localized area of reduced birefringence in the spindle fibers. The birefringence was reduced only at the site irradiated, and only on the spindle fibers irradiated. Areas of reduced birefringence, whether produced during metaphase or during anaphase, immediately began to move toward the pole in the direction of the chromosomal fiber, even though the associated chromosomes did not necessarily move poleward. Both the poleward and the chromosomal sides of the area of reduced birefringence on each chromosomal fiber moved poleward with about the same, constant, velocity. On the average, the areas of reduced birefringence moved poleward with about the same velocities as did the chromosomes during anaphase. The area of reduced birefringence was interpreted as a region in which most, though not necessarily all, of the previously oriented material was disoriented by the irradiation. The poleward movement of the areas of reduced birefringence indicates that the spindle fibers are not static, non-changeable structures. The poleward movement possibly represents the manner in which the birefringent spindle fibers normally become organized. All the experiments reported were on primary spermatocytes which completed the second meiotic division subsequent to the experimentation. Since both the irradiated and the control cells completed the two meiotic divisions, the movement and irradiation effects studied in the first division were non-degenerative.

INTRODUCTION
Though there has been much cytological work done on spindles (see E. B. Wilson, 1928; Schrader, 1953), the achromatic spindle fiber components were not conclusively shown to exist in living cells until 1953 (Inoué, 1953; see Schrader, 1953, and Mazia, 1961, for reviews). Using a highly sensitive polarizing microscope, Inoué showed that the chromosomal fibers (from the chromosomes to the poles), continuous fibers (from pole-to-pole), and asters are present in living cells, the birefringent fibers corresponding exactly to the appearance of the fibers seen in the best fixed and stained preparations (Inoué, 1953; Schrader, 1953). Though spindle fibers do exist in living cells, and though many theories attribute to these fibers a major role in the movement of chromosomes during
mitosis (Schrader, 1953; Dietz, 1958; Östergren et al., 1960; Mazia, 1961; Inoué, 1964; Roth, 1964), there is no direct evidence that the spindle fibers have such a role, and there is only scant knowledge of the physical and chemical nature of the fibers themselves. Most of the evidence for their role in chromosome movement is circumstantial, relying on the facts that kinetochores are necessary for normal anaphase movement to occur (Cornman, 1944; Ris, 1949; Schrader, 1953; Mazia, 1961), and that during prometaphase movement kinetochores are often stretched in the direction of the movement (Hughes-Schrader, 1943, 1947; Cooper, 1951; Dietz, 1956; Bajer and Molé-Bajer, 1963; Nicklas, 1963); also, various experimental agents which destroy spindle structure concomitantly stop chromosome movement (Pease, 1946; Cornman and Cornman, 1951; Inoué, 1964; Zimmerman and Marsland, 1964).

The best evidence that spindle fibers have an important role in chromosome movement is of the last kind. Inoué (1964) showed that the spindle fiber birefringence disappeared when the temperature was lowered during anaphase, and that the chromosomes stopped moving when the birefringence disappeared. Since the chromosomes did not resume movement until the birefringence reappeared, he concluded that apparently there is a direct relationship between birefringence and movement. Also, Inoué (1952) showed that the chromosomes moved toward the periphery of the cell during colchicine-induced shortening of the spindle fibers, and that such movement stopped when the birefringence disappeared, and therefore that a shortening of the spindle fibers could cause the chromosomes to move. While these evidences are suggestive of the interpretations outlined, those interpretations are not the only ones possible, because the experimental agents do not affect the spindle alone. Temperature changes would affect all the cellular processes. And, since colchicine is applied to the entire cell, colchicine too could affect components of the cell other than the birefringent spindle fibers. For example, colchicine is known to inhibit some dehydrogenases (Gal, 1938), and to alter chromosome structure (Eigsti, 1940), nucleolar structure (Herich, 1963), lysosome and Golgi apparatus structure (Robbins and Gonatas, 1964), DNA synthesis (LaCour and Pelc, 1959; Hell and Cox, 1963; Sriramula, 1963), RNA synthesis (Creasy and Markiw, 1964), cell nucleotide content (Wang, Greenbaum, and Harkness, 1963), and muscle excitability (Lecomte, 1949). Thus, while circumstantial evidences do implicate the spindle fibers, the possibility of non-specific action by the experimental agents prevents unambiguous interpretation of the data.

The objection of non-specific action can be overcome if one uses an experimental tool which affects only the spindle, or a part of the spindle, without affecting other cellular processes. The ultraviolet microbeam is such a tool (see Zirkle, 1957; Smith, 1964; for reviews). When the ultraviolet microbeam is focused to a small part of the spindle, ultraviolet light passes through only that part of the spindle, the cell membrane, and the cytoplasm above and below the spindle. The irradiation of the cell membrane and the cytoplasm near the spindle is unavoidable, but extra-spindle irradiation serves as a control for effects due to cytoplasmic and membrane irradiation. Thus, parts of the spindle can be selectively irradiated, and changes in function specifically due to irradiation of those spindle parts can be measured. Direct information regarding the role of the spindle fibers in chromosome movement can be obtained, therefore, by irradiating spindle fibers with a microbeam, while following the spindle fiber birefringence with a sensitive polarizing microscope, by following chromosome movement before and after irradiation, and by comparing chromosome movement in these irradiated cells with that in non-irradiated cells, and with that in cells irradiated in extra-spindle areas. Such experiments have been performed (Forer, 1964), and this paper is the first of two papers reporting the results.

This paper describes the behavior of the areas of reduced birefringence which are produced by ultraviolet microbeam irradiation; some implications of this behavior to the nature of normal, non-irradiated spindle fibers are considered in the discussion. Chromosome movements in these irradiated cells will be described in detail in a subsequent report (Forer, 1964, and manuscript in preparation).

MATERIALS AND METHODS

Crane flies (Nephrotoma suturealis, Loew)1 of all stages were maintained in the laboratory using the method

1 I would like to thank Dr. George W. Byers of the University of Kansas, Lawrence, Kansas, for identifying the species. The stocks derive from a single female caught by Dr. P. R. Dietz in Durham, North Carolina in the spring of 1961.
Last-instar larvae of the proper stage were chosen, surface-sterilized with 70 per cent ethanol, and then covered with Kel-F 10 fluorocarbon oil (Minnesota Mining and Mfg. Co., St. Paul, Minnesota). Each testis was dissected out under Kel-F 10 and smeared onto a clean quartz coverslip (A. D. Jones, Cambridge, Massachusetts). 70 µ thick Fluorglas (Commercial Plastics, New York) spacers were placed on the quartz, a clean glass coverslip was placed on top of that, and the preparation was partially sealed with dentist's wax (Conger, 1960). Using this method, the cells completed the 2 meiotic divisions (prophase I to telophase II) in over 3/4 of the preparations.

Chromosome behavior in normal cells will be described elsewhere (Forer, manuscript in preparation); it is similar to that described by Dietz (1956, 1959, 1963) for other species of crane flies. The three autosomal bivalents (Fig. 2 A1 and Fig. 2 B2) divide at anaphase, and the daughter dyads move to the poles (Fig. 13 A1) while the 2 unpaired sex chromosome univalents (Fig. 13 A2) remain at the equator. The univalents do not move poleward until the autosomes have neared the pole. The individual chromosomal spindle fibers are clearly visible in metaphase and anaphase with a sensitive polarizing microscope (e.g., Fig. 4 A2 and Fig. 13 A1).

Phase contrast microscope observations were made with a Carl Zeiss phase contrast microscope (Model KF124-202), using the 40 X, 0.65 na objective.

Polarizing microscope observations were made with an American Optical Company (New York) Super-Bio Polarizing Microscope with rectified optics (Inoué and Hyde, 1957; Shurcliff, 1962, pp. 154–155), or with a Model P-42 polarizing microscope modified such that a 20 X, 0.5 na strain-free objective (Swann and Mitchison, 1950; Inoué, 1961) was used as a condenser, a 43 X, 0.66 na rectified objective was used as the objective, a 17 m µ retardation compensator (E. Leitz, New York; see Inoué, 1961) was inserted between the objective and the analyzer, and Polaroid sheets (HN-22 from the Polaroid Corporation, Cambridge, Massachusetts) were used as analyzer and polarizer. An Osram HBO-200 high-pressure mercury lamp (Hanovia, New York) served as the light source.

Fig. 1 A schematic diagram of the polarizing microscope-ultraviolet microbeam arrangement. For simplicity, the substage front-surface mirror between the focusing cell and the reflecting condenser was omitted from the diagram.

3 Made available through the courtesy of the Marine Biological Laboratories, Woods Hole, Massachusetts, and Dr. Shinya Inoué.

4 Loaned by the American Optical Company to Dr. Shinya Inoué.

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pressure mercury arc was used as the light source for observation, and wavelengths other than the mercury green line (546 μm) were removed from the beam by a combination of Corning glass filters (No. 4600 and No. 3387, from Corning Glass Works, Corning, New York), and an interference filter with peak transmission of 70 per cent at 546 μm (Baird-Atomic, Cambridge, Massachusetts).

Photographs were taken with an AO Spencer No. 658 35 mm camera back with compensator lens and shutter, using KB-17 film (ADOX Fotowerke, Frankfurt, Germany). Photographs of living cells were taken at various time intervals, distance measurements were made from positive prints at a final magnification of 1000, and these measurements were used to make graphs of position versus time. Distances were measured from one pole chosen as a reference point, and were measured in the pole-to-pole direction.

The microbeam irradiations were from the condenser side, using one microscope for both observation and irradiation. (A similar system is described by Inoué, 1964.) For the irradiations the substage condenser was replaced by an American Optical 50 X, 0.56 na reflecting lens, and a small aluminum front-surface mirror (2.0 mm X 0.2 mm) was inserted into the system such that the light from the ultraviolet source was reflected from it and into the reflecting lens (Fig. 1). Portions of the small mirror were masked by painting with India ink; in Fig. 4 A3, for example, the bright area (UV) is visible light reflecting from the unpainted portion of the mirror and the dark area surrounding it is the painted portion.

The ultraviolet source was a General Electric

Such a system has been suggested by Zamenhof (1943) and used by Inoué (1964).

Explanation of Figures

B, bivalent
CF, chromosomal fiber
D, dyad
U, univalent
UV, image of the ultraviolet

Figs. 2 A1, 2 C1, 10 B2, and 10 B4 were photographed through a phase contrast microscope. The chromosomes appear dark against the non-granular spindle area (Fig. 2 A1-arrows).

The other photographs (Figs. 2, 4, 6, 8, 10, 11, 13, 15) were taken through a polarizing microscope. The birefringent spindle fibers (e.g., the chromosomal fibers labeled CF in Fig. 4 A3, and Fig. 13 A1) appear bright or dark against the background, depending on whether there is additive or subtractive compensation, respectively (Swann and Mitchison, 1950; Inoué and Dan, 1951; Inoué, 1961). In the polarizing microscope the bivalents and dyads have low contrast (Figs. 2 B2; 4 A2; 13 A1; labeled), and they are easiest seen as the termination of the chromosomal fibers. (The kinetochores are, by definition, the chromosomal position at which the chromosomal spindle fibers terminate.)

All photographs are printed at X 1000. The scale in the lower right corner is: 9.5μm in Figs. 13 and 15; 10μ in Figs. 2, 6, 8, 10 and 11; and 10.5μ in Fig. 4.

Figs. 3, 5, 7, 9, 12, 14, and 16 are graphs of distance from the pole on the irradiated side (ordinate), versus time (abscissa), the times being plotted with respect to the time at which the cell was irradiated (UV). In the schematic diagram at the top of each graph, the solid black area represents the area of reduced birefringence, a black line represents a chromosomal fiber, an ellipse represents a bivalent, the univalents are not shown, a half-ellipse represents a dyad, the pole on the irradiated side is labeled P1, and the areas of reduced birefringence, the kinetochores, and the opposite pole are labeled with the geometrical objects by which they are represented in the graphs. The geometrical objects which represent the kinetochores are closed (circles, and triangles), and those which represent the areas of reduced birefringence and the pole on the non-irradiated side are open (circles, and squares). In the graphs, pole P1 is a horizontal straight line. The points representing the area of reduced birefringence distances from P1 are connected by a solid curve, as are the points representing the distances from P1 of the other pole; the points representing the distances of the kinetochores from P1 are connected by a dashed curve.

The vertical arrow in Figs. 3, 5, 9, 12, 14, and 16 represents the time of dyad separation.

The position of the focused ultraviolet source measured from a picture taken 1.0 to 0.5 minutes before the irradiation is plotted on the graphs at the time of irradiation (UV).
AH-4 lamp with its glass cover removed. The measured relative line energies were as follows:

<table>
<thead>
<tr>
<th>Energy (m/ž)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>254</td>
<td>1.5</td>
</tr>
<tr>
<td>265</td>
<td>2</td>
</tr>
<tr>
<td>280</td>
<td>1</td>
</tr>
<tr>
<td>297</td>
<td>2</td>
</tr>
<tr>
<td>303</td>
<td>3.5</td>
</tr>
</tbody>
</table>

(See also Baum and Dunkelman, 1950.)

The rationale and the method used to focus the microbeam are discussed in detail in the Appendix. Describing the procedure briefly, the ultraviolet wavelengths were removed with a filter, a hexylene glycol-containing cell was inserted between the small mirror and the reflecting lens, the reflected visible image of the mirror was focused onto the

Figure 2  Cell 63727.C. Irradiated during metaphase. A1: each of the three autosomal bivalents is labeled with an arrow. A2, A3: the position to be irradiated (on the chromosomal fiber of the left bivalent) is indicated by a bracket. A4: The ultraviolet source is labeled UV. B1, B2, B3, B4: The position of the area of reduced birefringence (on the chromosomal fiber of the left bivalent) is indicated by a bracket. There is weak birefringence inside the area of reduced birefringence. B2: the three autosomal bivalents are labeled with arrows, which correspond to the arrows in A1.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

<table>
<thead>
<tr>
<th>Time</th>
<th>A1,</th>
<th>A2,</th>
<th>A3,</th>
<th>A4,</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-11</td>
<td>-7</td>
<td>-5</td>
<td>-0.5</td>
</tr>
<tr>
<td>B1,</td>
<td>+2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1,</td>
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<td>C2,</td>
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<td></td>
</tr>
<tr>
<td>B3,</td>
<td>+6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3,</td>
<td>+18.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4,</td>
<td>+7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4,</td>
<td>+19.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The area of reduced birefringence moved to the pole, and did not displace the pole when it reached the pole.
specimen, and the filter and the hexylene glycol were removed before the irradiation (Fig. 1). The focus was corrected for 275 μμ.

The ultraviolet output from the AH-4 lamp was monitored with a General Electric PV-10 ultraviolet sensitive photovoltaic cell (Jagger, 1961) used in conjunction with filters and a microvoltmeter (Leeds and Northrup, Philadelphia, Pennsylvania). The irradiation times were controlled, and were adjusted (between 14 and 20 seconds) to give a constant irradiation dose (energy/area). (The AH-4 output did not vary much in the first 100 hours of use.)

The total ultraviolet energy incident upon the cell was measured by placing the circular photovoltaic cell on the stage of the microscope and defocusing the reflecting lens such that the ultraviolet beam covered 0.9 of the surface of the photovoltaic cell (Uretz, 1962). The measurements indicate that the ultraviolet energies at the focus point in air were of the order of 10 ergs/μμ. This heterochromatic ultraviolet dose was used in all irradiation experiments.

The effective wavelengths for the irradiation effects described are less than 320 μμ. This was determined by prolonged irradiation through a filter which had zero transmission for wavelengths less than 320 μμ.

RESULTS

Ultraviolet microbeam irradiation of spindle fibers in living Nephrotoma suturalis (Loew) primary spermatocytes produced areas of reduced birefringence. Each discrete area of reduced birefringence was about the same size and shape as the image of the irradiation source aperture (Figs. 4, 6, 8, 10, 13, and 15), and the chromosomal fiber birefringence on both the poleward and chromosomal sides of the area remained essentially unchanged by the irradiation (Figs. 2, 6, 8, 10, 13, and 15). Each area of reduced birefringence remained localized on the irradiated fibers; the affected area did not expand to include non-irradiated regions. Irradiation with the same dose outside the spindle region had no effect on spindle birefringence. The continuous fiber birefringence is very weak and hard to detect at the meiotic stages when spindle fibers were irradiated (Forer, manuscript in preparation), so the experiments are concerned mainly with areas of reduced birefringence on chromosomal fibers; however, when

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Figure 3 The graph is for the cell photographed in Fig. 2. The distances from pole P1 of both sides of the area of reduced birefringence are plotted. Both sides of the area moved poleward with about the same, constant, velocity while the associated chromosome remained at the equator. (For simplicity, the kinetochore positions of only the left bivalent are plotted; the other two bivalents acted in the same manner.)

Figure 8 The graph is for the cell photographed in Fig. 2. The distances from pole P1 of both sides of the area of reduced birefringence are plotted. Both sides of the area moved poleward with about the same, constant, velocity while the associated chromosome remained at the equator. (For simplicity, the kinetochore positions of only the left bivalent are plotted; the other two bivalents acted in the same manner.)
The area of reduced birefringence moved to the pole, and did not displace the pole.

The area of reduced birefringence could be seen in the polarizing microscope, but could not be distinguished in the phase contrast microscope as being either lighter or darker than its surroundings (Figs. 2 and 10).

The first postirradiation observations were made at 20 seconds after the irradiation was finished; at this time the area of reduced birefringence was visible (Forer, 1964). Therefore, if there is a time lag between irradiation and formation of the area of reduced birefringence, this figure (20 seconds) is the upper limit for such a time lag.

Each area of reduced birefringence moved toward the pole immediately after it was formed,
and it continued to move until it reached the pole. This poleward movement occurred without exception in each of the chromosomal fiber areas of reduced birefringence studied, even though the chromosomes associated with the same chromosomal fibers did not necessarily move poleward at that time (Figs. 2, 4, 6, 8, and 10), and even though the cell was in metaphase at the time of irradiation (Figs. 2, 4, 6, and 8). The relation between movement of the areas of reduced birefringence and movement of the chromosomes will be discussed in detail in a subsequent paper.

"Movement" is defined operationally: as time proceeds, the distance from the area of reduced birefringence to the pole changes. The area "moves" toward the poles. Both sides of the area of reduced birefringence on each chromosomal spindle fiber moved poleward in the direction of the chromosomal fiber, and both sides of the area moved toward the pole with about the same velocity (Figs. 2, 8, 13, and 15). In some cells the reduced birefringence on different chromosomal fibers moved poleward with different velocities, and when this happened, the area of reduced birefringence changed in shape or in angle relative to the spindle axis as the area moved toward the pole (Figs. 10, 11, and 15).

The areas did not deform the adjacent unaffected fibers as they moved toward the pole (Figs. 2, 6, 8, 13, and 15), nor did they push away the asters once they reached the pole (Figs. 2, 6, 8, 11, 13, and 15). Rather, the size of each area gradually

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Figure 6 Cell 68j8, C. Irradiated during metaphase. A1, A2: The position to be irradiated (on the chromosomal fibers of the two left bivalents) is indicated by a bracket. A3: The ultraviolet source is labeled UV. A4, B1, B2: The position of the area of reduced birefringence is indicated by a bracket.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

<table>
<thead>
<tr>
<th>Time</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-5.5</td>
<td>-4.5</td>
<td>-0.5</td>
<td>+2</td>
</tr>
<tr>
<td></td>
<td>+2.5</td>
<td>+3.5</td>
<td>+9.5</td>
<td>+13.5</td>
</tr>
</tbody>
</table>
FIGURE 7 The graph is for the cell photographed in Fig. 6. The area of reduced birefringence distances from $P_1$, which are shown are of the chromosomal side of the area associated with the left bivalent. The area of reduced birefringence moved poleward with a constant velocity, while the associated chromosome remained at the equator. (The other chromosomes, and area of reduced birefringence, acted in the same manner.)

FIGURE 8 Cell 68h13.B. Irradiated during metaphase. A1, A2: The position to be irradiated (on the chromosomal fibers of the two left bivalents) is indicated by a bracket. A3: The ultraviolet source is labeled UV. A4, B1, B2, B3, B4: The position of the area of reduced birefringence is indicated by a bracket.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

<table>
<thead>
<tr>
<th>A1</th>
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<th>A2</th>
<th>-5</th>
<th>A3</th>
<th>-0.5</th>
<th>A4</th>
<th>+3</th>
<th>A5</th>
<th>+3</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>+4.5</td>
<td>B2</td>
<td>+6</td>
<td>B3</td>
<td>+11</td>
<td>B4</td>
<td>+16</td>
<td>B5</td>
<td>+17</td>
</tr>
</tbody>
</table>

The area of reduced birefringence moved to the pole, and did not displace the pole.

decreased after the area reached the pole until such an area was no longer distinguishable.

The primary interest was in effects on the chromosomal fibers of the autosomes. Areas of reduced birefringence on 99 such chromosomal fibers were studied; 39 were in cells in metaphase at the time of irradiation, and 60 were in cells in anaphase at the time of irradiation. (Figs. 2, 4, 6, and 8 show...
areas of reduced birefringence in metaphase cells, and Figs. 10, 11, 13, and 15 show areas of reduced birefringence in anaphase cells. Areas of reduced birefringence on chromosomal fibers of univalents also moved toward the pole immediately after being formed.

There was a change in the birefringence of both the areas of reduced birefringence, and the chromosomal fibers immediately adjacent to the areas of reduced birefringence, as the areas moved toward the pole. The birefringence inside each area of reduced birefringence often increased in magnitude as the area moved to the poles (Figs. 2, 4, and 13). The birefringence of the chromosomal fiber immediately adjacent to each area decreased in magnitude as the area moved to the pole. (That the last statement must be true can be seen from the following considerations. Since an area of reduced birefringence moves toward the pole, with time, the position of the chromosomal fiber adjacent to the area moves toward the pole, with time. After the area of reduced birefringence has reached the pole, the consecutive positions along the fiber, from the initial position of the area of reduced birefringence to the pole, represent the time course of the position of the fiber adjacent to the area of reduced birefringence. Since in these cells the spindle fiber birefringence is strong near the kinetochores and weaker toward the poles (Figs. 2, 6, and 13), and since after the area reached the pole the irradiated chromosomal fiber was indistinguishable from non-irradiated fibers, the birefringence of the chromosomal fiber adjacent to the area of reduced birefringence decreased in magnitude as the area moved to the pole.)

The distance between the area of reduced birefringence and the spindle pole on the irradiated side was measured on each photograph in the series of photographs of each cell. In favorable cases the distances from the pole of both sides of the area (of reduced birefringence) were measured (Figs. 3 and 16), but in general the distances from the pole of only the side of the area closest to the chromosomes were measured. The chromosomal side of an area could be followed until it was 3 to 4 μ from the pole, but because of the increasing birefringence inside the area, because the chromosomal fibers have less birefringence near the poles, and because the chromosomal fibers converge toward the poles, it was difficult to distinguish the area after this point.

The poleward velocity of the area of reduced birefringence on each chromosomal fiber was calculated from the distance measurements. The dis-
tance of each area from the pole was plotted with respect to the time elapsed since the fiber was irradiated, and the slope of this curve is the poleward velocity.

Each area of reduced birefringence moved poleward with a constant velocity (Figs. 3, 5, 7, 9, 12, 14, and 16), but different areas did not necessarily move with the same velocity, even in the same cell (Figs. 10, 11, and 15). The distribution of the poleward velocities of the areas (of reduced birefringence) is shown in Fig. 17 A, shaded, for cells irradiated before anaphase, and in Fig. 17 B, shaded, for cells irradiated during anaphase. The anaphase poleward velocities of chromosomes in non-irradiated (control) cells adjacent to the irradiated cells is the non-shaded area of the same figures. (The chromosome velocities were measured in the same way as were the area of reduced birefringence velocities.) These comparisons show that after irradiation either in metaphase or in anaphase, the poleward velocities of the areas of reduced birefringence are, on the average, about the same as the anaphase poleward velocities of chromosomes in non-irradiated control cells. Before making statements regarding the equivalence of area of reduced birefringence velocities and chromosome velocities, the velocities of individual areas of reduced birefringence should be compared directly with the velocities of the chromosomes associated with the same fiber, or with the velocities of individual control chromosomes. When this is done (Forer, manuscript in preparation), it is seen that the velocities of the areas are often different from

![Figure 10](cell6sL7_4.png)

**Figure 10** Cell 6sL7.4. Irradiated during anaphase. A1, A2: The position to be irradiated (across the entire half-spindle) is indicated by a bracket. A3: The ultraviolet source is labeled UV. A4, B1: The position of the area of reduced birefringence is indicated by a bracket.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

<table>
<thead>
<tr>
<th>Time</th>
<th>A1</th>
<th>A2</th>
<th>A3</th>
<th>A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-9</td>
<td>-7</td>
<td>-0.5</td>
<td>+1.5</td>
</tr>
<tr>
<td>B1</td>
<td>+3.5</td>
<td>B2</td>
<td>+4.5</td>
<td>B3</td>
</tr>
</tbody>
</table>

The area of reduced birefringence moved to the pole and did not displace the pole. In A4 the chromosomal side of the area of reduced birefringence makes a straight line; in B1 the chromosomal side of the area of reduced birefringence does not make a straight line, because the areas on different fibers moved poleward at different rates.

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FIGURE 11 Cell 68h8A. Irradiated during anaphase. A1, A2: The position to be irradiated (across the entire half-spindle) is indicated by a bracket. A4: The ultraviolet source is labeled UV. B1, B2, B4: The position of the area of reduced birefringence is indicated by a bracket.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

A1, −8 A2, −5.5 A3, −4.5 A4, −0.5 A5, +4
B1, +4.5 B2, +5.5 B3, +13 B4, +16.5 B5, +21

The area of reduced birefringence moved to the pole and did not displace the pole. In A5 and B1, the chromosomal side of the area of reduced birefringence makes a straight line; in B3, and B4 the area of reduced birefringence does not make a straight line because the areas on different fibers moved poleward at different rates.

The velocities of the chromosomes, by as much as a factor of 3. These results will be given in detail in a subsequent report.

In the experiments reported here, both the irradiated cells and the non-irradiated control cells completed the two meiotic divisions. (In five cases the data include irradiated cells which did not complete division II. In these cases there was indication of preparation for division II such as aster formation, or spindle formation, and the data were not in variance with other cases where the irradiated cells completed the second division.)

It is necessary to follow the cells through the completed second division to ensure that the movements observed in non-irradiated cells were normal and not degenerative, and to ensure that the differences between the irradiated and the control cells were not due to lethal or semilethal effects of the irradiation (e.g., Uretz and Zirkle, 1955; Izutsu, 1961; Bajer and Molé-Bajer, 1961). Since the cells completed a second division, the movements and irradiation effects studied in the first division were non-degenerative.

DISCUSSION

The birefringence of the irradiated part of a spindle can be greatly reduced without changing the birefringence of other parts of the spindle. Though this is not the first time such results have been reported (see Inoué, 1964), most ultraviolet microbeam workers report that the entire spindle structure disappears after irradiation, even after irradiation of the cytoplasm (Zirkle, 1957; Smith, 1964). The difference between the findings of those workers...
and the localized disappearance reported here is most likely due to a difference in dose rather than a peculiarity of crane fly spermatocytes, for the following reasons: (a) While cytoplasmic irradiation of Tradescantia stamen hair cells could cause the entire spindle structure to disappear, Otroshchenko and Sakharov (1964) showed that this did not happen at lower doses, and that depending on the dose used the spindle might or might not disappear. (b) Inoue’s results on Haemanthus endosperm (Inoué, 1964) are similar to those on Nephrotoma, yet it had been reported by other workers that cytoplasmic irradiation destroyed the entire Haemanthus spindle (Zirkle, Uretz, and Haynes, 1960). (c) The entire Nephrotoma spindle birefringence will disappear if the dose (energy/area) used for irradiation of spindle fibers is three times that used here (Forer, 1964).

Since the birefringence was changed only at the site of the microbeam irradiation, experiments were performed to test the role of the birefringent spindle fibers in chromosome movement. These results will be given in detail elsewhere. In this paper we will consider only the movement behavior of the areas of reduced birefringence, and the implications of this behavior toward the nature of normal (non-irradiated) birefringent spindle fibers. Since such inferences depend greatly on the exact nature of the area of reduced birefringence, I will first discuss the nature of the areas of reduced birefringence, and then discuss some implications of the behavior of these areas to the properties of non-irradiated spindle fibers.

**Nature of the Areas of Reduced Birefringence**

Material is birefringent when the refractive index for light whose electric vector is polarized in a certain direction is different from the refractive index for light polarized in a perpendicular direction. In an area of reduced birefringence the refractive indices are roughly the same for all orientations of the electric vector. The ultraviolet microbeam could cause the birefringence to be reduced (a) by causing the molecules which were previously oriented to become disoriented, or (b) by changing the intrinsic birefringence of the oriented molecules without greatly disorienting them, either through cross-linking or denaturation,

![Figure 12](image)

**Figure 12** The graph is for the cell photographed in Fig. 11. The area of reduced birefringence distances from $P_1$ which are plotted are of the chromosomal side of the area on the right dyad’s chromosomal fiber. The area of reduced birefringence moved to the pole with a constant velocity even though the two associated dyads temporarily stopped moving poleward.
or (c) by both denaturation and disorientation, or (d) if the spindle birefringence is mostly form-birefringence, by changing the relative volumes of oriented versus non-oriented material. Information to distinguish between these possibilities might be obtained by light microscope and electron microscope studies of fixed and stained areas of reduced birefringence, by micromanipulator studies of such areas compared with non-irradiated spindle fibers, by radioactive labeling of spindles and determining if areas of reduced birefringence lose label, or by following the movement of granules found in such areas; but such information is not available at the moment.

However, since the amount of birefringence inside the area increased as the area moved toward the pole, and since the area deformed neither the adjacent non-irradiated fibers nor the pole, the area of reduced birefringence is more likely material which is disoriented rather than material cross-linked or denatured, for I would not expect cross-linked or denatured material to gain birefringence as it moved toward the pole, and I would expect cross-linked or denatured material to push the pole away rather than be “disassembled” when it reached the pole.

Such an interpretation does not imply that all the structure or orientation is absent from an area of reduced birefringence, (a) because weak birefringence is often present inside the area after irradiation (Figs. 2 and 13), implying some remaining oriented material, and (b) because there could be oriented material in such an area which would not be detected in the polarizing microscope. This interpretation does imply that much or most of the oriented structure was destroyed by the irradiation. Thus, I interpret the area of reduced birefringence to be an area in which most, though not necessarily all, of the previously oriented material has been disoriented by the irradiation.

**Interpretations of the Behavior of the Areas of Reduced Birefringence**

In *Nephotoma* spermatocytes the birefringence poleward from the area of reduced birefringence was unaffected by the irradiation which produced the area of reduced birefringence (this report). Inoué (1964) reported that the birefringence pole-
ward (distal) from the irradiation site disappeared after ultraviolet microbeam irradiation of chromosomal spindle fibers in Haemanthus endosperm. He attributed the poleward disappearance of birefringence to the absence of an "organizing center" poleward from the irradiated site. If the poleward birefringence is unaffected in Nephrotoma because of the influence of the centriole acting as an "orienting center," the poleward side of the area of reduced birefringence must be given "disorienting" attributes as well, for the fiber between the area of reduced birefringence and the pole decreases in length as the area moves toward the pole. It is surprising that the centriole would have an influence in maintaining the birefringence, for both chromosome movement and spindle fiber birefringence are normal even when there is no centriole at the pole of Nephrotoma suturalis spermatocytes (Forer, 1964; this was originally demonstrated by Dietz, 1959, 1963 for spermatocytes of the crane fly, Pales crocata). The role of the centriole in maintaining the birefringence poleward from the irradiated site could be directly tested by microbeam irradiation of chromosomal fibers in spermatocytes in which the centriole has been experimentally dissociated from the pole.

The poleward movement of the area of reduced birefringence is defined operationally: as time proceeds, the distance from the area to the pole becomes smaller. Such behavior might mean that there is actual movement of the fiber material to the pole; i.e., that the birefringent material, and the non-birefringent material in the area of reduced birefringence, both move to the pole, are broken down at the pole (i.e., are disoriented and made non-birefringent), and are then re-cycled, and that the area of reduced birefringence is just a marker in this continuously moving system. This is not the only possibility, however. For example, it is possible that without some accessory component the oriented spindle fiber material will revert to the disoriented state, and that it is this component and not the birefringent fiber material which moves to the pole; with this interpretation, the ultraviolet inactivates this accessory component, the ultraviolet inactivated component is unable to cause orientation, disorientation ensues, and an area of reduced birefringence is formed. The area of reduced birefringence moves not when non-birefringent fiber material moves poleward, as in the previous interpretation, but rather when the non-functional accessory component moves poleward and displaces the functional component. As another alternative, it is also possible that no material moves poleward at all, but rather the area of reduced birefringence movement indicates a wave of organization which moves to the pole.

Pease (1946) found that spindle fibers grew from the chromosomes after removal from the high pressure which had caused spindle breakdown. While this might suggest that the area of reduced birefringence movement is an actual movement of fiber material, the data presented in this paper do not rule out the other possibilities. However, if the poleward movement of the area of reduced birefringence does indicate movement of the fiber material, this fiber material must change state, or orientation, as it moves, for as described in the Results section, there are changes in the birefringence of both the area of reduced birefringence, and the chromosomal fiber adjacent to the area, as the area moves to the pole.

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FIGURE 15 Cell 63h22.1, Irradiated during anaphase. A$: The position to be irradiated (on the chromosomal fibers of the two left dyads) is indicated by a bracket. A$: The ultraviolet source is labeled UV. A4, B1, B3: The position of the area of reduced birefringence is indicated by a bracket.

The times of the photographs follow; they are given in minutes with respect to the time at which the cell was irradiated:

A1, --8  A2, --5.5  A3, --0.5  A4, +2
B1, +3  B2, +5  B3, +6  B4, +16

The area of reduced birefringence moved to the pole and did not displace the pole. The chromosomal side of the area of reduced birefringence is a straight line in A4, but not straight in B1, B2, and B3 because the areas of reduced birefringence on the different chromosomal fibers moved poleward at different rates.

The poleward movement of the areas of reduced birefringence, then, represents a poleward movement of some material, or a wave of organization. Regardless of which of the interpretations is true, this indicates that even when the chromosomes do not move, the spindle fibers are not static, non-changeable structures, for they can drastically change their organization (see Inoué, 1959, 1964, for reviews). One question which does arise, however, is whether this poleward movement, or wave, indicates a dynamic organization which was present before the irradiation, or whether the fibers are really basically static, and that in changing the birefringence the irradiation set in motion a process whereby the changed region is propagated to the pole. At present there are no data to distinguish between these two possibilities. It is clear that in Nephrotoma the spindle fiber organization changes even without irradiation, for the chromosomal fibers gradually increase in width and birefringence between nuclear membrane breakdown and anaphase (Forer, 1964, and manuscript in preparation; also Dietz, 1963; and Inoué’s evidence for dynamic equilibrium, 1964), and thus that even before anaphase the organization of the spindle fibers is not static. But it is not known whether the normal increase in width and birefringence is at all related to the movement of the area of reduced birefringence, nor if the area movement is indeed induced by the irradiation. The existence of movement might be ascertained from the following experiment: Östergren, Molè-Bajer, and Bajer (1960) hypothesized that the poleward movement of spindle inclusions (such as nucleoli, acentric chromosomes, granules,...) was due to extra-spindle-fiber “pumps,” which acted on spindle fibers in the same way as on the inclusions. According to the hypothesis, the poleward move-
Figure 16 The graph is for the cell photographed in Fig. 15. The distances from pole $P_1$ of both sides of the area of reduced birefringence are plotted for the area on the middle dyad's chromosomal fiber. Both sides of the area of reduced birefringence moved poleward with about the same, constant velocity, even though the two associated dyads temporarily stopped moving.

I interpret the poleward movement of the area of reduced birefringence to indicate a process which occurs even without induction by the irradiation, and which represents the way in which the birefringent spindle fibers are organized from disoriented material, in conjunction with an as yet morphologically unidentified traction element (Forer, 1964; and manuscript in preparation). Further evidence in support of this hypothesis will be considered in a subsequent publication.

In conclusion, the results reported here confirm the findings of Inoue (1964) that the birefringence of local parts of a spindle can be greatly reduced by ultraviolet microbeam irradiation without affecting the birefringence of non-irradiated parts. The area of reduced birefringence is interpreted as an area in which most, though not necessarily all, of the previously oriented material has been disoriented by the irradiation. Other alternatives are not ruled out by the data, however.

The poleward movement of the areas of reduced birefringence which occurs in both metaphase and anaphase probably represents a movement of material, either of oriented molecules themselves, or of molecules necessary for orientation. It is also possible that no such molecules move, but that a
The shaded areas are the number of autosomal chromosomal fibers on which an area of reduced birefringence was formed (ordinate), versus the poleward velocity of each such area (abscissa). Fig. 17 A, shaded, is for areas of reduced birefringence in cells irradiated before dyad separation (during late prometaphase and early metaphase), and Fig. 17 B, shaded, is for areas of reduced birefringence in cells irradiated after dyad separation (during anaphase). The non-shaded areas are the number of dyads (ordinate) versus the poleward velocity of each such dyad (abscissa) for dyads in the cells used as controls for the irradiation experiments.

wave of organization is propagated along the fiber. With either alternative the results indicate that the chromosomal fibers are not static, non-changeable structures.

The poleward movement of the areas of reduced birefringence is interpreted to indicate a movement which occurs all the time, and which indicates the manner in which the birefringent spindle fibers are organized. An alternative interpretation is possible, in which such movement or propagation is induced by the irradiation.

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**APPENDIX**

**Focusing the Microbeam**

The visible light image of the ultraviolet source is used to focus the ultraviolet microbeam into the specimen plane (see Zirkle, 1957). When a reflecting lens is used for this focusing, the ultraviolet and visible images should be focused to the same position. But since a refracting material is present between the lens and the focus point, namely the quartz coverslip, light of different wavelengths will be focused at different positions because of the refractive index dispersion of this material. Thus, when the visible image of the ultraviolet source is in focus, other wavelengths are not in focus (Norris *et al.*, 1951; Uretz and Perry, 1957). This focus shift is illustrated in Fig. 18 A and the calculated focus shift for various wavelengths with respect to the mercury green line (546 nm) is shown in Fig. 18 B for different thickness coverslips. (The calculation uses Snell's law, the refractive indices of fused quartz as given in the Chemical Rubber Publishing Company's Handbook of Chemistry and Physics, and the application of simple trigonometry.) The problem in focusing an ultraviolet microbeam, then, is to correct for this focus displacement between the visible and the ultraviolet images of the source.

Usual methods of refocusing require (a) the ultraviolet aperture on the optic axis, and (b) motion of this aperture (or the reflecting lens) along the optic axis for (c) a measurable distance (e.g., see Uretz and Perry, 1957). This situation is illustrated in Fig. 19 A: Δh is the displacement along the optic axis (oa) between the visible (v) and the ultraviolet (uv) images of the source (UV), which is caused by the quartz coverslip (gz); M is the lateral magnification and Mc the longitudinal magnification of the lens which is represented in the thin lens approximation (Hall, 1953, chapter 3; Hardy and Perrin, 1932) as a straight line, L, with focal points at F. When the ultraviolet source aperture is moved a distance McΔh along the axis away from the lens to a new position (UV'), the ultraviolet image (uv') for this new position is brought into focus in the specimen plane (sp) and the corresponding visible image (v') is in focus.
between the lens and the specimen plane. Refocusing is accomplished by moving the aperture by $M\Delta h$ in a direction along the optic axis, or, equivalently, by moving the lens by a distance $\Delta h$.

In Fig. 19 A the ultraviolet aperture is drawn very large, such that the correction which focuses the ultraviolet image in the specimen plane introduces a lateral shift in the image position for those points of the source which are off the optic axis (cf. $uv$ and $u'v'$ in Fig. 19 A). Since the ultraviolet apertures used as sources are less than a millimeter, the lateral shift is no problem, if the apertures are placed on the optic axis of the system.

An optical method for refocusing the microbeam was devised which eliminates the need for accurate mechanical movement of the lens or aperture, and eliminates the need for the ultraviolet source to be on the optic axis. In this method, a flat cell is inserted between the ultraviolet source aperture and the reflecting lens while the aperture is focused onto the specimen with visible light; the cell is removed after focusing, prior to irradiation with ultraviolet. The effect of inserting and removing the flat is illustrated in Fig. 19 B. Interposing the flat makes the actual source aperture ($UV'$) appear to come from a position, $(UV)$, $M\Delta h$ closer to the lens. There is no lateral shift (i.e., in a direction perpendicular to the optic axis) between either the two visible images ($v, v'$) of the source aperture or the two ultraviolet images ($uv, uv'$) of the source aperture when the surfaces of the flat are perpendicular to the line between the ultraviolet source aperture ($UV'$) and the focal point $F$, even when the source aperture is not on the optic axis (Fig. 19 B). The flat position is adjusted such that by inserting and removing the flat the two visible images of the aperture occur at the same position on an ocular grid, the visible images being focused upon by changing the fine adjustment of the microscope. (This adjustment

\[
\Delta h = T \left( \frac{\tan \theta_2 - \tan \theta_1}{\tan \theta} \right)
\]

where $T = 0.170$, $T = 0.175$, and $T = 0.180$.
relies on the mechanical stability of the microscope fine adjustment, which, in our experience, is quite reliable, and much better than that of the substage lens-focusing system.) The flat was a machined cylinder of Plexiglass (Commercial Plastics, New York) to which was cemented two microscope slides, and into which was added aqueous solutions of hexylene glycol. The exact concentration varied depending on the optical path desired. The optical path of such a flat is:

\[ \Delta h' = \left( \frac{N_g - 1}{N_s} \right) T_f + \left( \frac{N_r - 1}{N_s} \right) T_s \]

where \( T \) is thickness, and \( N \) is refractive index. \( N_{\text{glass}} \), \( T_{\text{glass}} \), and \( T_{\text{solution}} \), were measured, and \( N_{\text{solution}} \) was adjusted (by changing the ratios of hexylene glycol and water) such that:

\[ \Delta h' = M^2 \Delta h \]

where \( M \) is the lateral magnification of the reflecting lens, and \( \Delta h \) is the focal shift due to the quartz coverslip as shown in Fig. 18 B. \( M \) was measured for the aperture position in question, and \( \Delta h \) was chosen for the wavelength desired, usually 275 m\( \mu \). The advantages of the refocusing system which used the flat are (a) it can be used with common laboratory microscopes, since it does not rely on well-machined aperture movement or lens movement devices; (b) it can be used with a minimum
of expensive equipment, and is as reliable as the expensive equipment; (c) it does not require that the ultraviolet aperture be placed on the optic axis; and (d) it permits very quick change of the focus correction, for no aperture position changes need be made but only a new solution, or a new cell, be inserted between the aperture and the reflecting lens. Brumberg (1943) and Norris et al. (1951) had previously corrected for the dispersion of the coverslip by using a substage plano-convex lens made from the same material as the coverslip. The optical refocusing method described here is similar in principle to their correction, but is much simpler in fabrication.