VIDEO DIGITIZER ANALYSIS OF BIREFRINGENCE ALONG THE LENGTHS OF SINGLE CHROMOSOMAL SPINDLE FIBRES

I. DESCRIPTION OF THE SYSTEM AND GENERAL RESULTS

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SUMMARY

A new system, based on a video digitizer interfaced to a microcomputer, has been developed to quantify birefringence of individual chromosomal spindle fibres from videotaped images of spindles. (The system also can be used for any other purpose that requires the analysis of video intensities.) Retardations along the lengths of single chromosomal spindle fibres have been studied throughout metaphase and anaphase in cells kept at constant temperatures. The instrumental readings are accurate to within less than 0.06 nm retardation, but operationally the retardation values along a single chromosomal spindle fibre can vary by up to 0.15 nm, primarily because of variation in operator definition of the spindle fibre. Retardations vary with position along the fibre. During anaphase the retardations along a given chromosomal spindle fibre do not move poleward, but rather change as if the oriented material is disorganized at the kinetochore. The retardation at the kinetochore of a chromosomal spindle fibre does not change during anaphase, except for non-predictable jumps of 20–30% that sometimes occur. Thus there is no 'decay of birefringence' during anaphase, such as has been described in other species. In this regard our data, that pertain only to single chromosomal spindle fibres, differ from those previously published; we argue that this is because the published data deal with mixtures of chromosomal and continuous spindle fibres, and because changes in birefringence can appear to occur, artefactually, when measurements of birefringence are made at a single spot in a spindle.

INTRODUCTION

Chromosomal spindle fibres extend between kinetochores and spindle poles. Each chromosomal spindle fibre contains components that produce the force to move the attached chromosome during anaphase of mitosis (see reviews by Schrader, 1953; Nicklas, 1975; Forer, 1981; McIntosh, 1982). We have studied chromosomal spindle fibres using polarization microscopy: chromosomal spindle fibres are birefringent and hence they can be observed using polarization microscopy (e.g. see Swann, 1951a, b; Inoué, 1953, 1964).

Spindle fibre birefringence can be quantified. The measured retardation is equal to the coefficient of birefringence times the thickness of the object; the coefficient of birefringence in turn is directly proportional to the concentration of the birefringent material (discussed, e.g., by Forer, 1976). In the usual method for measuring spindle birefringence the compensator is rotated so that the spindle becomes dark; the person making the measurement judges by eye when the spindle is maximally dark and that compensator angle is used to calculate the retardation of that maximally dark area of...
the spindle (e.g. see Bennett, 1950; Inoué, Fuseler, Salmon & Ellis, 1975).

We have developed a new method to quantify spindle fibre birefringence because for several reasons the usual method of measurement is unsatisfactory. For example, the judgement of maximum darkness is subjective, so that different observers of the same specimen often make considerably different measurements (discussed by Forer & Goldman, 1972; Forer, 1976). Therefore, the values obtained by the usual method, that involves judgement by eye, are not quantitatively accurate, especially at small retardations. Thus one criterion in developing a new method was that it should be objective rather than subjective.

As a second criterion, we wanted to be able to measure the birefringence simultaneously at different regions of the same spindle. For example, we wanted to be able to measure chromosomal spindle fibre birefringence simultaneously with but separately from continuous spindle fibre birefringence, or to measure birefringence along the length of a single spindle fibre. One cannot do this with any subjective or objective method that reads one 'spot' in a living cell (e.g. see Hiramoto, Hamaguchi, Shōji & Shimoda, 19816), but this capability is important for certain kinds of experiments (e.g. see Forer, 1976; Schaap & Forer, 1983). Thus we wanted to develop a method that is both objective and able to measure simultaneously the retardations at different regions of the spindle.

Photographic methods have been used to quantify the birefringence of different regions of the same spindle (e.g. see Swann, 1951a,6; Forer, 1976). We decided to use video methods instead of photographic methods because the former are faster, less tedious and more sensitive than photographic film (e.g. see Inoué, 1981a), and because the data extracted from the video images would be available immediately for computer processing, unlike the curves produced from the microdensitometry of negatives (e.g. see Forer, 1976). In this paper we describe the system that we developed to quantify birefringence objectively from videotape-recorded images. In the following paper (Schaap & Forer, 1983) we describe the effects of temperature on the birefringence of individual chromosomal spindle fibres.

MATERIALS AND METHODS

Animals and living spermatocyte preparations

Crane flies (Nephrotoma suturalis Loew and Nephrotoma ferruginea Fabricius) were reared as described by Forer (1982). Testes were dissected from male larvae and spermatocytes were prepared for microscopy using the techniques described by Schaap & Forer (1979). Because the experiments required a different microscope and a different temperature-control slide the following minor modifications were made. The dissected testes were punctured (under halocarbon oil) and the cells spread (under oil) on a large coverslip (35 mm × 50 mm, no. 1). A small coverslip (22 mm × 22 mm) was placed on top of the oil and then the preparation was sealed with dentist's wax and left to flatten for at least 30 min before it was scanned.

All coverslips were wiped clean with ethanol. Occasionally, coverslips were soaked in dimethyl sulphoxide prior to being cleaned in ethanol, if the ethanol cleaning alone was not sufficient. Coverslips were usually also passed several times through a low flame.

Temperature-control slide and supporting apparatus

The temperature-control slide was similar to that used by Stephens (1973) except that the inlet
Chromosomal spindle fibre birefringence

and outlet ducts were on the same side of the slide. The 35 mm × 50 mm coverslip with the cells on it was glued to the top of the slide and a larger coverslip (43 mm × 70 mm, no. 1) was glued to the bottom, both with a cyanoacrylate glue (Zip Grip 10, Devcon). The coolant (50% ethanol) flowed directly between the two sealing coverslips, maintaining the temperature of the cells by directly cooling the coverslips to which they were attached. The coolant was circulated and maintained to within 0.03 °C by a Lauda K2/RD (Brinkman Instruments) constant temperature circulator. To remove pressure vibrations the diameter of the tubing going into the slide was much smaller than that leaving the slide. A small column filled with clean sand filtered small birefringent particulate matter from the coolant before it entered the slide. After each experiment the coverslips were broken off and the glued portions removed from the temperature-control slide by soaking the slide in dichloromethane for 24–48 h.

The temperature was monitored during experiments by a YSI Tele-Thermometer (model 43 TZ) connected to a YSI surface thermistor (no. 427, Yellow Springs Instrument Company, Yellow Springs, Ohio) placed on the metal between the inlet and outlet ducts (these are on the same side of the circular viewing hole of the slide). Based on measurements made with the thermistor in the position of the cells, the temperature monitored between the inlet and outlet ducts was within 0.5 °C of that at the cells.

In the results described here the cells were initially at room temperature (22–24 °C), with no coolant flowing; they were taken to the experimental temperature (by allowing the coolant to enter the slide) at least 5 min before the onset of anaphase.

Microscopy

We used an Amplival pol.d (Carl Zeiss, Jena) polarizing microscope. The light from a 200 W mercury arc lamp (Illumination Industries Inc., Sunnyvale, Cal.) passed through a heat filter and a green interference filter (546 nm) before entering the strain-free Zeiss-Jena condensor that was used at a numerical aperture of 0.55. For polarizing microscopy the objective was a Zeiss (Oberkochen) strain-free 40× water-immersion lens (NA 0.75). The rotating mica (λ/32) compensator (Brace-Kohler) was supplied and calibrated by Zeiss/Jena. A 10× projecting ocular was used to project the light to the video camera.

Recording of data

A low light-level, silicon-intensified target TV camera (SIT camera, model TC1030/H, RCA) was attached to one outlet of the trinocular tube of the microscope; a 35 mm camera (Pentax) was at another outlet; and a viewfinder was at the third. Recordings generally were at intervals of 1 s with 3 degrees compensation using a video tape recorder (model XL8000, Javelin Electronics, Los Angeles, Cal.) and half-inch video tape. In some experiments cells were photographed on 35 mm film (Ilford HPS & FP4, 15 s exposure, 8 degrees compensation, developed in Diafine). For both video and film recordings the compensator was adjusted so that the spindle fibres were bright as viewed. The microscope stage was rotated until the spindle pole-to-pole axis was oriented at about 45° with respect to the crossed polars.

When recording, the video signals were transmitted through the video digitizer (model 270, Colorado Video Incorporated, Boulder, Colo.), the date–time generator (model V240T, Vicon Industries, Farmingdale, NY), video tape recorder (VTR), and then to the TV monitor (model VM15, Javelin Electronics) as shown schematically in Fig. 1. The digitizer is optional at this stage, but is useful as a check on video contrast. (The date–time generator labels each video field with the data and time.)

For recording polarizing microscopic data the video gain circuit of the camera was always off. The RCA camera has an 'auto black' circuit that automatically adjusts image contrast with respect to the blackest portion of the picture; to ensure that the black level remained constant an opaque dot on the edge of a round coverslip was inserted into the microscope’s projecting ocular and served as a black reference for the camera. After much of the experimental data were analysed we discovered that the VTR also has an automatic gain control circuit (AGC) that adjusts the video signals. The VTR was subsequently modified so that by bypassing a diode this AGC could be circumvented. As will be demonstrated in Results, the VTR AGC had little effect on our data because the digits of the date and time, the brightest spots in the field, serve as a constant upper light level for the VTR AGC circuit.
Data recording

![Diagram](image)

Fig. 1. Schematic diagram of our set-up when recording data and when analysing data. Arrows indicate flow of data. A plotter has not yet been incorporated into the set-up.

For recording phase-contrast images the light levels reaching the camera are too high for the camera, so additional green filters were placed in the light-path and the automatic gain circuit of the video camera was turned on.

Data analysis

The basis of the system we have developed to measure light intensities directly from video fields is a microcomputer-controlled video digitizer that can differentiate between 256 grey levels (Fig. 1). We have used two different systems. In the first, the digitizer was controlled by a Data General microNova computer (Toronto, Ont.) via an interface unit designed and built by M. Failes, K. Mueller and E. Cheung of Canadian Instrumentation and Research Limited (CIR, 2211 Dunwin Dr., Mississauga, Ont., L5L 1X1). In the present, upgraded system the digitizer is controlled by a Z80A microprocessor-based microcomputer (System IIIA, Arisia Microsystems, 1455 Gregwood Rd., Mississauga, Ont., L5H 2T5) via an interface unit designed and built by R. J. Planck and M. Gore of Limnoterra (275 Lancaster St, Kitchener, Ont., N2H 4V2). Some technical aspects of the systems are compared in Table 1. Both systems use the same digitizer so the data are directly comparable. Unless otherwise noted the following operational description applies to both systems.

Intensities were obtained from single video frames as follows. With the VTR in the 'stop frame' mode a white dot (cursor) superimposed on the video image (Fig. 2) is moved by the operator to any location. The operator moves the cursor to the start of a line that is to be scanned, pushes a button, and then moves the cursor to the end of the line and pushes the button again. The start and end points (pixel positions) of the line are stored by the computer. When the lines that are to be scanned have all been defined (up to 10 in any given field) the computer moves the cursor through each point of each line and accepts the intensity value for each point. (The Limnoterra interface allows 'previewing' and editing of the lines prior to the actual digitizing.) Cursor, kinetochore and pole positions are recorded on tracings for use during subsequent analysis of the computer-generated curves. The VTR records the odd and even lines of the video image on alternate video tape frames but the computer interlaces the intensities obtained from alternate frames, so there is no loss of information. To average out random electronic noise, lines are scanned from several consecutive pairs of odd and even frames and averaged by the computer. Typically five pairs of video frames are used. After the intensities are interlaced and averaged they are recorded on floppy diskettes. These 'raw data' are later converted to retardations as outlined in the next section.
Table 1. **Comparison of the two video analysis systems**

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<td>Analogue cursor control</td>
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<td>Coarse and fine voltage adjustments in X and Y directions are digitized and displayed as the ‘dot’</td>
<td>Interface ‘reads’ dot position</td>
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<td>Non-interlaced video field analysis only</td>
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<td>Separate cursor controls</td>
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Fig. 2. A series of photographs of the TV monitor taken at different times with the VTR in the stop frame mode, showing how the dot moves along a single *N. suturalis* spermatocyte chromosomal spindle fibre. ×1200, 19°C, AGC on.

**Method for the calculation of retardations**

Retardations are calculated using methods similar to those used by Swann & Mitchison (1950), Swann (1951a) and Inoué & Sato (1966). The basic formula (derived by Bear & Schmitt (1936) and discussed by Bennett (1950) and Inoué & Sato (1966)) can be simplified to become:
\[ R_o = \frac{(R_c) \sin(2B)}{\sin(2A)} \]  

\( R_o \) is the retardation of the object, \( R_c \) is the retardation of the compensator, \( A \) is the spindle-fibre angle with respect to the crossed polars and \( B \) is the angle of compensation required to achieve the maximally dark condition. Since the curve of intensity versus compensator angle for a blank field is exactly parallel to that when an object is in the field (Inoué & Sato, 1966), the retardation of an object can be determined by comparing the intensity of an object to its background and calculating the angle required to give that difference in intensity; that angle is the same as \( B \), the compensator angle that gives maximum darkness (Inoué & Sato, 1966). Since the retardation of the compensator \( R_c \) is known (in our case it is 17 nm) and since \( A \) and \( B \) can be calculated, the retardation of the spindle fibre, \( R_o \), can be determined using equation (1).

The fibre retardation is calculated by the computer software as follows. (1) A calibration curve is constructed, relating compensator angles to intensities of blank fields (areas of the cell preparation without cells); the latter intensities are averages of two scans through the middle of each blank field. (2) Using the end-point coordinates of each scanned line, the computer determines the angle \( A \) of each line with respect to the angle of the crossed polars. (The operator uses the angles of the astral Maltese cross configurations to define the angle of the crossed polars for the computer: the Maltese cross angles are taken as 45° to the polarizer/analyser angles.) (3) The user specifies scans that are through non-spindle fibre regions of the cell, and the computer calculates average intensities in these scans as 'average background intensities'. The net light intensity due to fibre retardation is calculated for each point along the fibre by subtracting average cell background intensity from total intensity at that point. (4) The intensity due to the given compensator angle (usually 3°) is calculated from the calibration curve (step (1)). (5) The intensity values calculated in steps (3) and (4) are added to give total intensity. (6) The equivalent compensator angle for the total intensity is determined from the calibration curve (step (1)). (7) The given compensator angle is subtracted from this angle to give \( B \), the equivalent compensator angle contributed by the spindle fibre at that point. (8) \( A \) and \( B \) are substituted into equation (1) and the retardation is calculated. The computer printout includes both the actual retardation values and a graph of retardation versus position along a spindle fibre. Subsequent analysis was done manually as described in Results.

Data

Data presented in the text are given as averages ± standard deviations.

RESULTS

Calibration curve

We periodically made calibration curves. The average intensities at different compensator angles remained relatively constant (Fig. 3), irrespective of temperature, most of the usable age of any given light bulb, thickness of the oil, thickness of the coverslip sandwich, and other slight variations in the experimental conditions. The intensities did vary under two conditions: (a) when there was strain in the coverslips and (b) sometimes when a new bulb was used. When coverslips were strained, but there was nonetheless an irresistibly photogenic cell, a separate calibration curve was taken for that cell, without rotating the stage, from an area directly adjacent to the cell. In all other cases the average calibration curve for each bulb was used to convert fibre intensities to retardation.

We studied single chromosomal spindle fibres, both to test the system and to study the effects of temperature on chromosomal spindle fibres (Schaap & Forer, 1983).
Chromosomal spindle fibre birefringence

Fig. 3. Standard calibration curves: for the AGC on (A) and for the AGC off (B). Light intensities are in arbitrary units and cannot be compared because the two curves were obtained using different microscope bulbs. The numbers above each average intensity value represent the number of measurements; the vertical bars represent the standard deviations. Each data point had the zero degree compensation intensity subtracted from it.

Chromosomal spindle fibres

We used stringent criteria to ensure that we studied individual autosomal spindle fibres. We chose late prometaphase or metaphase cells, identified the positions of all five chromosomes and their respective spindle fibres, and studied only cells in which at least one autosomal spindle fibre was free from underlying or overlying spindle fibres. Thus different numbers of fibres were studied in different cells depending on the degree of fibre overlap. For cells at different temperatures, each cell was videotaped from about the time of the temperature shift through late anaphase or early telophase.

We measured retardations along the lengths of individual autosomal spindle fibres, each of which is about 1 μm in diameter and about 10 μm long at metaphase. Individual autosomal spindle fibres were scanned from just beyond the pole to just beyond the kinetochore. Profiles of retardation versus position along a typical chromosomal spindle fibre (Fig. 4A, B) are clearly different from scans through the cytoplasm (Fig. 4C). (In all of the retardation profiles the computer prints a retardation value at each pixel position and in terms of distances in the cell there are 5–9 pixels per μm, depending on the angle of the fibre on the video monitor: the variation in number of pixels per μm is because in our set-up the inter-pixel distance is about 0.7 units in the Y (vertical) direction for every one unit in the X (horizontal) direction.)
The software takes this into account in calculating spindle-fibre angles. As confirmed from measurements made on the acetate tracings, the pole position on the retardation profile is the region in which the scan intercepts the baseline and the kinetochore position is that in which the retardation starts to decrease. Thus the birefringence is zero at the pole and increases linearly to a high value, after which the birefringence either levels off and is approximately constant to the kinetochore or increases more gradually to a maximum and then declines gradually to the kinetochore, forming a 'hump' (see Forer, 1976). (For ease of description we call the first, linearly changing part of the profile the 'slope' from the pole.) The slope region of the autosomal spindle fibre generally occupies 20–40 % of the length of the fibre.

![Diagram](image_url)

Fig. 4. A, B. Retardation profiles of one *N. ferruginea* spermatocyte autosomal spindle fibre; two different scans of the same fibre from the same video frames; the profiles are very close, but, as discussed in the text, they are not identical since they are defined by slightly different coordinates. C. The retardation profile of a scan through the cytoplasm beside the spindle area is very noisy because of granular material in the cytoplasm. The long rectangle above A is a schematic representation of one chromosomal spindle fibre; the solid circles represent the approximate size of a pixel (i.e., electronic cursor size) in relation to the fibre, whereas visually the cursor is nearly the width of the fibre. In this and subsequent figures P and K are the pole and kinetochore positions of the fibre, each + represents a pixel, and as described in the text there are five to nine pixels per μm. The fibre in A and B was at 10°C when recorded with the AGC off.
How accurate are the measurements?

Reliability of the system

One way to test the repeatability and accuracy of the system is to compare the average intensities from repeat scans of blank fields. When this was done the averages usually varied less than one unit of intensity and seldom two. (As can be seen from Fig. 3, one intensity unit corresponds to only about 0.1 degree of compensation, or 0.06 nm retardation.) Even this small variation is probably due not to the system but to irregularities in the field and the fact that the lines scanned were not identical: the coordinates of the lines were visually set by the operator, not by the software.

Another test of reliability is to repeat a scan of the same spindle fibre (e.g., Fig. 4). Repeat scans of the same spindle fibre are more dissimilar than repeat scans of a blank field. This is because spindle fibres must be visually redefined each time and, while the white dot used to determine the start and end points of a scan has a diameter equal to half or more of the width of a spindle fibre, electronically the dot is only about 1/4 to 1/7 of the width of a fibre. Thus, while the operator may attempt to scan down the centre of the fibre the scan may be slightly off; as shown at the top of Fig. 4, in which the two solid circles represent the electronic size of the dot, scans that appear to be along the length of a fibre may cross diagonally instead of down the middle. Visually redefining scans of fibres is complicated further, because in any given frame only half the video image is seen (either the even or the odd lines); thus parts of the fibre may not be clear on a single frame. Sometimes, in fact, the image of a fibre may even appear to shift slightly in advancing from an odd to an even frame. (These problems were minimized by playing the cell sequence to be analysed at 'fast' speed several times to give the operator visual familiarity with the fibre positions.) In addition, slight errors may be introduced in repeat scans as the operator visually redefines the 45° orientation of the spindle (or half-spindle if the mitotic apparatus is bent). In general, the main error in reproducibility seems to be in defining for the computer the coordinates of the line to be scanned. This is not a large error, though, since repeat scans are reproducible in profile and absolute values to within 0.15 nm (e.g., Fig. 4A, b).

As a further test, a 35 mm negative previously quantified using a Joyce Loebl microdensitometer (described by Forer, 1976) was reanalysed using the video digitizer system. (The negative was mounted on a light box together with a wedge composed of small strips of negatives of blank fields at different compensator angles. This composite of anaphase cell and wedge was analysed 'live' (i.e., without prior tape-recording) using a TV camera (RCA model TC1005/S01) with attached Nikon Micro-Nikkor 55 mm, 1:3.5 lens. Subsequent analysis was identical to that used for cells recorded directly on videotape.) While the profiles obtained by the two techniques have the same general shape the two techniques do not give identical retardations: those from the Joyce Loebl scans are about 10% higher than those from the video scans (e.g., Fig. 5). We think that this is due to different scanning areas in the two techniques: the slit for the densitometer was 0.09 μm along the length of a spindle fibre and 0.5 μm along the width, covering ≥1/2 the width of the spindle.
Fig. 5. Retardation profiles of the same autosomal spindle fibres, from a *N. suturalis* spermatocyte recorded on a negative and analysed with the video digitizer system described here (A, C) or densitometrically (B, D) as described by Forer (1976). The profiles in A and B, and in C and D, are from the same fibres. In A and C there are about 11 pixels per \( \mu m \). In B and D the horizontal length of each large square is 1.9 \( \mu m \). Room temperature.

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**Position**

in fibre, while the video pixel is only about 1/4 to 1/7 of the width of a fibre. This difference in size affects the data from both the fibre and the cytoplasm ('background'), since inhomogeneities due to 'granules' will be more pronounced in the video digitizer system. We think that such size differences account for the difference of 10% in absolute values that we regularly obtained.

As a further test, we compared videotape images with those of the same spindle fibres recorded photographically and then viewed via the video camera (Fig. 6). The profiles have similar shapes, and the retardations differ (in an inconsistent manner) by 10% at the most. We think that these variations are due to the fact that the scans are not from identical portions of the fibre. In addition, the images are separated by about one minute, and, as will be described later, chromosomal spindle fibre birefringence does vary with time.

In summary, we conclude that the measurements of birefringence are reproducible electronically to about 0.06 nm, but variations in operator definition of spindle fibres reduce the operational reproducibility to about 10% of the maximum retardation of a single autosomal spindle fibre, i.e. to about 0.15 nm.

We tested whether the VTR AGC circuit introduces errors. Blank fields at different angles of compensation were recorded with AGC 'on' and AGC 'off' (all other recording conditions were identical). As seen in Fig. 7, when the AGC is on the intensity tends to level off at a lower angle of compensation than when the AGC is off. Several cells were recorded with alternating sequences of AGC on and AGC off, and retardations were calculated using the corresponding calibration curve. The only difference between the two is that small differences in the highest light intensities are reflected
Chromosomal spindle fibre birefringence

Fig. 6. Retardation profiles of the same *N. suturalis* fibre recorded on videotape (A) or on a photographic negative (B), both analysed using the video digitizer system. In A and B there are 8 and 9.5 pixels per μm, respectively. The cell was at 20°C. AGC on.

Fig. 7. Calibration curves that directly compare intensity values (in arbitrary units) obtained from the same blank field but recorded with the AGC on (●) or off (■).
as larger differences in retardation with AGC on than with AGC off. That is to say, the upper retardations tend to be higher and noisier with the AGC on than with the AGC off (cf. Fig. 8A, B), but the other values are almost the same. Thus the data taken with AGC on may differ from those with AGC off (the latter is the more accurate), especially at higher retardations (>1 nm).

We should emphasize that data taken under any one condition (AGC on or AGC off) are consistent (within that set of data) to within 0.15 nm, as described above. The absolute values of retardations may be too high with AGC on, and thus absolute values are more reliable from recordings with AGC off. Nonetheless, the data are consistent within any one recording condition and can therefore be used for comparative purposes (e.g., for comparing birefringences at different stages of cell division).

**Spindle fibre retardation during metaphase and anaphase**

We followed individual spindle fibres as cells progressed from metaphase through anaphase. As each spindle fibre shortened during anaphase the 'slope' portion of the fibre remained of constant length as the 'flat' portion adjacent to the kinetochore shortened, as described previously (Forer, 1976; Salmon & Begg, 1980); that is to say, the spindle fibre profile changes during anaphase as if the spindle fibre shortens from the kinetochore end. The absolute value of birefringence at the kinetochore and along

Fig. 8. Retardation profiles of the same autosomal spindle fibre (N. suturalis) recorded about 1 min apart with the AGC on (a), or with the AGC off (b). In both cases the kinetochore retardation is about 1 nm, and in both cases the slope retardations are similar. Room temperature.
the entire spindle fibre often did not change during metaphase through anaphase, but sometimes the birefringence along the entire length of the spindle fibre jumped up or down, in an unpredictable fashion, by up to 25–30% (compare Fig. 9A, B with c). (In Fig. 9, kinetochore retardations were determined as the average retardations of several pixels in the region of the kinetochore. Each maximum retardation value was

![Graph showing average kinetochore and average maximum retardations of three fibres at different temperatures, at different times from the start of anaphase (t = 0). The numbers represent the number of repeat measurements of the same frames while the vertical bars represent the standard deviations of these measurements. N. suturalis; AGC on. A, 16°C; B, 25°C; c, 14°C.](image)
the maximum number for that profile on the computer printout.) When retardations did vary, the changes appeared to occur at random, although data from shorter intervals of time might reveal some regularities.

The jumps in birefringence seem to be temperature-independent, for they occur at all of the temperatures studied (between 8 and 30°C). Such jumps in birefringence were described previously in crane-fly spermatocytes kept at room temperature (Forer, 1976); our data on cells kept under constant temperature conditions show that these jumps in birefringence are not due to changes in temperature.

We measured the diameters of individual spindle fibres from late metaphase through mid-anaphase. Spindle fibre diameters did not change throughout this period or at different temperatures. The overall average chromosomal spindle fibre diameter is 0.8 ± 0.1 μm (n = 49 fibres from 14 cells between 10 and 25°C). Therefore, at all temperatures retardation can be converted to coefficient of birefringence by dividing by 0.8 μm.

DISCUSSION

Video digitizer analysis system

We developed a video digitizer system to quantify birefringence (retardation) objectively along the lengths of single chromosomal spindle fibres. Birefringence has been quantified previously by densitometric analysis of negatives (Swann & Mitchell, 1950; Swann, 1951a,b), but most subsequent work involved subjective visual estimates of birefringence (reviewed by Inoué, 1981b). Birefringence has also been quantified using photomultipliers (e.g. see Allen, Brault & Moore, 1963; Allen, Brault & Zeh, 1966; Taylor & Zeh, 1976; Hiramoto et al., 1981a); these systems measure average light intensities in single fixed regions of the cell (e.g., in an area of 1-3 μm in diameter (Allen et al., 1963), an area of 12 μm in diameter (Taylor & Zeh, 1976), or an area of 1 μm in diameter (Hiramoto et al., 1981b)), but, because the measurements are made from living dynamic systems, simultaneous measurements of different areas of the cell cannot be made. With the video analysis system we developed, on the other hand, one can quantify birefringence in very small regions of the cell (<0.1 μm), and one can measure all of these areas independently and simultaneously.

Photographic techniques, in theory, can give the same kinds of data that we obtain from our video system. Modern TV cameras can detect very low light levels, and therefore much better time resolution can be obtained using video rather than photographic methods (TV images can be recorded at 60 frames per s, whereas film exposures usually require about 10 s). As reviewed by Reynolds & Taylor (1980), Taylor & Wang (1980), Inoué (1981a) and Allen, Travis, Allen & Yilmaz (1981), newer TV technology has found widespread applications in many forms of light microscopy formerly limited by low light levels.

There are other advantages in using videotape, in addition to speed. For instance, it is much faster to align a cursor on the TV screen than to align a negative on the densitometer. In addition, the video data are digital and are immediately available for
computer manipulation: the densitometer produces curves of intensity versus position that subsequently need manual manipulation in order to obtain retardations, whereas the computer produces data that can be directly manipulated by computer.

Results from the video digitizer system are quite reliable. The electronic signal in itself is reproducible to about 0.06 nm retardation; scans of the same single spindle fibres from the same series of video frames are reproducible to within 0.15 nm. The main limit to reproducibility is the accuracy with which the operator can define the structure to be scanned, for two main reasons. One is that details of the video image may not be as clearly defined in single frames (which have only one set of lines) as in the original signal (which has both sets of lines). The other is that the cursor appears to be larger than it really is, and repeat scans therefore may not be of exactly the same coordinates. The definition of the image to be scanned remains the limit to reproducibility.

The video digitizer system can be adapted for use in any experiment in which one wants to measure and manipulate intensities (in straight lines or in defined areas) directly from videotape or via live video images. For example, it can be used to quantify images from fluorescence microscopy and from naturally occurring cellular luminescence or induced luminescence, and to quantify the amount of localized photobleaching.

Retardation of single autosomal spindle fibres

The birefringence of individual autosomal spindle fibres did not change from metaphase throughout as much of anaphase as we could follow single fibres, except sometimes for occasional 'random' changes (of up to about 20 or 30%) along the entire length of the fibre. This agrees with previous reports on single spindle fibres in crane-fly spermatocytes (e.g. see Forer, 1976; fig. 5 of Salmon & Begg, 1980). In other spindles, however, birefringence is reported to change during anaphase: in some cells spindle birefringence decreases during anaphase (e.g. see Swann, 1951b; Fuseler, 1973, 1975a,b; LaFountain, 1976), in some cells spindle birefringence first increases then decreases during anaphase (e.g. see Stephens, 1973; Hiramoto et al. 1981a), and in some cells spindle birefringence increases during anaphase (e.g. see Izutsu, Sato, Nakabayashi & Aoki, 1977). Why are the results from crane-fly spermatocytes different from those from other cell types? One difference might simply be that between single chromosomal fibres versus averages that include both chromosomal and continuous fibres; chromosomal spindle fibre birefringence could remain constant (our results) yet overall birefringence could vary because of changes in continuous fibre birefringence. Another difference might be between solely chromosome-to-pole movement (in crane-fly spermatocytes) versus simultaneous chromosome-to-pole movement and spindle elongation; we have no data on whether spindle fibre birefringence might change when chromosome-to-pole movement is accompanied by spindle elongation. Another difference might be that measurements of 'one-spot-in-the spindle' gave an artefactual appearance of birefringence change, as in the following analysis of published data.

In *Tilia americana* endosperm and *Asterias forbesii* eggs spindle birefringence was
maximal at the onset of anaphase and dropped exponentially through anaphase 
(Fuseler, 1973, 1975a,b), as deduced from retardation measurements at one spot at 
a constant 2–5 μm from the kinetochores. Assuming that the chromosomal fibres in 
these cells have retardation profiles that shorten from the kinetochore ends, as in 
crane-fly spermatocytes, birefringence measured at a constant distance from the 
chromosomes would decrease exponentially as the chromosomes move toward the 
pole (as illustrated in Fig. 10) even though the birefringence of an individual spindle 
fibre remained constant throughout anaphase. Since temperature affects the rate at 
which chromosomes move to the pole (Fuseler, 1973, 1975b; Schaap & Forer, 1979), 
and hence the rate at which a chromosomal fibre shortens, one would expect increased 
temperature to increase the **apparent** rate of decay, as has been indeed reported 
(Fuseler, 1973, 1975b). Further analysis of Fuseler’s data strengthens the argument 
that the 'exponential birefringence decay' is an artefact of the experimental technique. 

*Tilia* endosperm is described as having strong birefringence next to the 
kinetochore; the birefringence 'gradually' decreases poleward "until it drops more 
rapidly where the fibre begins to taper poleward" (Fuseler, 1975a, p. 161 and figs 1b, 
2b). The retardation remains constant during the first few minutes of anaphase, when 
chromosome separation is primarily due to spindle elongation (Fuseler, 1975a, fig. 3). 
The retardation decreases after this time, however, coinciding **exactly** with the short-
tening of the chromosome-to-pole distance (compare fig. 3a and b of Fuseler, 1975a). 
Thus there is no 'decay' of birefringence until the chromosomes move polewards, 
exactly as we would predict from our analysis, and thus a very strong argument can 
be made that the **apparent** decay of birefringence is an artefact of the measurement 
technique.

The idea that spindle fibre birefringence decays during anaphase has been used to

![Diagram](image-url)

**Fig. 10.** A cartoon of a fibre profile that shortens from the kinetochore end. If retardation 
is measured at the same position relative to the kinetochore as chromosome-to-pole move-
ment occurs, then the kinetochore is at position 0 when birefringence is read at position 
1, at position 1 when birefringence is read at position 2, etc., and the birefringence will 
appear to decay through anaphase.
argue that microtubule depolymerization causes chromosome-to-pole movement (Inoué, 1976, p. 1324). However, the argument is not valid because, as we have argued, the previously described birefringence decay is an artefact of the way the birefringence was measured, and because our data show that there is no decay of the birefringence of individual autosomal spindle fibres during chromosome-to-pole movements in crane-fly spermatocytes.

Individual chromosomal spindle fibres sometimes abruptly change birefringence by up to 20–30%. These changes occur along the length of the spindle fibre (see also Forer, 1976) but they do not occur in all cells and they are not temperature-dependent, since they occur at all temperatures studied. There are several possible explanations for these jumps in birefringence, including increases in oriented material or changes in strain birefringence due to fluctuations in tensile forces (e.g. see LaFountain, 1976). The various possibilities will be considered in detail elsewhere (Forer, unpublished). We think that the most likely explanation is that the jumps in birefringence represent changes in orientation of MAPs (microtubule-associated proteins); it is conceivable that local 'environmental' changes (e.g., ionic strength, pH, etc.) around the spindle fibres could change the orientation of some of the MAPs and such changes in orientation could give rise to the observed changes in spindle fibre birefringence. In myofibrils, for example, the S-1 and S-2 myosin subfragments undergo rotational changes in their attachments to actin filaments in response to release of Ca\(^{2+}\) (e.g. see Nihei, Mendelson & Botts, 1974; Huxley, 1976). It has been suggested that local variations in the release of membrane-sequestered Ca\(^{2+}\) play a regulatory role in chromosome movement (e.g. see Hepler, Wick & Wolniak, 1981), and such regulatory changes in the spindle could induce sudden coordinated changes in the binding orientation of MAPs to the microtubules that could result in coordinated fluctuations in birefringence along the length of a fibre.

It is relevant to compare our data on in vivo chromosomal spindle fibre birefringence with data on microtubules in crane-fly spermatocytes. (Heath (1981) and Roos (1981) have reviewed the ultrastructure of other mitotic cell systems.) Although there is considerable variability between individual kinetochores there does not appear to be a significant change in the number of microtubules inserting into the kinetochore from metaphase through anaphase (LaFountain, 1976; Forer & Brinkley, 1977; Fuge, 1980). This constancy fits well with the constancy of birefringence of individual chromosomal spindle fibres throughout anaphase, and argues that the 20–30% changes in chromosomal spindle fibre birefringence through metaphase and anaphase must be due to changes in something other than numbers of microtubules.

As observed electron microscopically, the microtubule orientations change during anaphase: the microtubules seen in the kinetochore bundle are more irregularly oriented (i.e. less nearly parallel to each other) during anaphase than during metaphase (LaFountain, 1976; Fuge, 1980). If the microtubules alone are responsible for birefringence then the birefringence should decrease in anaphase as the orientation becomes less regular. This is not seen: the birefringence remains at about the same level (except for the random jumps up or down). This suggests that components other than microtubules might contribute to the birefringence. Further, there are fewer
microtubules near the pole than near the kinetochore, and, although Fuge (1980) does not comment on it, it appears that the microtubules in the reconstructed autosomal fibre microtubule bundle are more fragmented and disorganized near the pole than in the rest of the bundle. (Indeed, Fuge (1980) comments that the microtubule bundle appears to end about 1–1.5 μm in front of the centrioles.) While these observations are all consistent with the experimentally observed decrease in retardation near the pole, other observations are not: Fuge (1980, p. 325) finds that "the distance between the distal termination of the bundle and the pole does not seem to remain unchanged but rather to increase to a certain degree due to the shortening (distal fragmentation) of the kMTs" (kinetochore microtubules). Thus if microtubules alone were responsible for the birefringence the polar slope region of chromosomal fibre retardation would move away from the pole during anaphase. But it does not: the length of the slope to the pole does not change during anaphase. These comparisons suggest that there may be components other than microtubules that contribute to spindle fibre birefringence.

In the following paper we describe temperature effects on spindle fibre birefringence and the implication of these data for thermodynamic parameters calculated from previous data.

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REFERENCES


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