A POLARIZED LIGHT ANALYSIS OF THE HUMAN RED CELL GHOST

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(Received 22 November 1952)

(With Plate 11)

INTRODUCTION

The structure and composition of the mammalian red blood cell ghost has been the subject of many investigations, mainly because the ghost is the only cell membrane which can be isolated in large quantities, and in what is thought to be a more or less intact condition. A summary of some of the earlier results on its thickness and composition will be given later. It suffices for the moment to say that the mammalian ghost is almost entirely composed of lipids and protein, and that there is probably enough lipid to form a continuous layer at the surface about 40Å thick (2 molecules), and enough protein to form a layer, when dry, from 50 to 150Å thick.

With one exception, however, there had been no measurements of the thickness of the ghost membrane in the normal hydrated state, when this work was started. A priori, it seemed quite possible that the membrane, and especially the protein of the membrane, would be considerably hydrated, since most cells contain from 60 to 90% of water. This was confirmed by the measurement mentioned above; the estimate of Seifriz (1927), by direct observation under the microscope, that the thickness of the membrane of amphibian red cells was 6000–8000 Å. Amphibian red cells, however, differ from mammalian red cells in size, shape and the presence of nucleus, so it seemed desirable to try to find whether mammalian ghosts showed a comparable degree of hydration.

A more important reason for trying to measure the thickness of the wet ghost membrane arose out of the work of Schmitt, Bear & Ponder (1936, 1938). They examined rabbit ghosts in polarized light and found that in glycerol the spherical ghosts showed negative tangential birefringence in their membranes.† They believed originally that this birefringence was due to radially oriented lipid molecules, and also that its strength indicated that it must be caused by lipid layers a great deal thicker than two molecules. Subsequently, they changed their views and

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† In a membrane with a radial optical axis, like the ghosts, it is a matter of convention whether the sign of birefringence is referred to the tangent or the radius (positive with respect to the tangent, and vice versa). The sign is given with respect to the tangent throughout this paper since it stresses the similarity between membranes and fibres or long plates. Extended protein chains would be positive in all these cases.
stated that this strength of birefringence could be caused by a few layers of lipids (footnote at the end of Harvey & Danielli, 1938). This was correct as regards the strength of birefringence of the ghost membrane, but it was still true that the thickness of the birefringent membrane seen down the microscope appeared to be much greater than a few molecular layers. Recent work, however, had established beyond doubt that there was only enough lipid in a ghost to form a continuous layer a few molecules in thickness, so that if this impression of a relatively thick membrane were confirmed, Schmitt et al's interpretation could not be correct.

The measurements described in this paper have confirmed that the birefringent ghost membrane in glycerol is relatively thick—about 0.5μ. A substantial portion of the birefringence must therefore be due mainly to protein, and, since the sign of the birefringence is negative, this protein must have a predominant radial orientation. This implies a new concept of the protein arrangement in the membrane.

A preliminary account of this work was published some years ago (Mitchison, 1950). This full account, however, has been delayed owing to the difficulty of getting the final computation of the diffraction formulae. In the interval Ponder (1951) has produced convincing evidence by another method that ghosts are thick-walled, or possibly solid, structures. He has shown that ghosts, when heated, break down into fragments whose total volume is approximately the same as that of the intact ghosts.

AN OPTICAL METHOD OF MEASURING MEMBRANE THICKNESS

The thickness of a spherical or cylindrical membrane cannot be measured directly under the microscope if this thickness is near or below the limit of resolution. The main reason for this is that diffraction obscures the inner and outer edges of the membrane. Under certain circumstances, however, there is a pattern of light intensity at the edge which can be measured. The thickness of the membrane can then be found by comparing this measured pattern with other patterns calculated from theory for membranes of varying thickness, and seeing which of these gives the best fit.

If a membrane absorbs light (e.g. a dyed membrane) or appears to emit it (e.g. a birefringent membrane), it will have a pattern of light intensity at the edge. It is relatively easy to photograph this pattern, measure the density of the photograph at various points and then express the pattern as a curve of light intensity (or retardation, in a polarizing microscope) against radial distance. The difficulty, however, comes in calculating the appropriate theoretical curves with which the measured curve can be compared. A general description of how this is done is given below, but the detailed formulae can be found in Appendix 1.

The first part of the problem is to derive the ‘undiffracted’ curve which gives the light intensity or retardation from the object before the pattern has been ‘diffracted’ by the microscope objective. Let us consider a spherical, weakly birefringent membrane (with a radial optical axis) in a medium of the same
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refractive index (to eliminate reflexion and refraction effects) and illuminated by a parallel beam of light in a polarizing microscope (Text-fig. 1 a). The retardation of a ray which has passed through the membrane will be the product of the coefficient of birefringence and the thickness of membrane traversed by the ray (as in any birefringent object); but there will also be a factor which allows for the varying orientation of the birefringent elements at different points in the membrane. It is convenient to combine this factor with the actual geometrical thickness of membrane traversed, so as to give a term which can be called the 'effective thickness'. The 'effective thickness' multiplied by the coefficient of birefringence gives the retardation. For a membrane of given radius and thickness, the retardation for any ray can be calculated from equation (1) in Appendix 1 (a) and an 'undiffracted' retardation curve can then be drawn (Text-fig. 1 b). It should be noted that since the coefficient of birefringence is a simple multiplying term it will only affect the height of the final retardation curve, and not its shape.

The second part of the problem is to take diffraction into account, and so to derive a 'diffracted' curve from the 'undiffracted' one. A point object in focus under a microscope gives a diffraction pattern in the image which is a function of
the objective aperture and the wavelength of the light (Text-fig. 1c). If the point is out of focus, the diffraction pattern depends not only on the objective aperture and the wave-length but also on the distance out of focus. The whole object can be regarded as a large number of points, and therefore the whole image can be constructed by summing a large number of point-diffraction patterns. Thus the diffracted retardation curve can be constructed by substituting a diffraction pattern for every point on the undiffracted retardation curve, and then summing all the patterns. The resulting diffracted curve (Text-fig. 1d) is broader and flatter than the undiffracted curve, though the areas underneath them are the same. A diffracted retardation curve, derived in this way, is the appropriate theoretical curve for comparing with the measured curve of a birefringent membrane.*

This method involves a number of optical assumptions which are considered in Appendix 2. There are, however, certain general points which are worth mentioning here. The advantage of the method is that it can be used to measure the thickness of a membrane which is near the limit of resolution. If the membrane is thick the method, though applicable, is usually unnecessary. It has, however, a number of limitations. First, the membrane must be spherical or cylindrical. Secondly, the equations in Appendix 1 will not be correct if the medium on either side of the membrane affects the light intensity, though it might be possible to allow for this. Thirdly, the equations only apply to a membrane of uniform structure throughout its thickness. It is possible to modify the equations to allow for a membrane of varying structure, but only when this variation is known and can be expressed quantitatively (e.g. an orientation falling off as the square of the distance from the edge). Fourthly, the surrounding medium must have nearly the same refractive index as the membrane, since otherwise difficulties with reflexion and refraction will arise.

The equations only apply to birefringent membranes, but it is quite easy to write down similar ones which apply to light-absorbing membranes under an ordinary microscope. The practical difficulty of using them to measure thickness is that the contrast from absorption is usually too low to measure accurately, owing to the bright background. With a polarizing microscope, on the other hand, the background brightness is so low that a birefringent object can have a very high contrast.

MEASUREMENTS ON RED CELL GHOSTS IN GLYCEROL

One volume of human blood was mixed with about a hundred volumes of glycerol. The glycerol haemolysed the red cells and also acted as an immersion liquid of high refractive index. The resulting ghosts were smooth spheres with an external diameter of 6-0 µ, and they showed a negative birefringence at the edge (Pl. 1a). A drop of the suspension of ghosts in glycerol was then mounted in a small cell made by cutting a 1 mm. hole in a no. 0 cover-slip with a diamond, waxing the

* In the calculation in this paper, line diffraction patterns were used rather than point patterns, since this simplifies the computation. This is discussed in Appendix 1.
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cover-slip to a slide, and then covering with another no. 0 cover-slip. It was important to ensure that there was no movement while the ghost was being photographed. If the ghost drifted this showed up in a photograph as an obvious broadening and blurring of the edge in the direction of movement. There was no sign, however, of this after the ghosts had been in the cell for half an hour. This was confirmed by finding no drift of the ghosts over a period of minutes under a micrometer eyepiece which would have shown movements down to about 0.1 μm. There was also no detectable brownian movement. A number of the ghosts were then photographed under a sensitive polarizing microscope (Swann & Mitchison, 1950). Careful focusing was necessary, but this was not difficult since the pattern at the edge of the ghost broadens quickly on either side of the focus, and it was relatively easy to focus the narrowest and sharpest pattern. The details of the optical system were as follows: Cooke, Troughton and Simms polarizing microscope with double thickness polaroids; ×95 'bloomed' achromatic 0.1 objective N.A. 1.30, with an internal stop reducing the N.A. to 1.0 (measured with an apertometer); ×10 compensating eyepiece; two lens 'bloomed' Abbe condenser stopped down to N.A. 0.25, and oiled to the slide; λ/117 mica plate compensator used 5° from its zero position, giving a background retardation of λ/672;* 5 amp. carbon arc with Kohler illumination and a narrow iris; exposures of 1 min. on Kodak Plus-X 35 mm. film; magnification of ×180 on the film. Each ghost was positioned in the centre of the field before being photographed. A series of calibration photographs were also taken on the same length of film. These were taken with no ghosts in the field and with varying amounts of compensation, so as to give the film density for known retardations.

The size of the ghosts on the negatives was too small for measurement, so the negatives were enlarged upon lantern plates to give a total magnification of ×1000 (i.e. the diameter of each ghost was 6 mm.). The calibration negatives were also enlarged, and all the plates were processed together.

The plates were measured with a densitometer designed to work with small areas of film (Swann & Mitchison, 1950). Readings were taken along a radial line traversing the membrane edge. The lightest part of one of the light quadrants of the membrane was chosen, where the tangent to the membrane was at 45° to the polarizer, and where the retardation of the compensator added to that of the membrane. Readings were taken on 100μ square areas of plate every 100μ along the traverse (=0.1 μ on the ghost). Three traverses were taken in each of the two light quadrants of eight ghosts, each of the three traverses being separated by a tangential distance of 200μ on the plate.

Each traverse gave a curve of radial distance against arbitrary units of film density and these units had to be converted to retardations from a calibration curve. This was obtained by measuring the calibration photographs, and then plotting film density against retardation. Each set of three traverses was drawn together on graph paper, and a smoothed curve was then drawn through them by hand.

* Compensation increases contrast, and enables the sign of retardation in the image to be determined.
A sample set is given in Text-fig. 2 to show the degree of smoothing that was necessary. The sixteen smoothed curves were equalized at their average peak value (3.77 A.), and averaged to give a final experimental curve. This curve is shown in Text-figs. 3 and 4, and is an average from forty-eight traverses. The left-hand side of the curve is the outside of the ghost. The standard deviation (σ) of the sixteen smoothed curves at a retardation of 1 A. on the left-hand side was 0.060 μ, and at 2 A. on the right-hand side was 0.056 μ. This gives an indication of the degree of variation of the smoothed curves, and is marked on the measured curve in the figures by the two cross-bars whose total lengths are 2 × σ.

![Image of smoothed curve and three traverses](image_url)

Text-fig. 2. Set of three traverses across the membrane of a ghost, and a smoothed curve.

It is not possible to work back directly from the experimental curve to the membrane thickness. The only way of finding the thickness is to calculate the theoretical curves for membranes of known thickness and then to see which of them fits best with the experimental curve. Accordingly, three theoretical curves were calculated from the out-of-focus equations (1), (2) and (6) in Appendix 1 for membranes of the same radius of the ghosts (3.0 μ), and with thicknesses of 0.1, 0.5 and 0.7 μ. The other variables in the equations were taken as follows: \( \rho = 0.1 \mu \), \( \lambda = 0.55 \mu \), N.A. = 1.0 and \( n = 1.473 \) (refractive index of glycerol). In order to compare them with the experimental curve, they were adjusted to the same peak value of 3.77 A. by taking the following values for their coefficients of birefringence \((n_p - n_o)\):

<table>
<thead>
<tr>
<th>Thickness in μ</th>
<th>((n_p - n_o) \times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5.90</td>
</tr>
<tr>
<td>0.5</td>
<td>1.47</td>
</tr>
<tr>
<td>0.7</td>
<td>1.29</td>
</tr>
</tbody>
</table>
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It should be noted that changing the coefficient of birefringence alters only the height of a retardation curve, and not its shape. These three theoretical retardation curves are shown in Text-fig. 3.

A fourth theoretical curve was also computed for a very thin membrane using equations (6) and (7) in Appendix 1. With this method of working, the coefficient of birefringence required for a given peak value varies inversely with the thickness assumed for the membrane. For a thickness of 40Å, (which, as will be seen later, is about the thickness of the lipid layer in a ghost) the coefficient for a peak value of 377Å. is $1.43 \times 10^{-8}$. This curve has not been shown in Text-fig. 3 because it would be indistinguishable from the 0.1µ curve. This means that the 0.1µ curve is a limiting case and that any membrane thinner than 0.1µ would, under these conditions, give the same shape of retardation curve, though its height would of course vary with the coefficient of birefringence.

The next problem is to consider how far the experimental curve fits any of the theoretical curves in Text-fig. 3. It is too broad to be produced by a membrane of 0.1µ or thinner, and judging by its breadth it might come from a membrane between 0.5 and 0.7µ. It is obvious, however, that its shape is wrong for either of these curves—the outer (left) part drops down from the peak too slowly and the first minimum comes too far out.

So far, the model membranes used to give the theoretical curves have been assumed to be uniform and to have the same birefringence throughout. In fact this assumption is very probably wrong, but in order to produce a more plausible model membrane it will be necessary to anticipate some of the later discussion and state that there is fairly good evidence of a lipid layer about 40Å. thick at the outer
surface of a ghost. Most of the rest of a thick ghost would be a protein gel. The retardation curve which would be produced by such a membrane can be made up by taking the curves for a 40A. membrane (the lipid) and for a 0.5μ membrane (the protein), displacing the lipid curve 0.5μ outwards (this is equivalent to having the lipid outside the protein), and then adding the two curves together.* The two curves can be added in different proportions, so the values taken for the coefficients of birefringence determine the shape of the final curve in this case, as well as its height. Three different cases are shown in Text-fig. 4, together with the experimental curve. In each case the membrane curve is calculated for a 40A. layer on

![Text-fig. 4. Experimental curve for ghosts in glycerol and three theoretical curves for a membrane with an inner layer 0.5μ thick and an outer layer 40A. thick.](image)

The outside of a 0.5μ layer, but different values for the coefficients of birefringence of the layers are assumed in each case:

<table>
<thead>
<tr>
<th>Curve</th>
<th>( n_\text{e} - n_\text{t} ) for 40A. layer</th>
<th>( n_\text{e} - n_\text{t} ) for 0.5μ layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.52 \times 10^{-8}</td>
<td>1.07 \times 10^{-4}</td>
</tr>
<tr>
<td>B</td>
<td>0.85 \times 10^{-8}</td>
<td>0.88 \times 10^{-4}</td>
</tr>
<tr>
<td>C</td>
<td>1.25 \times 10^{-8}</td>
<td>0.64 \times 10^{-4}</td>
</tr>
</tbody>
</table>

It can be seen that curve B gives a satisfactory fit with the experimental curve. It is not perfect but it is well within the errors of measurement and averaging of the experimental curve. Curves A and C do not give a satisfactory fit nor do combinations of a 40A. curve with curves thicker or thinner than 0.5μ.

These results show that the ghost membrane can be adequately represented by

* Radial orientation, producing negative birefringence, being assumed for both layers.
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A model which has one layer about 0.5 μ thick surrounded by a second layer about one-hundredth of the thickness of the first layer, but 100 times more birefringent. This is of course very similar to a model membrane in which the orientation and birefringence decrease on moving inwards (Ponder, 1951). There are almost certainly a number of functions of distance and orientation which would give as good a fit as curve B, if not better, but the choice of such a function would be so arbitrary that it is not worth doing with our very limited knowledge of membrane structure. The only definite thing that can be said about membrane models in which the orientation falls off, is that the results in this paper show that the membrane must be about 0.5 μ thick and also that there must be a reasonably large degree of orientation some distance inwards, i.e. the orientation must not become vanishingly small at 0.2 or 0.3 μ inwards.

Finally, it should be said that there is one other model membrane structure which gives a good fit to the experimental curve. This is a membrane composed of two 40 Å layers of equal birefringence separated by an isotropic layer exactly 0.3 μ thick. However, such a model would not give the results which are found on photographing ghosts in water, as will be described in the next section, and in any case would not agree with other experimental work on red cells.

MEASUREMENTS ON RED CELL GHOSTS IN WATER

Schmitt et al. (1936) found that red cell ghosts in distilled water showed a weak positive birefringence (a negative cross in their terminology). It seemed desirable to confirm this and also to see what information could be gathered by measuring the breadth of the diffraction pattern at the edge of the ghosts. Since the medium has a different refractive index from the ghosts, light will be reflected from their surfaces. This light will have its plane of polarization rotated, and may possibly show a small amount of elliptical polarization. There may also be a Becke line effect from parts of the membrane which are out of focus. There is no easy way of distinguishing these effects from the retardation caused by rays travelling through the membrane, so it is inaccurate to express the light intensity of the pattern in terms of retardation and apply the full analysis used for the ghosts in glycerol. A limited amount of information, however, can still be got by measuring the breadth of the pattern.

Sphered ghosts were prepared by mixing one volume of human blood with ten volumes of distilled water, and then mounting under a cover-slip. When examined under the polarizing microscope they showed a very weak pattern at the edge. If this pattern is assumed to be due to birefringence, its inner ring was positive and narrower than the pattern in glycerol. Outside this there was a ring of the opposite sign of birefringence (as with the ghosts in glycerol), and its strength appeared to be about the same as that of the inner layer.

If the ghosts were transferred to 4% formalin or to saline (0.9% NaCl) saturated with butyl alcohol there was no change in their appearance, but in pure saline they showed a pattern which was even weaker though of the same shape. Schmitt et al.
(1936) found similar results with pure saline and with saline saturated with butyl and amyl alcohol.

The pattern of the edge of the ghosts in water was so weak that it proved impossible to get a photograph of them good enough for reproduction in this paper. It was, however, possible to measure the photographs on a densitometer, and four ghosts were photographed and measured in exactly the same way as the ghosts in glycerol, except that a stronger light source was used and the exposure reduced to 5 sec. The average curve from these ghosts is shown in Text-fig. 5, together with the experimental curve for the ghosts in glycerol. The ordinate of the curve for the ghosts in water is in arbitrary units of film density (very nearly proportional to light intensity) since a retardation curve would have little meaning for the reasons stated above. However, in order to give some standard of com-

Text-fig. 5. Experimental curves for ghosts in glycerol and in water.

parison with the curve for ghosts in glycerol, the peak value of film density (16) has been drawn as equivalent to a retardation of 1 A. This is because the peak value of light intensity at the edge of a ghost in water was the same as that produced by a retardation of 1 A, put in by a mica plate compensator.

The important fact that emerges from these measurements is that the inner lobe of the diffraction pattern at the edge of ghosts in water does not differ significantly in breadth from the outer lobe, and is considerably narrower than the equivalent part of the curve for the ghosts in glycerol. This implies that nearly all the thick inner layer of the ghost membrane which is responsible for the breadth of the main lobe of the retardation curve in glycerol must show no birefringence in water. Since it has a negative intrinsic birefringence in glycerol it must therefore have an equal and opposite positive form birefringence in water.

It is impossible to make any detailed analysis of the curve for ghosts in water for two reasons. First, the measured curve may well be broader than the true one, both because aberrations in the objective may become significant when a low refractive index mounting medium is used, and because brownian movement in
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A low viscosity medium may cause the ghosts to shift during the exposure. Secondly, as stated above, it is difficult to know how much of the pattern is due to rotation and other effects rather than to birefringence.

If, however, the pattern at the edge of the ghosts is assumed to be a true positive birefringence with a maximum retardation of about 1 A., it is tempting to consider it as due to the form birefringence of a thin lipid layer at the surface. The normal Wiener formula for form birefringence cannot be applied to a single sheet, but it is possible to derive an appropriate formula using the same basic assumptions as Wiener (1912). This has been done by Dr R. O. Gandy, and the working is given in Appendix 3. The formula is calculated for a single layer of small plates arranged edge to edge, and is probably the right formula to use for a thin lipid layer with holes in it. This gives a positive form birefringence in water of about 1.1 x 10^-2 if the refractive index of the layer is taken as 1.46 (the refractive index of human fat). If the layer has a negative intrinsic birefringence of 0.85 x 10^-2 (p. 404), the total positive birefringence in water will be 0.25 x 10^-2. Since a layer with a birefringence of 1.43 x 10^-2 would give a retardation curve with a peak value of 3.77 A. (p. 403), a layer with a birefringence of 0.25 x 10^-2 would give a peak value of 0.66 A. This is of the same order as the peak value of 1 A. which is given by the ghosts in water. The objection to this argument is that the pattern for the ghosts in water is narrower than the one for a very thin membrane, so presumably the pattern has been distorted by some of the errors mentioned above.

One other deduction that can be made is that a membrane consisting of two thin lipid layers separated by 0.3 μ (which would fit the experimental curve in glycerol, p. 405) would very probably not give this pattern in water. There would be both rotation and retardation at the inner layer, and it is almost certain that the resulting pattern would be broader than the one that is actually found.

The Effect of Glycerol

It will be argued in the discussion that the ghost membrane is probably a 2% protein gel. The argument assumes that glycerol replaces water in the intermicellar spaces of the membrane, and does not swell or shrink the structure. The grounds for this assumption must therefore be considered, especially since there have been some objections to it (Ponder, 1951).

Probably the best evidence that glycerol does not affect the membrane structure is that ghosts in glycerol have the same shape as sphered red cells (or ghosts) in saline, and very nearly the same size. The radius of ghosts in glycerol (3.9 μ) is only 8% greater than that of sphered red cells (2.77 μ from volume of 89 cu. μ). It is unlikely that glycerol would cause any significant change in the membrane structure without affecting shape or size.

An experiment was also done to test the effect of glycerol on the size of weak protein gels. Plasma was prepared from fresh oxalated bull's blood. The plasma was allowed to clot by neutralizing the oxalate with CaCl₂. Two small blocks (about 2 x 1 x 1 cm.) were cut out of the clot, placed in glycerol, and measured at
intervals. After 4 days in glycerol one block showed a decrease in length of 6\%, and the other showed a decrease in volume of 17\%. These are relatively small changes, and are in a gel which has a lower protein content than that of the ghost membrane (the fibrinogen content of cow's plasma is 0.72\% (Howe, 1925), so the clot must be a gel of fibrin at about this concentration). The clot became almost transparent, showing that the glycerol had replaced the water. A similar experiment done on a 20\% gelatin gel gave almost the same result—a linear shrinkage of 5\% after 3 days in glycerol, and the gel turning transparent. Until, therefore, there is further evidence, it is reasonable to assume that immersion in glycerol for a few hours leaves the ghost membrane substantially unaltered.

It should be added that Schmitt et al. (1936) found that concentrated solutions of urea also produced a negative birefringence in ghosts, and pointed out that this indicated that glycerol was not causing birefringence by dehydration.

Glycerol is not entirely satisfactory as an immersion liquid for removing all the form birefringence, because its refractive index (R.I. = 1.47) is lower than that of protein (about 1.55). This is not, however, a serious disadvantage since form birefringence curves (birefringence plotted against R.I. of immersion medium) show that there is usually less than 10\% of the form birefringence left when the R.I. of the medium is ±0.075 from that of the micelles. Glycerol also has the advantage that its R.I. is very near that of most lipids, so that reflexion from the surface of a lipid layer will be largely eliminated. In any case, there is no other possible immersion liquid. Non-aqueous liquids are useless, and solutions of potassium mercuric iodide cause fixed ghosts to shrivel, and unfixed ones to disintegrate. Even glycerol is unsuitable with fixed ghosts as they do not become smooth spheres.

DISCUSSION

(a) Summary of results

Before starting the discussion it is worth recapitulating the results of the experimental work, and commenting very briefly on their validity. The main results are as follows:

1. The birefringent membrane of ghosts in glycerol is relatively thick—of the order of 0.5\μ.

2. The outer part of this membrane is more birefringent than the inner part. A good agreement with the experimental results is given by a model membrane consisting of a layer 0.5\μ thick with a negative intrinsic birefringence of 0.88 \times 10^{-4}, covered by a layer of 40\AA. thick with a negative birefringence of 0.85 \times 10^{-3}. Other model membranes with the birefringence stronger in the outer regions might, however, give an equally good agreement.

3. The appearance of ghosts in water indicates that most of the membrane must have a positive form birefringence equal and opposite to the negative intrinsic birefringence. It is possible that there is a very thin outer layer which has a positive form birefringence greater than its intrinsic birefringence, so that it shows a total positive birefringence in water.
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The validity of these results depend on calculations made with diffraction optics, and, as with nearly all such calculations for microscopes, a number of simplifying assumptions have to be made. These assumptions have been justified as far as possible, and are discussed in Appendix 2. It is very difficult, however, to allow for all possible errors, especially in the calculation of out-of-focus diffraction patterns, and if these errors were serious they might cause a broadening of the pattern at the edge of the ghosts, and therefore an overestimate of the membrane thickness. On the other hand, the membrane curves are comparatively insensitive to changes in the aperture of the system and to changes in shape of the out of focus patterns. Furthermore, the narrow pattern at the edge of ghosts in water indicates that errors in the optical system or in photographic technique are probably not responsible for the broad pattern in glycerol. To summarize, it is almost certain that the ghost membrane in glycerol is relatively thick, and that most of this membrane has negative intrinsic birefringence and an equal positive form birefringence. The exact thickness and strength of birefringence of the layers of this membrane, are, however, less certain.

(b) The components, and the dry thickness of the ghost membrane

It is known from chemical analysis that the two main components of washed ghosts are lipids and a protein which has usually been called 'stromatin' in the past. Boehm (1935) analysed the properties of stromatin and, although some of his results are in doubt, his general view is likely to be correct—that stromatin is fibrous in form and can, like nucleo-proteins, form gels at very low concentrations. In chemical composition, stromatin differs from other known proteins (Ballentine, 1944). Recent workers believe that there are at least two proteins in ghosts, apart from any contaminating haemoglobin (Howe, 1951; Moskowitz & Calvin, 1952). However, as Howe (1951) points out, although there may be a number of separate proteins in ghosts, it is equally true that the process of preparation involves the tearing of a tissue unit into fragments. In any case the problem is not relevant here since the argument that will be presented is equally valid whether ghost protein is a mixture or not.

Phospholipids and cholesterol make up 85% of the lipids in human ghosts (Williams, Erickson & Macy, 1941), and it is likely that a large proportion of these lipids are bound to protein (Parpart & Dziemian, 1940). Moskowitz & Calvin (1952) have separated a lipid-carbohydrate-protein complex called 'elinin' from ghosts. About 25% of elinin is alcohol-ether extractable material (presumably mainly lipids) and the rest is stromatin. Elinin is a long rod-shaped molecule with a high molecular weight. It is combined with the ether extractable lipids to form 'stromin' which is roughly equivalent to washed ghosts.

Carbohydrate is present in elinin and it has also been found before in red cells (Morgan, 1947; De Burgh et al. 1948). It appears, however, to be present only in small quantities.

Earlier work on the thickness (and composition) of the membranes of red cells
and ghosts has been well reviewed by Ponder (1948, 1949a) and it is only necessary here to give a summary in Table 1 of some of the more important results.

The amounts of dry protein in section B of Table 1, and of the 'fixed framework' in section C, are possibly underestimates since there may be a loss of matter during haemolysis and during washing (Howe, 1951). The results of Waugh & Schmitt (1940) indicate that protein and lipid can be lost, and both the anti-sphering protein and an unidentified globulin can be detected in the supernatant fluid during

<table>
<thead>
<tr>
<th>Reference</th>
<th>Thickness of layer in A</th>
<th>Material</th>
<th>Method</th>
<th>Comments</th>
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<tr>
<td>A. Lipids</td>
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</tr>
<tr>
<td>Gortet &amp; Grendel (1925)</td>
<td>c. 40</td>
<td>Various R.B.C.</td>
<td>Extraction and spreading at interface</td>
<td>Assuming a continuous surface layer</td>
</tr>
<tr>
<td>Fricke (1925)</td>
<td>33</td>
<td>Dog R.B.C.</td>
<td>Capacity measurements</td>
<td>Assuming dielectric constant = 3</td>
</tr>
<tr>
<td>Waugh &amp; Schmitt (1940)</td>
<td>60</td>
<td>Rabbit ghosts</td>
<td>'Leptoscope'</td>
<td>Haemolysis at pH 7.4</td>
</tr>
<tr>
<td>Waugh &amp; Schmitt (1940)</td>
<td>70</td>
<td>Rabbit ghosts</td>
<td>'Leptoscope'</td>
<td>Haemolysis at pH 6.0</td>
</tr>
<tr>
<td>Parpart &amp; Dziemian (1940)</td>
<td>38</td>
<td>Human ghosts</td>
<td>Chemical analysis</td>
<td>From 3.94 x 10^{-10} mg. lipid/cell*</td>
</tr>
<tr>
<td>Parpart &amp; Dziemian (1940)</td>
<td>37</td>
<td>Rabbit ghosts</td>
<td>Chemical analysis</td>
<td>From 3.50 x 10^{-10} mg. lipid/cell*</td>
</tr>
<tr>
<td>Pfeiffer (1949)</td>
<td>139-160</td>
<td>Amphibian R.B.C.</td>
<td>Reflected polarized light</td>
<td>See p. 411</td>
</tr>
<tr>
<td>B. Dry protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>60</td>
<td>Rabbit ghosts</td>
<td>Leptoscope</td>
<td>Haemolysis at pH 7.4</td>
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<td>Waugh &amp; Schmitt (1940)</td>
<td>70</td>
<td>Rabbit ghosts</td>
<td>Leptoscope</td>
<td>Haemolysis at pH 6.0</td>
</tr>
<tr>
<td>Ponder (1949)</td>
<td>37</td>
<td>Human ghosts</td>
<td>Chemical analysis</td>
<td>From 7.8 x 10^{-10} mg. stomatin/cell*</td>
</tr>
<tr>
<td>C. Total dry thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waugh &amp; Schmitt (1940)</td>
<td>120</td>
<td>Rabbit ghosts</td>
<td>Leptoscope</td>
<td>Haemolysis at pH 7.4</td>
</tr>
<tr>
<td>Waugh &amp; Schmitt (1940)</td>
<td>215-230</td>
<td>Rabbit ghosts</td>
<td>Leptoscope</td>
<td>Haemolysis at pH 6.0</td>
</tr>
<tr>
<td>Fricke, Parker &amp; Ponder (1939)</td>
<td>120</td>
<td>Rabbit ghosts</td>
<td>Conductivity and weighing</td>
<td>From 'fixed framework' 2.2%*</td>
</tr>
<tr>
<td>Williams, Erickson &amp; Macy (1941)</td>
<td>180</td>
<td>Human ghosts</td>
<td>From phospholipid content</td>
<td>From 'fixed framework' 3.3%*</td>
</tr>
<tr>
<td>Wolpers (1941)</td>
<td>150-250</td>
<td>Human ghosts</td>
<td>Electron micr.</td>
<td>By density</td>
</tr>
<tr>
<td>Zwickau (1941)</td>
<td>200-300</td>
<td>Human ghosts</td>
<td>Electron micr.</td>
<td>By density</td>
</tr>
<tr>
<td>Bessis &amp; Bricka (1949)</td>
<td>500-1000</td>
<td>Mammalian ghosts</td>
<td>Electron micr.</td>
<td>By shadowing</td>
</tr>
<tr>
<td>Hillier, in Parpart &amp; Ballentine (1952)</td>
<td>50-60</td>
<td>?</td>
<td>Electron micr.</td>
<td>?</td>
</tr>
<tr>
<td>Parpart &amp; Ballentine (1952)</td>
<td>55</td>
<td>Rabbit ghosts</td>
<td>Weighing</td>
<td>From 'fixed framework' 1.1%*</td>
</tr>
<tr>
<td>D. Total wet thickness</td>
<td>6000-8000</td>
<td>Amphibian R.B.C.</td>
<td>Direct observation</td>
<td></td>
</tr>
</tbody>
</table>

* These calculations of thickness assume: (1) a continuous surface layer; (2) density of lipids = 0.85, density of protein density of 'fixed framework' = 1.0; (3) surface area of rabbit R.B.C. = 110 sq.μ, surface area of human R.B.C. = 16; volume of rabbit R.B.C. = 60 cu.μ, volume of human R.B.C. = 90 cu.μ.

Table 1. Thickness of red cell and ghost membranes
the washing of ghosts (Ponder, 1948; Moskowitz & Calvin, 1952). It is possible, however, that this loss may be compensated by contamination with haemoglobin.

It is uncertain exactly what layer was measured by Pfeiffer (1949), but his results have been tentatively placed with those on the lipids. He found the thickness of the layer by measuring the changes in ellipticity and vibration direction of polarized light reflected from the surface of amphibian red cells. The cells were stretched between micro-needles in various salt solutions. If the hypothesis about the membrane structure which is suggested later in this discussion is correct, most of the reflexion should take place at the inside and outside of the lipid layer, where there is likely to be a large change of refractive index.

The electron microscope measurements are not likely to be exact, since shadowing is difficult to interpret with a membrane which may have a curved edge, and density comparisons are never very accurate.

Bearing these comments in mind, it seems reasonable to make the following estimates of thickness for the mammalian ghost membrane from the results in Table 1:

- Thickness of lipid layer: 30–50 Å (approx. 2 molecules)
- Dry thickness of protein layer: 50–150 Å
- Total dry thickness: 100–200 Å

These are only rough figures, and there are likely to be large differences both between different animals, and according to the method of preparation.*

(c) The wet thickness of the ghost membrane

The measurements in this paper have shown that the thickness of the human ghost membrane in glycerol is about 0.5 μ or 5000 Å. If it is assumed that glycerol replaces water in the membrane, but otherwise does not affect its structure (p. 407), then the hydrated ghost membrane is also 0.5 μ thick. Although this value agrees roughly with Seifriz's figure of 0.6–0.8 μ for amphibian red cells, it is a good deal larger than most other estimates that have been made in the past.

A rough check on this figure was obtained by centrifuging human ghosts up to 116,000 g. (Mitchison, 1953). They were found to pack down into a layer whose thickness indicated that each ghost had a volume of 75–81 cu.μ. Results of the same order (human ghost volume of about 45 cu.μ) were also obtained in the more thorough centrifuging experiments of Ponder (1950). The volume of a spheroid ghost membrane 0.5 μ thick will be nearly 50 cu.μ. The volume of a packed ghost should therefore be of the same order as the volume found in these centrifuging experiments, if it is simply a collapsed membrane 0.5 μ in thickness. If, on the other hand, the ghost were a thin walled bag with a fluid interior, it might well be

* According to Ponder (private communication), these figures are probably underestimates rather than overestimates. Many of the measurements have been made with washed ghosts, and it seems likely that the more a ghost is washed the thinner it becomes. The true thicknesses, however, are unlikely to exceed twice the value above.
expected to collapse under strong centrifuging and reach a very much smaller final volume.

Recently, Ponder (1951) has provided further confirmation of the idea that ghosts have thick walls. He has heated ghosts to about 50° C. and found that they break up into fragments whose total volume, measured in a conductivity cell, is approximately the same as that of the intact ghosts. This indicates that the ghosts are thick-walled or solid structures rather than thin hollow shells.

Since the wet thickness of the membrane is about 5000 A., and the dry thickness is 100–200 A., the membrane must contain at least 96% of water. Such a hydrated structure could not be produced by the lipids alone, and we must therefore conclude that it is a protein or lipoprotein gel. Taking the dry thickness of the ghost protein as 100 A., its concentration in this gel must be about 2%. Although this is quite a low concentration for a gel, it is by no means unusual for a protein.

(d) The structure of the ghost membrane

In order to elucidate the structure of the ghost membrane, it is essential to establish which of the components is responsible for the birefringence. There are four possible substances: glycerol, haemoglobin, lipids and stromatin, and they will be considered in this order.

Glycerol can be dismissed at once, as there is no evidence from other work that glycerol can show birefringence. In any case Schmitt et al. (1936) found that urea solutions produced the same birefringence in ghosts as glycerol.

At first sight it appears that haemoglobin might be responsible, since it has a strong birefringence when crystalline, and it was certainly present in the ghosts prepared by mixing blood and glycerol. There are, however, two pieces of conclusive evidence against this possibility. First, Perutz & Mitchison (1950) have shown that when a small thickness of haemoglobin shows birefringence, it will also show anomalous colours on compensation. There was no trace of these colours in the ghosts. Secondly, there was no apparent difference between the haemoglobin-contaminated ghosts prepared by simple mixing and those prepared by the more complicated method used by Schmitt et al. (1936) to free them from haemoglobin. It would perhaps be desirable to check the thickness of these haemoglobin-free ghosts. They are not, however, more suitable than ghosts prepared by simple mixing because there is evidence that the process of washing out the haemoglobin also washes out some of the membrane constituents (Howe, 1951).

Having dismissed the possibility that the birefringence is due to glycerol or haemoglobin, we are left with the problem of deciding whether it is due to lipids or protein. It has been shown that the optical measurements agree satisfactorily with a model consisting of a layer 0.5μ thick with a negative birefringence of 0.88 x 10^-4 surrounded by a thin layer 40 A. thick with a negative birefringence of 0.85 x 10^-8. We have also seen that there is enough lipid in ghosts to form a layer about 40 A. thick. This suggests that the surface layer is composed of radially oriented lipids. Such a layer would agree with electrical capacity measurements (Fricke, 1925; Cole, 1940), and there is also a good deal of evidence from other
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sources that the surface layer of the membrane is predominantly lipid, though with holes which would presumably contain or expose protein. First, such a structure fits many of the facts of permeability (Davson & Danielli, 1943). Parpart & Ballentine (1952) have emphasized the need for postulating water channels or pores in the membrane in order to explain the rapid penetration of water into red cells, but they point out that such pores would only account for a small part of the surface area—0.1% in rabbit red cells. Secondly, the electrophoresis measurements of Winkler & Bungenberg de Jong (1940) show that the iso-electric point of ghosts is intermediate between that of stromatin and that of the lipids. Furchgott & Ponder (1941) conclude from I.E.P. measurements that the surface of the intact red cell is dominated by the phosphoric acid groups of kephalin and related lipids. Thirdly, the wetting properties of red cells indicate a mainly lipophilic surface (Mudd & Mudd, 1926). Fourthly, a mixed surface is indicated by the fact that it is damaged by both lipases and proteases.

Returning to the question of the birefringence, let us first consider the possibility that radially oriented lipids are responsible for all the birefringence. We can postulate a membrane structure in which the total quantity of lipids is divided into two parts. The first part is concentrated as a nearly continuous layer at the surface with a thickness of 1 molecule or about 20 Å. The second part is distributed through the 0.5μ thick protein jelly in the form of little radially oriented micelles. At first sight, such a structure is not incompatible with the optical measurements. A 20 Å. layer would produce the same effect as a 40 Å. layer provided it had double the birefringence, i.e. 1.7 × 10⁻². If the remaining 20 Å. were spread out through 0.5μ it would give a birefringence of 0.68 × 10⁻⁴, which is not greatly different from 0.88 × 10⁻⁴. There is however a serious objection to this idea. Both the lipid micelles and the monomolecular surface layer would almost certainly have to be stabilized and kept in position by a well-oriented protein framework with the chains lying at right angles to the direction of the lipid molecules (i.e. tangentially in the ghost), as in other known protein-lipid structures such as the nerve myelin sheath (Schmitt, 1950; Fernández-Morán, 1950) or retinal rods (Schmidt, 1951). Since the birefringence of oriented protein is as large, if not larger than that of lipid, and there is twice as much protein as lipid in the ghost, the presence of such a protein structure would certainly reduce the negative intrinsic birefringence drastically and very probably reverse the sign of birefringence.

If we reject the idea of scattered lipid micelles being responsible for the birefringence of the 0.5μ layer, we must conclude that this birefringence is due to the protein. Most of the lipids can then be regarded as forming a bimolecular layer of 40 Å. at the surface of the protein layer. This is a widely accepted idea, both because of the inherent stability of such a layer and because it agrees with the electrical measurements. A birefringence of 0.85 × 10⁻² is a reasonable figure for lipids. There are no figures for the birefringence of the isolated membrane lipids, but Schmitt & Bear (1937) give a value of 1.1 × 10⁻² for the birefringence of myelin. It is difficult to draw any conclusions from the birefringence of 0.88 × 10⁻⁴ for the protein layer, since very little is known about the relation between birefringence
and the details of protein structure, but the degree of orientation is probably similar to that of muscle since the latter has very roughly ten times the amount of protein and ten times the intrinsic birefringence of the thick layer of the ghost membrane.

We have seen that the evidence strongly suggests that the protein layer shows positive form* and negative intrinsic birefringence, which implies radial molecular orientation and tangential micellar orientation. Now there are only two protein arrangements in a cell membrane which have been suggested in the past. One arrangement is with extended protein chains running all ways in the plane of the surface. This will not fit the facts above, unless it is assumed that the side chains are lying radially and that they determine the sign of birefringence. The evidence is

against this, because in all extended proteins whose birefringence has been measured, the sign is determined by the direction of the long backbones of the polypeptide chains and not by the side chains. The second arrangement is that of globular proteins adsorbed on the surface. These molecules, however, have usually been regarded as statistically isotropic, like balls of string.

A new model of the protein structure is therefore necessary. One possible model would have the protein micelles running all ways in the plane of the surface, and composed of long polypeptide chains looped like a Chinese cracker or squib with their main orientation radial. A cross-section of such an arrangement is shown in Text-fig. 6a. For ease of illustration, the micelles are shown as running only in

* The positive form birefringence of ghosts is shown in a striking way in photographs of ghosts prepared for the electron microscope by Williams (1953). These are freeze-dried in a special way so that they maintain their spherical shape when dry in air. They show the strong positive form birefringence which would be expected if air replaced water as the intermicellar medium.
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the plane of the paper, whereas in the membrane they would have to run all ways in the surface, in order to give statistical isotropy when viewed from above. Such an arrangement would have the required positive form birefringence due to the tangentially oriented crackers or micelles, and negative intrinsic birefringence due to the radial orientation of the polypeptide chains in the crackers.

This arrangement of looped protein chains is more than a speculation. There is a considerable amount of evidence from X-ray crystallography that proteins can form structures in which the main chains appear to lie at right angles to the length of the micelles (the 'cross-β' pattern). Such evidence is given for egg albumin by Astbury, Dickinson & Bailey (1935); for epidermin by Rudall (1949); for keratin by Mercer (1949); and for casein, haemoglobin, zein, edestin and peanut protein by Senti, Eddy & Nutting (1943).

Evidence, mostly from the electron microscope, has been accumulating for some time that protein fibres may consist of a linear sequence of globular or corpuscular particles, e.g. Waugh (1944) for insulin; Farrant, Rees & Mercer (1947) for keratin; Jakus & Hall (1947) for actin. Recently, Perutz (1949), by a detailed X-ray analysis of haemoglobin, provided a possible molecular basis for this arrangement. He found the haemoglobin molecule to be a cylinder with folded polypeptide chains running parallel to the base. If these cylinders could aggregate end to end, they would give just such a linear sequence of corpuscular particles. These cylindrical columns would have the same optical properties as the looped crackers—a negative intrinsic birefringence from the looped chains and a positive form birefringence (haemoglobin crystals show a birefringence which is positive with respect to the cylinder length, but this is probably caused by the haem groups rather than by the protein). A second model of the ghost membrane can be made with these cylindrical columns instead of the looped crackers (Text-fig. 6b). It is a less likely arrangement than the first because the elasticity (Beams, 1947; and Norris, 1939, for amphibian R.B.C.), the gelation at low concentrations, and the properties of the extracted components (Moskowitz & Calvin, 1952), seem to indicate a more fibrous arrangement.

In view of the increasing interest in the possibilities of spirals in protein structure, it is worth pointing out that a third model can be made with the protein chains coiled in tight spirals like springs (Text-fig. 6c). This would also give positive form and negative intrinsic birefringence if the spirals were large enough.

The main points which have so far been made in this discussion can be summarized in a simplified model of the structure of the ghost membrane (Text-fig. 7). Most of the membrane would consist of looped bundles of protein chains forming a 2% gel about 0.5 μ thick. The size of the bundles is quite uncertain. Outside this would be a bimolecular lipid layer 30–50 A. thick, with a certain number of holes in it. Ponder (1948) has suggested that there might also be an incomplete layer of proteins outside the lipid layer, perhaps 20–50 A. thick and probably not continuous, which would be responsible for anti-sphering and antigenic properties. The intact red cell membrane would be similar except that the protein layer would contain large numbers of haemoglobin molecules between,
and possibly within, the bundles. This is in many ways similar to the model suggested by Ponder (1948); the main difference lies in the arrangement of the protein.

This model is both speculative and simplified. The detailed structure is undoubtedly more complicated, and differs from one animal to another. This point is emphasized by Jacobs (1950) from permeability studies. The surface lipids probably form lipo-protein complexes with the surrounding proteins, which might be lying tangentially in this region. It is also uncertain whether the proteins responsible for the anti-sphering and antigenic properties form a separate layer, or are located in the holes in the lipid layer. The justification for this type of simple model is that it explains some of the facts about the general structure of the membrane, and also serves as a basis for argument and criticism.

In the literature on ghosts and other cell membranes, there is a considerable difference of opinion about the use of the words 'cell membrane', 'plasmalemma', 'ultrastructure', 'cortex', etc. If the model suggested above is correct in outline, it seems reasonable to suggest the following terminology. The 'cell membrane' would include the whole membrane. The 'structural layer' would be the thick protein gel responsible for the mechanical properties of the membrane (equivalent to the 'ultrastructure' of some workers). The 'permeability layer' would be the thin perforated lipid layer (and possibly some associated protein) which is responsible for the permeability and electrical properties of the membrane.
Implication of the suggested membrane structure

A controversy has been going on for many years as to whether the red cell is a thin-walled balloon containing haemoglobin in solution, or whether it has a solid gel-like interior. Details of the controversy are given in Ponder (1948), and only a brief summary will be given here. Examination under the microscope, whether with ordinary or dark-ground illumination, shows no internal structure and favours the balloon hypothesis. Electron microscope photographs also show the red cell as a collapsed balloon with folds in its walls (e.g. Wolpers, 1941; Bessis & Bricka, 1950). Evidence in favour of a gel-like interior comes from several sources. Red cells can be cut by microdissection into what are apparently lumps of jelly containing haemoglobin (Mason & Rockwood, 1925). Appreciable quantities of a clear jelly appear in red cells stratified by ultracentrifuging (Beams, 1947), and also in sickle cells (Ponder, 1948). If red cells are haemolysed by hypotonic media, the ghosts contain more haemoglobin than is likely to remain on the surface from simple adsorption (Ponder, 1942). The concentration of haemoglobin in the ghosts is 1·3–3·8 times that in the surrounding medium, and Ponder believes it necessary to postulate an internal structure in the ghosts holding the haemoglobin. Finally, from a consideration of the shape transformations of heated human red cells, Ponder (1949b) suggests "that the phenomena observed...are part of a process of disintegration of a plastic Hb-bearing "solid", rather than manifestations of the breakdown of a balloon-like structure in which the Hb is held in solution".

This controversy can be settled if the intact red cell has a membrane of the same thickness and structure as that of the ghost. It can then be regarded as a balloon surrounded not by a thin membrane, but by one 0·5μ thick. The intact red cell would have a structure whose cross-section is shown in Text-fig. 8 (the external dimensions are those for the average human red cell taken from Ponder, 1948). The thickness at the biconcavity is 1·0μ, so the two internal edges of the membrane would be touching at this point. Within the membrane there would be an annular space with a maximum thickness of 1·4μ. The haemoglobin would have to be present not only in this space, but also throughout the weak protein gel of the membrane. This model will explain all the facts above. It would show no internal

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**Text-fig. 8.** Cross-section of the human red cell with membrane 0·5μ thick.
structure under the light microscope, and would dry down to give the appearance of a thin-walled balloon under the electron microscope. On the other hand, the haemoglobin-containing jelly of the membrane would explain the evidence for a gel-like interior. It is possible that the annular space within the membrane, or the corresponding spherical space in a sphered cell, also contains a weak protein gel structure, though the absence of birefringence towards the centre of a ghost shows that such an interior gel must either lack orientation or disappear on haemolysis.

If the inner edges of the membrane touch in the biconcavity, it is much easier to understand how the red cell maintains its disc shape. It is obviously a far more stable shape with this thick membrane than it would be with a thin one.

The looped structure in the membrane may help to explain the disk=sphere transformation in red cells. During the transformation from disk to sphere the volume of the cell remains constant, but its surface diminishes by about one-third. It is not unreasonable to assume that the looped membrane structure which has been suggested for the ghost is also present in the sphered red cell. If so, there may be a tendency for the membrane to expand in area by an unlooping of the protein chains. If the surface area expands, and the volume remains constant, the sphere will start to dimple, and the dimples will deepen until their inside edges touch. This will produce a structure very similar to the normal disk shape. A further expansion might account for the long streamers in sickle cells. In a similar way, any factor which encourages the formation of the loops will cause the membrane to contract in area, and change from a disk to a sphere.

The difficulty with this scheme is that if the cell behaves like a rubber ball and the membrane keeps a constant volume, then the thickness of the membrane should diminish by one-third when the cell changes from a sphere to a disk. In that case, the inside edges of the membrane would not touch in the biconcavity. A partial solution of this difficulty comes from the observation of Waugh & Schmitt (1940) that the dried ghost shows an increased thickness of at least 15% in the region of the biconcavity* (the optical method described above is not sufficiently sensitive to detect differences of thickness of this amount). The presence of these thicker patches implies that the diagram in Text-fig. 8 is too simple, but it may explain the observation of Furchgott (1940) that in a series of disk=sphere transformations the biconcavity always reforms at the same place. It is possible, however, that this difficulty may not arise, as it may be wrong to assume that the membrane keeps a constant volume on expansion.

Since the preliminary account of this work (Mitchison, 1950) appeared three years ago, a number of comments on it have been published by other workers which ought to be discussed briefly. Ponder (1951) thinks that more weight should be placed on the results of Schmitt et al. (1936) on the birefringence of ghosts placed in different agents. These workers made the initial discovery of the negative birefringence of ghosts in glycerol on which this work is based. They also found a very

* This is disputed by Bessis & Bricka (1950) who find that ghosts under the electron microscope are thicker at the edge.
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weak positive birefringence in saline, and a stronger positive birefringence in distilled water and in saline saturated with lipid solvents (butyl and amyl alcohol). They interpreted the increases in positive birefringence as being due to a disorientation or solution of the lipids which had been responsible for the negative birefringence in glycerol. The measurements in this paper, however, show that the thick layer which produces the birefringence in glycerol is different from the thin layer which produces the birefringence in water or the other media. It is doubtful, in any case, whether this latter birefringence is a true birefringence or a spurious one due to a rotation of the polarized light on reflexion. If we suppose that it is a true birefringence, the increase in positive birefringence on treatment with alcohol-saturated saline might be due to lipid disorientation, but it might equally well be due to a change of state of the protein, since these alcohols are also protein denaturing agents. The same effect is also obtained with formalin which is not a lipid solvent, but does affect protein structure.

Ponder (1951) gives a model of the red cell ghost which has a fairly thick lipoprotein membrane in which the concentration and orientation of lipoprotein falls off on moving inwards from the edge, and the concentration of haemoglobin increases. Such a model fits most of the data on ghosts and is perfectly consistent with the optical measurements (p. 405), subject to two provisos. First, the lipid is probably concentrated at the surface of the ghost and not spread through the membrane for the reasons given earlier (p. 413), so the material of the membrane should be protein, and not lipoprotein (except for the surface layer). Secondly, the concentration must not fall off too rapidly. The model membrane in this paper has been assumed to end abruptly 0.5 μ from the edge, but this is mainly for reasons of convenience in the mathematics, and because the choice of any particular function defining the fall off in orientation or concentration would be somewhat arbitrary. In fact, Ponder's model may well be nearer the truth.

Moskowitz & Calvin (1952) believe that what is being measured in the ghost in glycerol is an 'internal structure', probably composed of 'haemoglobin, S-protein and other materials' and not the true cell membrane. This is largely a matter of terminology, and there is no reason why the S-protein and 'other material' should not be regarded as part of the structural membrane. Haemoglobin is unlikely to be responsible for the birefringence of the thick layer for reasons given earlier, though it is presumably present in this layer. It is possible that Moskowitz & Calvin's elinin consists of most of the looped protein suggested here, both from the main part of the membrane, and from the surface layer where it would be combined with lipid to form a lipo-protein complex. These loops would almost certainly extend out on extraction of the elinin to give the long rods found with the electron microscope. It is, however, also possible that the elinin is only the extreme outer layer of the membrane, since the quantitative yield is not given; certainly elinin contains some of the outer layer because it contains surface antigens.

Finally, Parpart & Ballentine (1952) object to the idea of a thick watery membrane on the grounds that this does not fit the permeability and electrical data. This objection, however, should not apply to the model suggested above. The thin layer
at the surface would be responsible for the permeability and electrical properties
of the membrane, while the thick watery layer within would not affect them.

This study of the ghost membrane has a number of implications in cell biology. It
seems likely that other cell membranes may have a similar structure, and if so,
the possibility of active membrane expansion which follows from a looped protein
structure may be of importance in explaining the mechanism of cell cleavage and
certain other processes (Mitchison, 1952). It is also possible that similar protein
structures occur in cytoplasm (Swann & Mitchison, 1951).

SUMMARY

1. A new method is described for measuring the thickness of thin spherical bire-
fringent membranes. It consists of measuring a curve of retardation against radial
distance at the edge of the membrane, and comparing this curve with other curves
calculated from theory for membranes of known thickness. Diffraction is taken
into account.

2. This method shows that the wet thickness of the human red cell ghost in
glycerol is about 0.5 μ. A good agreement with the experimental results would be
given by a model membrane consisting of a weakly birefringent layer 0.5 μ thick
surrounded by a strongly birefringent layer 40 A. thick. It is suggested that the
thick layer is a 2% protein gel, and that the thin layer is a bimolecular layer of lipids.

3. The birefringence indicates that there is radial molecular and tangential
micellar orientation in the protein gel. This can be explained by an arrangement of
the protein chains in looped bundles.

4. On the basis of these results a new model is put forward for the structure of
the red cell membrane, and some of its implications are discussed.

I am especially grateful to Dr R. O. Gandy, Mr A. F. Huxley and Prof. D. R.
Hartree for their invaluable help with the optics and mathematics in this paper.
I am also much indebted to Dr L. E. R. Picken and Prof. M. M. Swann for the
many useful discussions we have had on this subject.
APPENDIX 1. FORMULAE FOR RETARDATION CURVES

(a) 'Undiffracted' retardation curves

This equation has been derived by Bear & Schmitt (1936) in the first half of a paper on the optics of the nerve myelin sheath, but it seems worth giving a simple proof of it below.

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

In Text-fig. 9 the ray \( AB \), at a distance \( x \) from the centre \( C \), traverses a spherical membrane of birefringent material (with a radial optical axis) from \( S_1 \) to \( S_2 \), and has previously traversed an equal amount below. The external radius of the membrane is \( r \) and its thickness \( t \). \( S_1 \) and \( S_2 \) subtend an angle \( \phi \) at the centre. \( dS \) is a small element of the path, at a distance \( r' \) from the centre, and subtending \( d\theta \) at the centre. If \( \Gamma' \) is the retardation along \( AB \) then:

\[
\Gamma' = 2 \sum \frac{s_i}{s_o} \sin^2 \theta dS (n_s - n_o).
\]

Now
\[
r' = x/sin \theta,
\]
and
\[
dS = (r'd\theta)/\sin \theta.
\]
Therefore
\[
dS = (x d\theta)/\sin^2 \theta.
\]
Therefore
\[
\Gamma' = 2x \int_{\theta_o}^{\theta_1} d\theta (n_s - n_o)
\]
\[
= 2x[sin^{-1}[x/(r-t)] - sin^{-1}[x/r]](n_s - n_o),
\]
where
\[
sin^{-1}[x/(r-t)] = \frac{1}{2} \pi \text{ when } (r-t) < x < r.
\]

Text-fig. 9.
It should be noted that the associated ordinary and extraordinary rays are assumed to follow the same path. Bear & Schmitt (1936) conclude that for small values of \(n_e - n_o\) the errors introduced by this assumption are negligible.

The first half of the right-hand side of equation (1) can be regarded as the 'effective thickness' of the membrane, corresponding to the thickness \(d\) in the normal retardation relation \(\Gamma = d(n_e - n_o)\).

For spherical or cylindrical membranes where \(t < 0.62r\) the maximum retardation \((\Gamma'_{\text{max}})\) is at the inner tangent ray, i.e. \(x = (r - t)\) and its value can be found by solving equation (1) with \(x = (r - t)\).

For membranes where \(t > 0.62r\) (up to a solid sphere or cylinder, where \(t = r\)), \(\Gamma'_{\text{max}}\) is at the point \(x = 0.62r\) and its value can be found from

\[
\Gamma'_{\text{max}} = 1.12r(n_e - n_o) .
\]

This is given by Frey-Wyssling (1948), who also points out that the widely quoted equation for \(\Gamma'_{\text{max}}\) given by Bear & Schmitt (1936) is incorrect.

It can be shown that

\[
\int_0^r \Gamma'_v dx = \pi t(2r - t)(n_e - n_o)/4
\]

\[
= \pi r(n_e - n_o)/2, \quad \text{if } t \leq r .
\]

This means that the area under the retardation curve for a thin membrane is directly proportional to the radius, the thickness, and the coefficient of birefringence. This is also true for the diffracted retardation curve.

(b) 'Diffracted' retardation curves

If a spherical birefringent membrane is illuminated by a coherent beam of light in a polarizing microscope, the retardation \((\Gamma_v)\) at each point on the 'diffracted' curve (retardation against radial distance) is given by the sum of the amplitudes, taking account of sign, of the contributions from neighbouring points. Each contribution will be the product of the retardation at the neighbouring point \((\Gamma'_v)\) times a factor given by the amplitude of the diffraction pattern at the distance away of the point \((y - x)\).

\[
\Gamma_v = \sum \Gamma'_v D_{v-x} .
\]

\(\Gamma'_v\) can be calculated from equation (1). The diffraction function \(D_{v-x}\) is less straightforward, and three cases will be considered below. In each case the lens is assumed to be free from aberrations.

(i) General in-focus case

If all points of the membrane are assumed to be in focus the amplitude diffraction pattern will be the normal circular pattern associated with a point.

\[
D_v = \frac{1}{a} J_1\left[\frac{2\pi a (\text{N.A.)}}{\lambda}\right] .
\]

Where \(a(=y-x)\) is the distance from the centre of the pattern, (N.A.) is the numerical aperture of the objective, \(\lambda\) is the wave-length of light, and \(J_1\) is a first-order Bessel function.

The constant \(K\) in equation (2) is in this case \([(\text{N.A.})m^2/\lambda]\) if a square grid of points is used and \(m\) is the distance between points.
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(ii) In-focus case for a set of strips

If the membrane can be assumed to be cylindrical, with the long axis in the object plane, then the calculations can be considerably simplified by taking the contributions from a set of thin birefringent strips, instead of from a grid of points. If all the membrane is in focus, the appropriate diffraction pattern is that for a line (Rayleigh, 1896).

\[ D_a = \frac{1}{a} \sin \left( \frac{2\pi a (\text{N.A.})}{\lambda} \right) \tag{4} \]

In this case \( K \) is \( \rho/\pi \), where \( \rho \) is the width of the strips.

(iii) Out-of-focus case for a set of strips

If the membrane is assumed to be composed of a set of strips, as in case (ii), but these strips cannot be assumed to be in focus, then an out-of-focus line diffraction pattern must be used for each strip, and the pattern must be that appropriate for the average out-of-focus distance between this strip and the focal plane.

Gandy (1953) has shown that the amplitude diffraction pattern of a line both in and out of focus can be given by the integral:

\[ \frac{1}{2\beta} \int_{-\beta}^{\beta} \exp \left[ ikp \sin (\theta - \eta) \right] \cos \theta \, d\theta, \tag{5} \]

where \( \beta \) is the semi-angle of aperture of the objective and \( k \) is the propagation constant \( 2\pi/\lambda \). If \( a \) is the distance between a point on the pattern and the centre of the pattern and \( b \) is the distance out of focus, then \( \rho = \sqrt{(a^2 + b^2)} \) and \( \tan \eta = b/a \).

In the case of a cylindrical membrane with the centre axis lying in the focal plane, each of the birefringent strips is composed of two elements lying an equal distance above and below the focal plane. The appropriate diffraction pattern is therefore given by

\[ D_{a,\, z} = k\beta \sqrt{(R^2 + I^2)} \cos \left[ \tan^{-1}(I/R) - kb \right], \tag{6} \]

where \( R \) and \( I \) are the real and imaginary parts of the integral (5) and \( b \) is the distance out of focus of the membrane for the ray at distance \( z \) from the centre (in Text-fig. 9, \( b = A S_1 + B S_2 g/2 \)). In this case, as in case (ii), \( K \) is \( \rho/\pi \). It is worth noting that for the in-focus pattern where \( b = 0 \) equation (6) reduces to equation (4).

Unfortunately, the integral (5) is formally insoluble, and for large values of \( b \) and \( \beta \) numerical integration by hand takes a very long time. Prof. D. R. Hartree, however, has considerably simplified the computing (Hartree, 1953) and has prepared a programme for the automatic computer (the EDSAC) in the Mathematical Laboratory, Cambridge. This has been used to provide the numerical values used in computing the curves in this paper.

In any problem involving measurements of spherical membranes the appropriate diffraction pattern will depend primarily on the radius of the membrane and the N.A. of the objective. In the case of red cell ghosts, it is permissible to use line patterns rather than point patterns since the difference between curves calculated with equation (3) and with equation (4) is negligible. It is almost certain that there would be an equally small difference in the out-of-focus case between curves using equation (6) and those using equation (3) modified to allow for out-of-focus point patterns. No attempt, however, has been made to find these latter curves owing to the prohibitive length of the computing which would be needed.

On the other hand, it is not permissible to assume that all parts of the membrane are in focus, since substantial contributions to the retardation curve are made from parts of
the membrane well beyond the depth of focus of the objective used. All the theoretical
curves were therefore computed with the diffraction patterns from equation (6). It is
worth noting, however, that the broadening of the retardation curves when the out-of-
focus effects are allowed for is not as great as might perhaps be expected. If these effects
are ignored, and equation (4) used for the theoretical curves, the thickness of the ghost
membrane comes out at about \(0.7\mu\) rather than \(0.3\mu\).

It should be emphasised that two important assumptions have been made in deriving
the equations above. First, that the objective is free from aberrations, and secondly, that
the membrane is illuminated by coherent light. The validity of these assumptions is
considered in Appendix 2 with regard to the particular optical system used for the ghost
photographs.

(c) Retardation curves for very thin membranes

If a membrane is very thin compared to the breadth of the diffraction pattern, equation
(2) will tend to give undue weight to the maximum retardation at the inner edge. Instead of using the height of
a strip under the retardation curve as the multiplying factor for the diffraction pattern, it is better to use the
area of the strip in the following way. This approximation is only valid for very thin membranes.

In Text-fig. 10, the 'effective thickness' \(E = 2x\delta\theta\) (from equation (1)). For a thin membrane \((t \ll r)\),

\[
\delta\theta = \sin \delta\theta = \frac{y}{r} = t \cot \theta/r.
\]

But \(\cot \theta = x/\sqrt{(r^2 - x^2)}\).

Therefore \(\delta\theta = tx/(r\sqrt{(r^2 - x^2)})\).

Therefore \(E = 2x^2t/(r\sqrt{(r^2 - x^2)})\).

Let the area under the undiffracted retardation curve from \(x = a\) to \(x = b\) be \(A\).

Then

\[
A = (n_s - n_u) \frac{2t}{r} \int_a^b \frac{x^2}{\sqrt{(r^2 - x^2)}} \, dx
\]

\[
= (n_s - n_u) \frac{2t}{r} \left[ \frac{r^2}{2} \sin^{-1} \frac{x}{r} - \frac{x}{2} \sqrt{(r^2 - x^2)} \right]_b^a.
\]

Then the diffracted retardation curve is given by

\[
\Gamma_x = K \sum_{\Delta x} A_x D_{x-\Delta x}.
\]

APPENDIX 2. VALIDITY OF THE OPTICAL ASSUMPTIONS

A number of optical assumptions were made in the derivation of the membrane equations
and their application to the ghosts. The most satisfactory way of confirming the validity of
these assumptions would have been to test the theoretical curves against a model
membrane of known thickness and of the same dimensions as the ghosts. Such a pro-
cedure would have given a simultaneous check on the validity of all the assumptions,
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But unfortunately it proved impossible to find a suitable model membrane. As a result, it was necessary to test some of the assumptions separately.

The first main problem was to see whether the in-focus line diffraction pattern defined by equation (4) in Appendix 1 was in fact the diffraction pattern given by a line under the optical system used. It was possible that this pattern might have been seriously distorted by wrong assumptions about the N.A. of the system, the presence of lens aberrations, or the use of white light rather than monochromatic light. The best method of testing this seemed to be to photograph a linear diffraction pattern and see whether it agreed with theory. The main piece of the tail of a bull sperm makes a good test object, since it acts as a birefringent line whose thickness is small compared to the diffraction pattern.*

Accordingly, dried bull sperm were photographed (Pl. 1b) and measured in the same way as the red cell ghosts except in two respects. First, a mixture of paraffin and methyl salicylate (R.I. = 1.50) was used instead of immersion oil (R.I. = 1.52) to allow for the fact that some of the ghosts may have been photographed through a layer of glycerol. Secondly, the plates were measured on a Hilger microphotometer with a long thin slit (0.05μ by 1μ at object), which is more suitable for a linear diffraction pattern than the densitometer used for the ghosts. The average experimental curve is shown in Text-fig. 11, together with two theoretical curves for N.A. of 0.8 and 1.0.

* Randall & Friedlander (1950) from EM photographs of ram sperm give diameters of 0.50–0.53μ for the main piece, and 0.15–0.20μ for the tail end or axial filament. If a bull sperm is similar to a ram sperm, the main piece would seem to be too broad for a line source. It does, however, appear to act as a line source, because it gives a linear diffraction pattern which is the same as that produced by the thinner tail end. We must therefore presume that only a part of the main piece is acting as the major source of birefringence. This may well be the axial filament, but, even if the whole tail is birefringent, most of the birefringence would appear to come from a strip narrower than its diameter, since the tail is circular in cross-section (possibly semicircular when dried on to a surface).
with two theoretical linear diffraction patterns calculated from the \( \sin \pi x/\lambda \) formula (equation (4), Appendix 1) with N.A.'s of 1.0 and 0.8, and \( \lambda = 0.55 \mu \). The average curve would give the best fit to a theoretical curve calculated for N.A. 0.9, though the outer lobes are rather smaller in amplitude.

A confirmation of these results was obtained by measurements on teased asbestos fibres which, when thin enough, also yield linear diffraction patterns. The fibres were mounted in Canada Balsam (R.I. = 1.53) and Euparal (R.I. = 1.48-9), and the distance between the two first minima of the diffraction pattern was measured with a screw micrometer eyepiece (the rest of the optical system being the same as for the ghosts and the sperm). This was much quicker than measuring photographs, though a photographic check on five fibres showed that the micrometer measurements were consistently less than the photographic ones by an average of 5%. Thirty-five fibres were measured, and the results for the lowest ten in each medium are given below (the lowest ten were taken because the distance between minima cannot be smaller than in the theoretical diffraction pattern, but can become larger as the fibre thickness increases):

<table>
<thead>
<tr>
<th>Medium</th>
<th>Av. distance between minima in ( \mu )</th>
<th>Standard deviation in ( \mu )</th>
<th>Av. distance plus 5%</th>
<th>Equivalent N.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balsam</td>
<td>0.89</td>
<td>0.02</td>
<td>0.93</td>
<td>0.86</td>
</tr>
<tr>
<td>Euparal</td>
<td>0.88</td>
<td>0.05</td>
<td>0.92</td>
<td>0.87</td>
</tr>
</tbody>
</table>

These results agree with those from the sperm where the measured curves have an equivalent N.A. of about 0.9.

These two experiments show that in this optical system the in-focus diffraction pattern of a line does not show a large departure from what should be expected from theory, and that lens aberrations and white light do not cause serious errors. It was, in any case, unlikely that lens aberrations would have been large. The objective was stopped down from N.A. 1.3 to 1.0 which would make a considerable reduction in spherical and chromatic aberration. Coma and chromatic difference of magnification were likely to be negligible since the ghosts were always centred in the field, and only occupied a small part of it. As for the question of light, although it would have been better in theory to have used monochromatic light, in practice the ghost photographs could not have been taken in monochromatic light owing to the much longer time exposures which would have been necessary.

When calculating the theoretical membrane curves with the out-of-focus diffraction formula it seemed better to use the true N.A. of the objective (1.0) rather than the empirical value of 0.9 found above, since this broadening of the linear patterns might not have been due to a real decrease of the N.A. Even if the lower N.A. had been used, however, it would have made a negligible difference to the final theoretical curves. An earlier calculation with an N.A. of 0.625—40% lower than N.A. 1.0—gave a curve for a 0.5 \( \mu \) membrane only 11% broader at half peak value. The reason for this is that although the breadth of the in-focus pattern is inversely proportional to the N.A., the broadening of the pattern as it goes out of focus is less with a smaller N.A.

In deriving the equations in Appendix 1 it was assumed that the membrane was illuminated by a parallel and coherent beam of light. Since a condenser was used in the microscope this was not strictly true, and it is therefore necessary to consider how much difference this would have made.

There are two effects of using a cone of light from a condenser rather than a parallel beam. The first effect is on the summation of diffraction patterns, which with coherent
A polarized light analysis of the human red cell ghost

Illumination should be added in amplitude, and with random phase illumination should be added in intensity. In a microscope it is usually assumed that if the condenser N.A. is small compared to the objective N.A. the illumination is coherent, while if the two N.A.'s are equal the illumination approaches random phase. With the optical system used for photographing the ghosts the condenser N.A. was \( \frac{1}{4} \) of the objective N.A., which would probably be regarded as small. It is impossible to be strictly quantitative about this, but there are two indications that the assumption of coherence in this case is justified. First, Hopkins & Barham (1950) have developed the theory of a 'partial coherence factor'. This varies from 1 when the illumination is coherent (condenser N.A. very small) to 0 when the illumination is in random phase. They consider an area to be coherently illuminated when this factor is greater than 0.88. With the optical system used for the ghosts this factor is 0.85 for points 0.4/1 apart (and increases as the separation decreases). In the case of the theoretical membrane curve for a 0.5μ thick membrane, 70% of the contributions to the peak value come from parts of the membrane which are 0.4μ apart and less. Secondly, the diffraction pattern given by the sperm tails in Text-fig. 11 resembles the theoretical pattern calculated from the sine function which assumes coherence. The theoretical pattern for a line in random phase illumination is given by a Struve function and is quite different from the sperm tail curves, e.g. the tail of such a pattern never descends below zero.

The second effect of using a cone of light from a condenser is not connected with diffraction and is a purely geometrical one. Beams of light coming through the membrane at different angles will produce undiffracted retardation curves which are displaced relative to each other, and it is possible that this might broaden the diffracted curves. Bear & Schmitt (1936) concluded that this effect could be ignored with a small condenser N.A., but it seemed desirable to confirm this point by an approximate calculation with ray optics.

Consider an oblique ray of light \( AB \) at an angle \( \theta \) to the vertical, and touching a birefringent cylindrical membrane of radius \( r \) at the point \( A \) (Text-fig. 12a). This ray will produce the same retardation (zero in this case) as the vertical ray touching the membrane at \( C \), but the retardation will appear to come from the point \( B \) in the focal plane. A similar argument applies to all rays parallel to \( AB \). They will produce a retardation curve identical to that produced by vertical rays (neglecting questions of focus), but which will appear to be displaced outwards from the centre by the distance \( BC \). It can be seen that

\[
BC = r(\sec \theta - 1).
\]
In the case of the ghosts, each point in the focal plane was assumed to be illuminated by a cone of light from the condenser. The apex of the cone was at the point in the focal plane, and the apex angle was the angle of aperture of the condenser. A circular cross-section of the cone was divided into six strips of equal breadth, as in Text-fig. 12b. The strips were assumed to be parallel to the straight edge of the cylindrical membrane. The light from each strip (from a series of cones) would produce a retardation curve displaced by an average amount equal to $r(\sec \phi - 1)$, where $\phi$ was the half angle subtended at the apex of the cone from the mid-point of the strip ($P_a$, $P_b$ or $P_c$). Values of the displacement were calculated for each of the strips $A$, $B$ and $C$, assuming a half angle of the condenser of 9.5° (n.a. 0.25 in oil), and $r$ as 3$\mu$. These were:

<table>
<thead>
<tr>
<th>Strip</th>
<th>Displacement in $\mu$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>0.028</td>
</tr>
<tr>
<td>$B$</td>
<td>0.011</td>
</tr>
<tr>
<td>$C$</td>
<td>0.001</td>
</tr>
</tbody>
</table>

A series of six undiffracted retardation curves were then drawn, calculated from equation (1) for a membrane 0.5$\mu$ thick. The curves were displaced by the amounts above, and were also weighted by the areas of the strips ($A:B:C = 0.525:0.875:1$). They were then summed, and the resulting curve was 'diffracted' with the linear in-focus function (equation (4), Appendix 1). The final diffracted curve was so nearly identical with a curve calculated in the same way but assuming parallel light, that they would not be separable at the scale used for the graphs in this paper. It is reasonable to assume that the difference would also be negligibly small if the out-of-focus diffraction function was used. We can therefore conclude that the effect of correcting for an illuminating cone is small enough to be ignored, and that the illumination can be treated as parallel.

There are two main limitations in this calculation. First, it was done with ray optics where it is probably correct to assume that the cone of light should be that delivered by the condenser. It would be better if it could have been done with wave optics where the acceptance cone of the objective could also have been considered. Secondly, it neglected the fact that the contributions from the oblique rays were not only displaced, but might also have been out of focus. It can be shown, however, that the maximum distance out of focus ($AB$ in Text-fig. 12a) for the outer strip $A$ is within the depth of focus of the objective, even with the stringent quarter-wave criterion.

There is one final question—whether the out-of-focus diffraction patterns might not be distorted by lens aberrations. It is impossible to test this experimentally with a line source like the sperm tails because the formula for the out-of-focus pattern (equation (6), Appendix 1) assumes an object symmetrically placed above and below the focus. However, this effect is not likely to be serious since the in-focus pattern does not seem to be distorted by lens aberrations. Also it would need a very large distortion of the out-of-focus patterns to make any significant difference to the theoretical curves.
APPENDIX 3. FORM BIREFRINGENCE OF A SMALL THIN PLATE

By R. O. Gandy

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If an object whose dimensions are small compared with a wave-length is placed in a monochromatic electromagnetic field \( E_0 \), then the resultant field \( E_r \) may be regarded as the combination of the incident field \( E_0 \) and the 'scattered field' \( E_s \); and it is well known that the scattered field is that which would be produced by a dipole source of a strength \( d \) situated within the object. If the object is being examined in a polarizing microscope only the field produced by the component of this dipole which is perpendicular to the incident field will contribute to the image. By comparing the strengths of this component of the equivalent dipole for a refractive and for a birefringent object we arrive at the formula for the effective birefringence (form birefringence) of the refractive object. Since the dimensions of the object are small compared with a wave-length the calculations can be carried out using only electrostatic theory.

In Text-fig. 13 we suppose that the incident field is propagated along the \( x \) axis, and that its electric vector \( E_0 \) parallel to \( OP \), makes an angle of 45° with the \( x \) axis. The dielectric constant of the surrounding medium is \( k_0 \). The object considered is a parallelepiped with dimensions \( e, \rho, t \) along the \( x, y, z \) axes respectively, and we assume: \( e \ll \rho \ll \lambda \) and \( t \ll \lambda \), where \( \lambda \) is the wave-length.

We require the component of the equivalent dipole in the direction \( OA \) for two cases:

Case A. The object is isotropic, with dielectric constant \( k_0 \).

Case B. The object is uniaxially birefringent with its optical axis parallel to the \( x \) axis; its dielectric constants are \( k_0, k_1, k_2 \) in the \( x, y, z \) directions.

The incident field may be resolved into fields of strength \( E_0/\sqrt{2} \) parallel to the \( x \) and \( y \) axes, and these two fields can be dealt with separately. For the field parallel to the \( x \) axis, it can be shown that the moment \( d_x \) of the equivalent dipole is given approximately by

\[
\text{case A } d_x = \frac{E_0 V}{4\pi\sqrt{2}} \left( 1 - \frac{k_1}{k_2} \right),
\]

\[
\text{case B } d_x = \frac{E_0 V}{4\pi\sqrt{2}} \left( 1 - \frac{k_1}{k_0} \right),
\]

where \( V \) is the volume of the object (\( = \rho \times e \times t \)). For the field parallel to the \( y \) axis, it can be shown similarly that the moment is given approximately by

\[
\text{case A } d_y = \frac{E_0 V}{4\pi\sqrt{2}} \frac{k_2 - k_1}{k_2 + k_1},
\]

\[
\text{case B } d_y = 0.
\]
When the incident field is $E_0$ parallel to $OP$ then the total resultant dipole has components $d_x$, $d_y$ parallel to the $x$, $y$ axes. Hence the component parallel to $OA$ is

$$d_A = d_x \sqrt{2} - d_y \sqrt{2}.$$ 

Then:

$$\text{case A: } d_A = \frac{E_0 k_3}{8\pi} \left( k_3 - k_1 \right)^2$$

$$\text{case B: } d_A = \frac{E_0 k_3}{8\pi} \left( k_3 - k_1 \right).$$

If the birefringent object is chosen so as to be equivalent to the refractive object then we must have

$$\frac{k_3 - k_1}{k_3} = \left( \frac{k_3 - k_1}{k_3} \right)^2.$$ 

Hence the form birefringence of the refractive object can be found in terms of the refractive index of the medium $n_0$, and of the object $n_k$ (provided $k_3 - k_1$ is small)

$$n_0^2 - n_k^2 = \left( \frac{\left( n_0^2 - n_k^2 \right)}{n_0^2 + n_k^2} \right) \frac{n_0^2}{n_k^2}.$$ 

This formula also applies to a number of small plates lying in the $y$, $z$ plane, and so approximates to the condition of a thin lipid layer with holes in it.

REFERENCES


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EXPLANATION OF PLATE

(a) Human red cell ghost in glycerol. Polarized light, with compensation. See p. 400.

(b) Tail of dried bull spermatozoon. Polarized light, with compensation. See p. 425.
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