PROTOPlasMIC STRUCTURE AND MITOSIS

I. THE BIREFRINGENCE OF THE METAPHASE SPINDLE AND ASTERS OF THE LIVING SEA-URCHIN EGG

By M. M. SWANN

From the Department of Zoology, University of Cambridge, and the Marine Station, Millport

(Received 15 March 1951)

(With Plate 8 and Nine Text-figures)

INTRODUCTION

The mitotic figures of many different living cells are now known to be birefringent. The first observation of this kind was made by Runnström (1928) on the eggs of the sea-urchin Psammechinus miliaris. Since then birefringence has been described for a number of mitotic figures: in the eggs of the same sea-urchin by Schmidt (1937, 1939) and Monné (1944); in the eggs of Chaetopterus by Inoué (1950); in the cells of the chick by Hughes & Swann (1948), and in the cells of Tradescantia by Kuwada & Nakamura (1934). I myself have seen birefringence in the mitotic figures of amphibian, bird and mammal tissue cultures; in the eggs of nematodes, annelids, molluscs and echinoderms, and in the spermatocytes of Orthoptera. Since this birefringence is so widespread, and since spindles and asters are, by definition, oriented structures, it is likely that all mitotic figures are birefringent. However, this may not always be apparent in living cells, because of the opposing effects of form and intrinsic birefringence (Swann & Mitchison, 1951).

The only detailed study of the birefringence of mitotic figures is that of Schmidt (1937, 1939). He found that the spindle of Psammechinus miliaris was most strongly birefringent at metaphase, being positive with respect to its long axes. Since protein fibres are also positive with respect to their long axis, he supposed the spindle to consist of fibres arranged lengthwise. The asters show positive radial birefringence, and he supposed that they consist of fibres arranged radially. He also found that the birefringence of the whole mitotic figure disappeared gradually in anaphase, and attributed this to a contraction of the fibres, since the birefringence of structures such as muscle also decrease on contraction. Though not conclusive, these observations have lent considerable weight to the traction fibre hypothesis of mitosis.

Many hypotheses have been put forward at various times to account for chromosome separation (Schrader, 1944). Most of them, however, are quite inadequate to account for the process, and in a valuable review, Cornman (1944) comes to the conclusion that only a traction fibre hypothesis is free from serious objection. A study by Hughes & Swann (1948) on the form of anaphase movement curves led to the same conclusion. On the other hand, there is evidence in many cells that the spindle elongates during anaphase, and that this elongation is partially, and in a few
cells even largely, responsible for chromosome separation (Ris, 1943). Traction and elongation, however, are not mutually exclusive, and as Ris has pointed out, it is possible to classify mitoses according to the relative contributions of the two mechanisms to the total chromosome movement.

Unlike some hypotheses of mitosis, which have supposed the spindle to be no more than a passive guide for chromosomes which are repelling each other, traction and elongation both imply an active function. It seemed important, therefore, to know something more of the changes that take place in the spindle, and it is for this reason that I have attempted a quantitative study of birefringence throughout the mitotic cycle. The results of this study will be presented in a number of separate papers.

In this, the first paper of the series, the structure of the spindle and asters at metaphase is analysed in a preliminary way. In the second paper (Swann, 1951a), the changes during anaphase are examined. These two papers will show that various structural changes take place in the spindle and asters during anaphase, probably caused by the release of certain substances from the chromosomes. Later papers will deal with structural changes in the cortex and cytoplasm at other points in the mitotic cycle, and with questions of form and intrinsic birefringence. From this evidence, a hypothesis of mitosis is developed (Swann, 1951b, 1952), based on the idea that structural agents, concerned with altering protoplasmic patterns, periodically condense on to, and diffuse away from, the chromosomes. In some joint work with J. M. Mitchison, these ideas are extended to the cleavage process (Mitchison, 1952).

**MATERIAL AND METHODS**

Most of the work described in these papers was done at the Marine Station, Millport, using the eggs of the *Psammechinus miliaris*. The metaphase spindle in these eggs has a maximum retardation of about \( \lambda/100 \), or 50 A, so that it is easily detectable with a polarizing microscope of ordinary sensitivity. In the early and late stages of mitosis, however, the retardations may be as low as 1 or 2 A, and increased sensitivity becomes essential. The means for obtaining this increased sensitivity have been described elsewhere (Swann & Mitchison, 1950). The sea-urchin egg, however, presents a particular difficulty, in that its numerous granules scatter a large amount of light. At objective N.A.'s of 0.65 or more, this scattered light entirely obscures the weaker retardations. All the observations were therefore made with a 10 x objective of N.A. 0.28. Under these conditions the scattered light is greatly reduced.

Most of the photographs were taken with a 16 mm. time-lapse camera. Using polarized light, the level of illumination at the image plane is very low, so that the fastest emulsions, such as Kodak Super XX or Kodak R 55 are essential. For the same reason a carbon arc was used as the light source, with a Chance ON 19 heat filter. The magnification on the film was usually about 100 x, a compensator was used, and the condenser N.A. was standardized at 0.12. Under these conditions the exposure required varied from 5 to 15 sec. Time-lapse rates varied between 2 and 6 frames per min. For still photography a 35 mm. camera was used.

* These and other terms in polarized light microscopy are briefly explained in Appendix 1.
The retardation at any point in the spindle and asters was calculated from the density of the film negative. To do this, a densitometer was built to work on patches of film of about 100μ square. This densitometer, and the methods of calculating retardations from density measurements, have been described by Swann & Mitchison (1950). The light scattered by the granules in the sea-urchin egg, however, raises certain special problems in the calculation of retardation which are discussed in Appendix 2.

**Text-fig. 1.** Diagram to illustrate the build-up of retardation in a radially symmetrical structure.

THE ANALYSIS OF ASTER BIREFRINGENCE

The structure of the aster has been the source of continual speculation. On one point only is there agreement: that it is radially symmetrical body. Whether it consists of discrete fibres radiating from a centre, or whether it is a homogeneous body with a radial structure, is uncertain. The simple fact that it is radially symmetrical, however, makes it possible to determine the coefficient of birefringence \( (n_e - n_o) \) at any point within it. As will become apparent, this information goes some way to settling the controversies about its structure.

The retardation of an aster, illuminated by a thin pencil of polarized light, is dependent on the birefringent elements traversed by the pencil. Pencil \( a \) in Text-fig. 1, for instance, traverses elements from the centre to the periphery of the aster, but always parallel to their long axes. Pencil \( b \) traverses elements near the centre more or less normally, and elements further out at an angle. Similarly for pencil \( c \).
material is the same at a given distance from the centre along any radius (because of the radial symmetry of the aster), it would be possible, if the variation in coefficient of birefringence with distance were known, to calculate by summation the retardation to be expected in any pencil \(a, b, c,\) etc., having regard to the angle at which the various elements were traversed. In fact the position is reversed. The retardations in pencils \(a, b, c,\) etc., are known, but not the variation in coefficient of birefringence with distance from the centre. Mathematically, however, the retardation of the aster can be expressed as an integral equation, involving the coefficient of birefringence of the astral elements. If this integral equation is solved, the coefficient of birefringence at any point in the aster can be determined.

By good fortune, the integral equation can be solved exactly.\(^*\) From a curve of aster retardation against distance from the centre, it is possible therefore, by applying the appropriate procedures, to construct a curve of the coefficient of birefringence against distance from the centre. The derivation of the integral equation and the solution are given in Appendix 3.

Metaphase mitotic figures of one- and two-cell stages are shown in Pl. 8, figs. 1–3. The asters are evident in each case, at either end of the strongly birefringent spindle. The centres are isotropic, but at a short distance out, the retardation rises to a maximum, and then falls gradually to nil. The continuous curve of Text-fig. 2 shows this variation of retardation with distance quantitatively. The dashed curve shows the coefficient of birefringence, derived from the retardation curve.

The close similarity between the curves of retardation and coefficient of birefringence is surprising at first sight. This similarity comes about because most of the retardation of an aster is produced by material more or less normal to the line of

\(^*\) I am indebted to Mr Freeman Dyson for solving this equation.
vision. The retardation due to elements at smaller angles to the line of vision is only slight, since they are for the most part at some distance from the centre, where there is a low coefficient of birefringence, while the fact that they are seen at a small angle still further reduces their contribution to the total retardation. As a result, the curve for retardation is, as regards its shape, a fair approximation to the curve for coefficient of birefringence.

In many types of cell, the asters and spindle are generated by sharply defined granules, the centrioles. In the sea-urchin egg, however, there is no distinct granule, but a diffuse body of a few microns in diameter, the centrosome. It might be expected that the aster curves would show no coefficient of birefringence for a short distance out from the centre, but would then rise abruptly to a maximum value. In fact, there is a gradual rise over the first 2 μ, a steeper rise between about 2 and 4 μ, and then a gradual rise to a maximum at about 5 μ. It is difficult to know what reliance to place on this part of the curve, since there are several effects in the optical system, discussed in detail in Appendix 4, that tend to smooth out sharp discontinuities in the object. Nevertheless, it is doubtful whether they could produce sufficient smoothing to convert a sharp discontinuity into the curve that is actually found. It is possible, therefore, that orientation is not built up suddenly at the edge of the centrosome, but more gradually over a distance of a few microns.

Having reached a maximum at about 5 μ from the centre, the coefficient of birefringence falls away steadily, reaching a minimum at about 15 μ. Before going on to consider the meaning of this steady fall, it is a matter of some importance to decide whether the figure of 15 μ, derived from birefringence measurements, represents the real limits of the metaphase aster. The fibrillar appearance of the aster in living and fixed cells, seen by ordinary light, extends only as far out as this. It is significant too, that Chambers (1924) finds that the metaphase mitotic figure is a relatively small gelated body which can be pushed round the fluid cytoplasm, while Heilbrunn (1943) finds that the bulk of the cytoplasm at metaphase has a low viscosity. The evidence, such as it is, suggests therefore that the limits of the aster as determined from birefringence measurements, do correspond to the limits of oriented structure.

The coefficient of birefringence of the aster falls from about 6 × 10⁻⁸ at 5 μ from the centre, to a much lower value at the edge. It is not possible to say exactly how low this value is, since it can only be calculated from the very low retardation at the periphery of the aster, and this cannot be measured with much accuracy. There is, however, at least a twenty-fold fall.

For any given material, presumably protein in the case of the aster, the coefficient of birefringence depends on a number of factors:

1. Amount of material present.
2. Proportion of the total material actually oriented.
3. Degree of molecular orientation.
4. Degree of micellar orientation.
5. Micellar volume (proportion of material organized into micelles).
In simple oriented high polymer systems, the amount of material present does not vary from point to point, and the whole of this material is oriented to a greater or lesser degree (Text-fig. 3a). There is no a priori reason, however, for supposing that the amount of material in different regions of the spindle or asters is the same. Nor is it certain that the oriented structure is homogeneous. Oriented fibrils lying in an unoriented matrix, for instance, might give a proportion of oriented material that varied from point to point (Text-fig. 3b). In some birefringent systems, particularly the strongly fibrous and rather inert proteins, it is possible to separate, by means of imbibition experiments, the relative contributions of form and intrinsic birefringence. Assuming that the amount of material present and the proportion of it that is oriented, remain constant, form birefringence then gives a measure of the degree of micellar orientation and the proportion of material organized into micelles (micellar volume), while intrinsic birefringence gives a measure of the degree of molecular orientation. In the case of the mitotic figures of the sea-urchin egg, however, the protoplasmic structure is labile, and the effect of fixatives and imbibition agents is drastic, so that it is not easy to carry out satisfactory imbibition experiments. A detailed interpretation of molecular and micellar arrangement is therefore difficult, and the problem is only examined in a preliminary way in the present paper. Questions of form and intrinsic birefringence will be considered at length in a later communication.

The amount of material in different regions of the aster is not easily determined. Living asters, examined in ordinary light, show a somewhat reduced refractive index

---

Text-fig. 3. Two types of structure built up from anisodiametric particles. a, amount of material and proportion of material oriented constant throughout; b, amount of material constant throughout, but only about 50% of material oriented (fibrils lying in an unoriented matrix). The particles shown are purely diagrammatic, and represent either molecules or micelles.
Protoplasmic structure and mitosis

at their centre, as do unstained sections in phase contrast (the well-known dumb-bell of metaphase) but this seems to be because the centres are free of granules. These granules are mostly basophilic, and readily seen by staining with basic dyes, either vitally or in sections. If, on the other hand, the ground cytoplasm is stained with acid dyes, it takes up the stain uniformly, and there is no sign of variation in protein density from point to point. It should be added that an examination in polarized light, using a suitably high aperture, leaves no doubt that the birefringence of the aster is caused by ground cytoplasm and not by granules. While, therefore, there may be slight differences in protein density, they are quite insufficient to cause the twenty-fold decrease in coefficient of birefringence between the peak and the edge of the aster.

The possibility that the fall in coefficient of birefringence is due to a decrease in the proportion of oriented material is particularly interesting, since one of the classical conceptions of aster structure, of fibres radiating from the centre like the spokes of a wheel, requires for simple geometrical reasons, that the proportion of oriented material should fall on an inverse square law. The coefficient of birefringence of a metaphase aster is therefore plotted beside inverse square and inverse fourth-power curves in Text-fig. 4.

Text-fig. 4. Coefficient of birefringence of a metaphase aster (a), compared with an inverse square curve (b) and an inverse fourth power curve (c). The metaphase aster curve is the same as in Text-fig. 2.

The coefficient of birefringence of the aster reaches a maximum value of $6 \times 10^{-6}$, at $5 \mu$ from the centre. If the molecular and micellar structure of the aster remained constant throughout, and if the proportion of oriented material did not vary, the coefficient of birefringence would of course remain constant at $6 \times 10^{-6}$ out to the periphery. If, on the other hand, the proportion of oriented material fell off as an inverse square, as the result of a radiating fibrillar structure, the coefficient of birefringence should fall to about $1.2 \times 10^{-6}$ at a distance of $12 \mu$. In fact, the coefficient of birefringence falls to about $0.3 \times 10^{-8}$ at this distance. If therefore the aster is a homogeneous body, with no fall in the proportion of oriented material,
the twenty-fold fall in coefficient of birefringence between 5 and 12 μ, has to be accounted for solely in terms of molecular and micellar changes. If, on the other hand, the proportion of oriented material falls on an inverse square, there is only a four-fold drop to be accounted for in these terms.

Although there is no reason why molecular and micellar changes should not combine to give a twenty-fold decrease in coefficient of birefringence over a distance of 7 μ, there is no obvious mechanism to account for such an effect. A radiating fibrillar structure, on the other hand, provides a simple explanation for the greater part of the fall in coefficient birefringence. It is apparent from Text-fig. 4 that the aster curve is not in fact an inverse square, since it falls away rather too rapidly, but if the fibrils in question were of uneven length, it might be possible to account even for this. Moreover, there are other reasons for thinking that a protoplasmic structure such as the asters and spindle might consist of fibrils, rather than being homogeneously oriented. Muscle, flagella and most oriented protoplasmic structures that have been examined in the electron microscope, have proved to contain discrete fibrils, often of great length. A further suggestive point is that in spermatocytes and in various protozoa, the same centres generate both the spindle and the flagella.

A decision as to whether the mitotic figure is homogeneously oriented, or whether it consists of definite fibrils, is not likely to be possible without direct evidence from the electron microscope. So far only one such study has been made, by Rozsa & Wyckoff (1950). These authors found that with the normal precipitating fixatives the spindle appeared coarsely fibrous, but that after treatment with neutral formalin, there was no sign of orientation whatsoever. Presumably, therefore, the living spindle contains no gross oriented structures, and fibrils if they exist, must be very fine. Rozsa & Wyckoff do not give their resolution, but it does not appear to have been very great.

**THE ANALYSIS OF SPINDLE BIREFRINGENCE**

The metaphase spindle (Pl. 8, figs. 1–3) is not unlike two asters side by side. This superficial similarity is borne out by the form of spindle retardation curves (Text-fig. 5), although the absolute values are considerably higher than in asters. There is the same rise between 2 and 4 μ, the same maximum at about 5 μ, and the same steady fall with increasing distance from the centrosomes. The similarity is somewhat deceptive however, as can be seen from the retardation curves for different stages in the early growth of the spindle (Text-fig. 6). Even at 50 min., when the spindle has only just become visible, there is appreciable birefringence at the equator. In fact, the equatorial birefringence is relatively as great at this stage as at any other, and there is no suggestion that the spindle arises from the fusion of two asters. This is not altogether surprising, since it is well known that the oriented structure of the spindle is generated not only by the centrosomes or centrioles, but also by the centromeres of the chromosomes. In many spindles there are in fact no centrosomes or centrioles.

The spindle, unlike the aster, is not radially symmetrical, so that it is not possible to formulate and solve an integral equation to calculate coefficient of birefringence. By assuming a definite shape, however, it is possible to calculate approximately the
Text-fig. 5. Curve of retardation (continuous line) and coefficient of birefringence (dashed line) of the metaphase spindle, in Pl. 8, fig. 1. The inset shows the shape of spindle assumed for the coefficient of birefringence calculations.

Text-fig. 6. The growing retardation of the spindle in prophase and metaphase. These curves are taken from figs. 4–6 and fig. 1 of Pl. 8, 50, 52, 54 and 56 min. after fertilization.
coefficient of birefringence. In Text-fig. 5 the curve of retardation of metaphase spindle is compared with the curve for coefficient of birefringence calculated in this way. As with the aster, there is a definite similarity between the two curves.

The calculation of coefficient of birefringence involves assuming a shape for the spindle (inset in Text-fig. 5), and allowing, as in the aster, for the fact that different elements of the spindle make different angles with the optical axis. The assumption of a definite shape is not likely to lead to serious error, but it is not strictly justified, as curves of retardation across the equator of the spindle show (Text-fig. 7). The spindle is evidently not clearly demarcated in this region, since the birefringence shades off gradually. Some allowance should also be made for birefringence outside the arbitrary limits of the spindle. This can be done, though it turns out that the correction is negligible, amounting at the most only to 5%.

Text-fig. 7. Curves of retardation across the equator of a spindle: at metaphase (continuous line) and 2 min. after the beginning of anaphase (dots and dashes).

The interpretation of the coefficient of birefringence curve for the spindle follows the same lines as for the asters. An examination of living cells and stained sections in ordinary light, and of unstained sections in phase contrast, shows that there are no significant variations of density within the spindle, so that once again, the fall in coefficient of birefringence must be due either to molecular and micellar changes or to a fall in the proportion of oriented material. A comparison of the fall in coefficient of birefringence with an inverse square is made in Text-fig. 8, and it is apparent that
there is close agreement. Once again, therefore, it seems possible that there is a structure of discrete submicroscopic fibrils radiating from the centrosomes. The oriented structure of the spindle, however, does not spread out freely into the cytoplasm as in the case of the asters, so that the coefficient of birefringence would hardly be expected to fall off quite as rapidly as an inverse square. Some of the orientation at the equator, moreover, is presumably due to the centromeres. The close agreement between the calculated curve and an inverse square may therefore be a little misleading, and it is likely that, but for these modifying factors, the coefficient of birefringence would fall off rather more rapidly than an inverse square, as it does in the case of the aster.

Text-fig. 8. Curves of coefficient of birefringence for each half spindle taken from Text-fig. 4 (continuous lines), and an inverse square curve (dashed line).

SUMMARY
1. The present paper is the first of a series dealing with the birefringence of mitotic figures in the eggs of the sea-urchin *Psammechinus miliaris*.
2. Living eggs have been examined using time-lapse photography, and retardation curves for the mitotic figures constructed from densitometric measurements made on the film negatives.
3. In the case of the aster, an integral equation relating retardation and coefficient of birefringence can be formulated and solved exactly to give coefficient of birefringence. In the case of the spindle, coefficient of birefringence can only be calculated approximately.
4. In both asters and spindles, the coefficient of birefringence is nil at the centres,
rises to a maximum at 5 or 6 µ out, and then falls to a minimum at the equator of the spindle or the periphery of the aster.

5. The rise in coefficient of birefringence round the centre is not as sharp as might be expected, and there is some evidence that orientation is built up gradually over a distance of a few microns.

6. The fall in coefficient of birefringence away from the maximum is approximately an inverse square in the case of the spindle. In the aster it falls off somewhat more rapidly. Since the density of material does not vary from point to point, this fall must be due to changes in molecular and micellar arrangement, or to a decreasing proportion of oriented material.

7. The classical conception of the spindle and asters as structures built up of discrete fibrils radiating from the centres, would be expected, for geometrical reasons, to give an inverse square fall in proportion of oriented material. While, therefore, a homogeneous structure with varying molecular and micellar arrangement cannot be ruled out, it is possible that the mitotic figure consists of definite fibrils radiating from the centres.

8. Evidence from other sources supports this view, and suggests that the fibrils must be submicroscopic in size.

I should like to thank the Director and Staff of the Marine Station, Millport, Scotland, for their help on many occasions.

APPENDIX 1

An outline of polarized light microscopy

A polarizing microscope is essentially only an ordinary microscope fitted with Nicol prisms or sheets of Polaroid. One prism or Polaroid is fitted below the condenser, and is known as the polarizer; another, known as the analyser, is fitted above the objective. Polarizing materials only transmit light vibrating in a particular plane, so that if the plane of the analyser is set at right angles to the plane of the polarizer, the microscope will transmit (theoretically at least) no light at all.

Consider now the effect of a birefringent object between the polarizer and analyser. Light traversing the object can be considered as being split up into two components, vibrating in planes at right angles to each other; but since in birefringent materials, the refractive index is not the same for light vibrating in different planes, the two components will travel at different speeds. When they emerge from the object, therefore, they will be to some extent out of phase, and instead of recombining to give a plane-polarized beam, they will produce ‘elliptically’ polarized light. A component of this light will vibrate parallel to the plane of the analyser, and will therefore get through to the field of the microscope.

The measure of birefringence is the extent to which the two components are out of phase when they emerge from the object. This is normally referred to as the retardation, and is measured in wave-lengths. In the present work, however, the retardations are so small that it is more convenient to measure them in Angstroms (wave-length of green light = 5500 Å).
Protoplasmic structure and mitosis

Retardations are caused, as we have seen, by objects whose refractive index is not the same for light vibrating in different planes. This difference in refractive index is known as the coefficient of birefringence \((n_e - n_o)\). Retardation and coefficient of birefringence are very simply related: the retardation of an object is the product of its coefficient of birefringence and its thickness. Thickness and retardation must, of course, be measured in the same units.

In most oriented biological structures, including mitotic figures, there are only two planes with different refractive indices, and these coincide with the ordinary axes of symmetry of the object, e.g. in the case of a fibre, parallel to and at right angles to its length. Birefringence may be either positive or negative with respect to a particular axis, depending on whether the refractive index parallel to that axis is the greater or lesser one. It is usual to refer the sign of birefringence of an object to its long axis.

The retardation produced by an object is a function of the angle between its axes and the planes of the polarizer and analyser. At 45° the retardation is at a maximum, while at 0 or 90° it falls to nil. For this reason birefringent objects are normally examined with their axes in the 45° position, and this has been done in all the photographs of the present work.

Much use is made, in polarized light microscopy, of a birefringent plate, or compensator, which is inserted between the polarizer and analyser, usually just above the polarizer. By this means, stray birefringence in lenses or slides can be counteracted, and strength and sign of birefringence can be determined. The compensator can also be used to increase the contrast of weakly birefringent objects (Swann & Mitchison, 1950). Because it adds either positive or negative birefringence to the whole field of the microscope, the compensator can make birefringent objects appear brighter or darker than the background. For the same reason it causes radially symmetrical objects like asters to appear bright in two quadrants and dark in the other two. This effect is evident in all the photographs of Pl. 8.

We have seen that both strength and sign of birefringence may vary. In addition, there are two distinct types of birefringence, known as form and intrinsic. Intrinsic birefringence is due solely to molecules, whereas form birefringence is produced by larger aggregates such as micelles. Form birefringence depends simply on the presence of asymmetrical particles of different refractive index from the surrounding medium, the particles themselves need not be birefringent. The majority of biological tissues show both types of birefringence; the relative contributions can usually be distinguished by immersing the tissue in a medium of high refractive index, when the form birefringence disappears.

A full account of the theory and practice of polarized light microscopy is given by Bennett (1950).

APPENDIX 2

The derivation of retardation from measurements of film density

The derivation of retardation from densitometer measurements, presents certain difficulties in the case of the sea-urchin egg. Normally there are two possible methods: either to calibrate the negative in terms of retardation, or to work from the characteristic curve of the film. In the first case it is only necessary to include a
number of blank frames at the end of a run, each illuminated with known amounts of retardation produced by means of a compensator. A curve relating retardation to the densitometer readings can then be constructed. In the second case, a number of blank frames are included in the same way, but with a constant setting of the compensator, and with exposure increasing in multiples. From these blank frames, the characteristic curve of the film can be constructed, and densitometer readings converted to light intensity. Since retardation is proportional to the square root of intensity, for small retardations, it is a simple matter, after converting the intensity values to a linear scale and establishing a zero line, to calculate retardation.

If the object scatters no light, the first of these methods is by far the simpler. The granules in the sea-urchin egg, however, scatter a certain amount of light even at low N.A.'s. This has the effect of raising slightly the whole level of the densitometer curves, and so making it impossible to read off retardation directly from a calibration curve.

The difficulty can be overcome using either method. In the second case it happens automatically, since a zero line has to be drawn; this line is simply drawn rather higher than would otherwise be the case. Using the first method it becomes necessary to draw a zero line, and subtract from the densitometer readings the amount by which the zero differs from the background. For this, of course, the densitometer readings must be converted to a linear scale. Since all the photographs of living asters and spindles are taken with a compensator in position, it is necessary, in both cases, to subtract the amount of compensation from the final figure. The effect of allowing for light scattering is to make the first method as long as the second, and in fact both of them have been used in working out the results. It should be added that they give the same answer.

These methods, though laborious, present no difficulty apart from the establishment of the zero line. In the case of the spindle this is simple, since it only involves drawing a straight line from the minimum of one centre, to the minimum of the other. This line should be horizontal, although occasionally the whole background may have a slight gradient, if the field illumination is not quite even. The zero line for asters is not so easily established. If there were no light scattering this also would be horizontal, but in fact the light scattered is a function of the optical section of the egg, so that the zero line is necessarily slightly tilted, and is strictly speaking not a straight line, but a curve. In practice, the curve is so gradual that a straight line is a reasonable approximation. Occasionally there is some doubt as to how this line should be drawn, though it is usually obvious. The precise position of the line has very little effect on the proximal parts of the aster, but affects the distal regions rather more seriously; hence the uncertainty referred to in the text about the precise values for retardation at the edge of the aster.

The only remaining operation is the smoothing of the retardation curves. The strongly birefringent regions of the spindle and asters are in fact so smooth that it is only necessary to draw a line through all the points. The peripheral regions of the asters and spindles in late anaphase, however, are liable to be bumpy. These bumps are not usually greater than 0.5 A., so that smoothing is not difficult.
APPENDIX 3

The integral equation of aster structure and its solution

$O$ is the centre of an aster;

$AB$ is the axis of vision;

$C_r$ is the coefficient of birefringence at any point in the aster, at distance $r$ from the centre;

$T(d)$ is the retardation of the aster at distance $d$ from the centre, seen in plan view, due to birefringent elements along the $AB$ axis;

$s$ and $\psi$ are as shown in Text-fig. 9.

If an oriented element, with $(n_e - n_o)$ small, lies with its optical axis at an angle $\theta$ to a ray, it can be shown by taking the polar equation for its velocity ellipsoid, that its effective birefringence along the ray path is $(n_e - n_o) \sin^2 \theta$. Thus:

$$T(d) = \int_0^d C_r \cos^2 \psi \, ds \quad (r = d \sec \psi, s = d \tan \psi, ds = d \sec^2 \psi \, d\psi).$$

Therefore

$$T(d) = \int_0^{\pi} C_r (d \sec \psi) \cos^2 \psi d \sec^2 \psi d\psi = d \int_0^{\pi} C_r (d \sec \psi) \, d\psi.$$

The solution of this equation, for which I am indebted to Mr Freeman Dyson, is

$$C_r = \frac{2}{\pi} \int_0^{\pi} \left[ \frac{2T(\rho \sec \psi)}{\rho \sec \psi} - T'(\rho \sec \psi) \right] \cos \psi \, d\psi,$$

where $C_r$ is the coefficient of birefringence at distance $\rho$ from the centre. The practical application of this solution involves first plotting a smooth retardation curve, and measuring the slope, $T'(\rho)$, for different values of $\rho$. Values of the function $R(\rho)$ are then calculated and plotted against $\rho$.

$$R(\rho) = \left[ \frac{2}{r} \frac{T(\rho)}{T'(\rho)} - 1 \right].$$
Then
\[ C(p) = \frac{2}{\pi} \int_0^{\pi} R(\rho \sec \psi) \cos \psi \, d\psi. \]

Values for \( C(p) \) for a sufficient number of values of \( p \) are worked out by numerical integration.

APPENDIX 4

The limitations of the optical system

Sharp changes of light intensity in the object are spread out in the image, to an extent determined primarily by the N.A. of the objective. All the photographs in the present work were taken with a 10\( \times \) objective of N.A. 0.28. Under these conditions a sharp edge is spread out to a more or less steady slope, the top and bottom of which are separated by about 2\( \mu \). Gradients of intensity in the object that are as steep as this will not therefore be accurately reproduced. The steepest gradients to be found in the retardation curves of the present work are those between the centres and the maxima of the asters and spindles. The distance between top and bottom, however, is about 5 or 6\( \mu \), so that the transitions are well within the capacity of the system.

A possible error is concealed in the assumption that the retardation measured at a particular point is the true retardation along the optical axis at that point. In fact, the light that traverses the object is not parallel, but in the form of a cone, whose angle is determined by the aperture of the condenser. As a result the marginal rays cut the aster or spindle slightly nearer the centre. The retardation as measured at any point is effectively therefore the retardation of a point slightly nearer the centre, so that the whole retardation curve is displaced outwards. The degree of displacement, however, is very slight, being only about 0.06\( \mu \) at 15\( \mu \) from the centre.

The last difficulty concerns depth of focus. It has been tacitly assumed that light from every point along the optical axis comes to a focus at the same point in the image plane. This, however, is only the case over a certain range known as the depth of focus. Unfortunately there is no entirely satisfactory formula for depth of focus, the standard quarter wave-length criterion of Rayleigh being generally recognized as too stringent. On this criterion, the depth of focus at the N.A. used in this work (0.28) would be about 10\( \mu \). While it is desirable that the whole object should fall within this distance, it is not essential, and provided that the transitions are not sharp, the object can be considerably out of focus, and yet be reproduced with reasonable accuracy.

The sharp transitions of light intensity in the spindle and asters are to be found in two different regions. The first is between 2 and 4\( \mu \) from the centres, where retardation is building up rapidly. This transition clearly falls well within the depth of focus limit, even on the Rayleigh criterion. The second region, where the transitions are rather less sharp than in the first, falls between about 5 and 8\( \mu \) in the aster, and 6 and 12\( \mu \) in the spindle. To reproduce the aster perfectly would therefore need a depth of focus of about 2 \( \times \) 8 = 16\( \mu \). But, as already pointed out, the main retardation is caused by elements lying more or less normally to the optical axis. Calculation shows in fact that the contributions of elements making an angle of less than 45° to the optical axis is negligible. Under these circumstances the required depth of focus is about 2 \( \times \) 8 sin 45° = 11\( \mu \). The aster is therefore likely to be reproduced
SWANN I—PROTOPLASMIC STRUCTURE AND MITOSIS
Protoplasmic structure and mitosis

fairly accurately by the present system. The spindle is a slightly different case, since it is not radially symmetrical about the centres. The traverses across the spindle in Text-fig. 7 show that its maximum breadth is about 18 μ, but the outer regions are not strongly birefringent. Most of the retardation lies within 5 μ of the axis, so that once again a depth of focus of 10 μ should be adequate. The correctness of these conclusions is borne out by the simple experiment of focusing up and down on a mitotic figure. Over a wide range there is no perceptible difference in appearance.

Although the various effects inherent in the optical system are not likely to be serious, they necessarily all tend towards smoothing out steep gradients in the true curves of retardation. For this reason it is unwise to place great reliance on the absolute values and precise shapes of the measured retardation curves. It is difficult, for instance, to be certain that the rather gradual rise in retardation round the centrosomes is not an artefact, though it seems unlikely. There can be no doubt, however, about the general shape of the curves.

REFERENCES


EXPLANATION OF PLATE 8


Figs. 4–6. Growth of the mitotic figure in prophase. ×400. Compensated. Photographs taken at 50, 52 and 54 min. after fertilization. Fig. 1 is the 56 min. stage of this series.