THE IMMERSION REFRACTOMETRY OF LIVING CELLS BY PHASE CONTRAST AND INTERFERENCE MICROSCOPY

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I. INTRODUCTION AND THEORY OF THE METHOD

In the field of mineralogy, methods of measuring the refractive indices of homogeneous crystals by examining them microscopically when immersed in liquids of similar refractive index, have been employed for over 100 years. Crystals, or other homogeneous transparent objects, are examined in a succession of mounting media, usually oils, of different refractive indices; and when two such fluids are miscible, a continuous range of media of intermediate refractive index can be made and used. Various optical criteria have been used to detect the presence of small optical path differences, or phase changes, that occur in the transmitted
light passing through the crystal when its refractive index is different to the mounting medium, notably the presence of a bright "Becke line" at the boundary of the object in convergent light with central illumination, and the appearance of an asymmetric border shadow under oblique illumination. The absence of such appearances in any one of the mounting media normally indicates that the object has a refractive index very close to that of the particular fluid in which it is immersed; and under these circumstances the crystal will appear almost invisible. The refractive index of the mounting medium can then be measured in a refractometer.

Living cells, like crystals, are also usually transparent, and frequently contain quite large amounts of optically homogeneous cytoplasm and other homogeneous material; and similar immersion methods have actually been used to measure their refractive indices for over 70 years. Vles (1911) pointed out that the immersion media for living cells "must not in any way change the protoplasm so as to alter the refractive index of the cell"; and this in effect means that they must be non-toxic, incapable of penetrating cells, and not cause changes in cell volume. This seems to have been appreciated by Exner who, as early as 1887, mounted living muscle fibres of the beetle *Hydrophilus* and of an unspecified mammal in liquid paraffin, solutions of egg albumin, and in the aqueous humour extracted from the eyes of freshly killed mammals. He used oblique illumination as an optical criterion for determining when the fibres had the same refractive index as the mounting medium, and obtained values for their mean refractive index closely comparable with those recently obtained by Huxley and Niedergerke (1958), and the present writer and Dr. Casselman (1960) for living muscle fibres from frogs and mice.

Fauré-Fremiet also, in 1929, used immersion refractometry to measure the refractive indices of the pseudopodia of living amoebocytes, and he observed that the mounting media "... should be free from any toxic action whatever, and that their molecular concentration should be near to that of the normal physiological medium of the cells in question". He mounted the amoebocytes of the starfish *Asterias* in sugar solutions of nearly the same tonicity as sea water, and those of the earthworm *Lumbricus* in solutions of accacia gum dissolved in 0.7% saline, which has the same tonicity as that earthworm's blood. He examined the cells so mounted with a microscope using vertical illumination, and was able to determine from the presence and the nature of the interference fringes in the pseudopodia whether the refractive index of the mounting medium was higher or lower, and from their absence to infer that the refractive indices of the mounting medium and pseudopodia were the same. This elegant method of showing up small refractive index differences was admirably suited to the material in question, i.e. thin homogeneous sheets...
of protoplasm in contact with a glass surface, but is not satisfactory for thicker regions or for the curved surfaces of a spherical cell, and cannot be applied to living cells in general.

The development of the Zernike phase-contrast microscope from 1941, however, provided an instrument that is capable of showing up small optical path differences, or phase changes, in a wide variety of different kinds of living cells more strikingly and critically than any previous optical system. In 1952 Dr. Barer and his colleagues at Oxford, the present writer and Mr. S. Joseph (né Tkaczyk), developed a method for measuring the refractive index of the cytoplasm of living cells by immersion refractometry, using phase contrast microscopy and isotonic solutions of bovine plasma albumin as immersion media (Barer and Ross, 1952; Barer, Ross and Tkaczyk, 1953). The principles of this technique have been very fully reported in a series of excellent articles and papers (Barer and Joseph, 1954, 1955a, b; Barer, 1956a), and only a fairly brief description of it need be given here, although certain of its practical aspects will be discussed in some detail. This article will be mainly concerned with the extension and wider applications of the method now made possible with the development of the interference microscope. This instrument is as sensitive as the phase contrast microscope for detecting small phase changes but is also capable of measuring them accurately as well; and this enables the scope of quantitative investigations on living cells that are possible with immersion refractometry, to be considerably extended.

II. THE INTERPRETATION OF REFRACTIVE INDEX MEASUREMENTS AS AN INDICATION OF HYDRATION

Prior to 1951 it seems that the full biological implications of making refractive index measurements on living cells had not been appreciated, and the measurements made by earlier workers were simply regarded as additional physical data. In 1951 and 1952, however, Davies and Wilkins, and Barer, apparently independently, pointed out that such measurements, when applied to living cytoplasm and many other cell constituents, could give a close indication of the concentrations of water and total solids present. This is because nearly all the substances that are commonly found dissolved or finely dispersed in an aqueous phase in living protoplasm, of which proteins, lipoproteins and amino acids normally form by far the greater part, all have very similar specific refraction increments, which do not deviate appreciably from 0.0018: that is to say, the refractive indices of their aqueous solutions increase by very nearly exactly 0.0018 for every 1% rise in their w/v concentration.
This means that the w/v concentration of the total solids in the cytoplasm, and other regions of living cells containing water soluble substances with similar refraction increments, \( C_s \), can be obtained from the formula:

\[
C_s = \frac{n_c - n_w}{0.0018}
\]

where \( n_c \) is the refractive of the region of the cell being measured, and \( n_w \) is the refractive index of water (usually taken as 1.333 at room temperature). It is often extremely convenient to express this relationship graphically so that refractive index measurements can be converted rapidly into values for the approximate per cent w/v solid concentration; and Fig. 1 shows a suitable graph for this purpose. This covers the ranges of refractive index normally found in living cells and bacterial vegetative cells, and includes the highest concentrations obtainable of the more frequently used immersion media. The hard line indicates refractive index plotted against per cent cell solids assuming a refraction increment \((\alpha)\) of 0.0018. The broken line is a similarly plotted relationship for a refraction increment of 0.0019 which is a closer approximation of the specific refraction increment of haemoglobin, and is therefore applicable in the special case when the refractive indices of red blood corpuscles are being measured.

**Fig. 1.** Graph for the quick conversion of refractive index measurements at 20°C into total cell solid concentrations in gm per 100 ml. Hard line: for all ordinary cell material assuming it has a mean refraction increment \((\alpha)\) of 0.0018. Dotted line: for the haemoglobin concentration in red blood corpuscles, assuming the specific refraction increments of haemoglobins approximate closely to 0.0019.
The values for the specific refraction increments of the wide variety of substances on which this generalization is based—many different proteins (including haemoglobin), lipoproteins, amino acids and carbohydrates—have been obtained by a number of different workers in the course of the last twenty years, and these are fully cited by Davies et al. (1954), Barer and Joseph (1954), Barer (1956a) and Davies (1959) and need not be quoted again here. It is worth mentioning, however, that, although all those workers always found the relationship between the refractive indices and w/v concentrations of the above substances to be linear, their measurements were nearly all made with concentrations of less than 50%; and, until recently, some doubt had been expressed as to whether the specific refraction increments of proteins were necessarily linear at very high concentrations and in the nearly solid state. A few measurements on seemingly solid proteins and products containing nearly pure protein, such as dried tobacco mosaic virus, had lower refractive indices than might be expected on this assumption, and a refraction increment 0.0015 for high concentrations of protein had been suggested by Davies et al. (1954) and Barer (1956b). Recently, however, Davies (1959) and Davies and Thornburg (1959) have made some very careful measurements of the refractive indices of some crystalline proteins (β-lactoglobulin and α-chymotripsinogen), containing very little water, and have obtained appreciably higher values than those obtained hitherto, which suggest that the specific refraction increments of proteins are in fact linear, and of a value close to 0.00185 over their whole range of concentrations. Jones's single measurement of the refractive index of air-dried crystalline protein (1946) is also in agreement with this. It consequently seems probable that many supposedly dried protein products contain small but appreciable amounts of “bound water” difficult to remove by ordinary desiccation processes; and the same would appear to apply in the case of bacterial spores (see p. 47).

An approximation of the w/v concentration in various regions of living cells can also be obtained from refractive index measurements; but this is not just 100 minus the per cent concentration of cell solids, because the specific volumes of proteins, and of some other water-soluble substances occurring in living cells, are less than one. One gram of dry protein, for example, does not occupy 1 c.c., but approximately 0.75 c.c. Consequently the w/v per cent water concentration in a protein solution, \( C_w \), is given by the formula:

\[
C_w = 100 - 0.75C_s
\]

where \( C_s \) is the w/v per cent solid concentration. Since protein is the principal solid constituent of protoplasm, an approximation for the water
concentrations can be obtained in this way; but such values will be less accurate than those of the per cent solid concentrations since they are derived from a second set of assumptions; for it is important to realize that although quite a number of non-proteins occurring in protoplasm have similar refraction increments, they do not necessarily have similar specific volumes.

In some cases, refractive index measurements on cytoplasmic inclusions may be interpreted with even greater precision than those made on cytoplasm, because, while cytoplasm is an extremely complex association of many substances, many inclusions can be demonstrated histochemically to consist of single substances or relatively simple mixtures of only a few substances. If the specific refraction increments of these substances are precisely known, their solid content can be more accurately determined. In other cases, even when the water and solid content of a cytoplasmic inclusion is not accurately determined, the refractive index measurements may clearly indicate the presence of water; and this in itself may provide an indication of its probable submicroscopic morphology. An example of this will be described below (p. 38).

Generally speaking, it is certainly true to say that, because refractive index measurements in themselves merely indicate the total solids in any region, the more completely the chemical composition of the region is known, the more precisely and fully can the refractive index measurements be interpreted. Quantitative measurements on cell inclusions of entirely unknown composition are usually of little value: but such measurements can be very informative when made in conjunction with specific histochemical tests.

III. SUITABLE MOUNTING MEDIA FOR THE IMMERSION REFRACTOMETRY OF LIVING CELLS

A. NECESSARY REQUIREMENTS FOR SUITABLE IMMERSION MEDIA

As has already been mentioned (p. 2), liquid mounting media for the immersion refractometry of living cells and organisms must be nontoxic, and must not penetrate the cells nor cause any alteration in cell volume. Their refractive indices must also be capable of being continuously variable over a range covering the refractive indices of the cytoplasm, and of any other optically homogeneous regions in living cells and organisms that are adjacent to their surface, and therefore accessible to measurement by immersion refractometry. Such variations can only be achieved by mixing two substances of different refractive indices in varying proportions—either two miscible liquids or a solid dissolved in
a liquid in varying concentrations. To match the refractive indices of every kind of viable cell and micro organism, the refractive indices of these media should be continuously variable over a range extending from little above that of water (1.333 at room temperature) up to values approaching those of dried proteins (e.g. c. 1.540 for some bacterial spores), and no single mixture of suitable substances is capable of compassing the whole of this range. Mixtures of some animal and vegetable fats and oils are suitable for a restricted part of the higher end of this range, and are sometimes helpful for the measuring of the refractive indices of bacterial and fungal spores; but the greater majority of cells, bacterial vegetative cells and protozoa have cytoplasmic refractive indices lower than 1.420, and can be measured in aqueous solutions of suitable solid substances.

A very full account of the necessary properties of such solutions and of the various substances tried by Dr. Barer, the present author, and others is given by Barer and Joseph (1955a): but the most notable thing is that very few substances appear to fulfil in all the exacting conditions mentioned above. Quite a number of reputedly non-toxic manufactured preparations containing molecules that are undoubtedly large enough to be incapable of passing through normally constituted cell membranes in life, such as peptones, dextran, polyvinyl-pyrollidone, and some polyglucose preparations, appear to penetrate almost all living cells either immediately or only a short time after the cells are mounted in them; and it is by no means always clear why this happens. In all probability, in most of these cases, the preparations in question contain traces of toxic substances that have a lytic effect on cell membranes when the solutions are of sufficient concentration for their refractive indices to exceed that of the cytoplasm of the cells being measured.

The present account need only be concerned with the substances that form solutions in which a large variety of different cells appears to remain in a completely viable condition for long periods in a wide range of concentrations: and, although there are many preparations of proteins and other non-toxic substances with large molecules that need to be tested, several have now been exhaustively investigated and found to fulfil these conditions satisfactorily.

B. Bovine Plasma Albumin Immersion Media

Of the successful immersion media made from aqueous solutions of solids, the one that has been most extensively used in the last seven years, by Dr. Barer and his colleagues, the present writer and a number of other workers (e.g. Mitchison, Passano and Smith, 1956; Allen, 1958;
King and Roe, 1958), is bovine plasma albumin, fraction V, manufactured by the Armour Laboratories, Kankakee, Illinois, U.S.A. (and also obtainable from the Armour Laboratories, Eastbourne, England). This dissolves equally readily in distilled water or saline to form solutions of concentrations up to about 50%, w/v, or a refractive index of 1·424. The following remarks apply particularly to these bovine plasma albumin solutions, but are, for the most part, just as true when solutions of other substances are being used.

1. Adjustment of the Tonicity of the Immersion Media

For measuring the refractive indices of fresh-water Protozoa, and organisms such as fungi and bacteria that do not appear to shrink or swell in solutions of quite widely different tonicity, the fraction V powder can be dissolved in distilled water. For the refractometry of animal tissue cells, however, it is necessary to make the solutions isotonic with the body fluid of the animal in question, in order that their volume should remain unchanged, and this means that the powder must be dissolved in a solution of salt of the right concentration.

Tonicity has been defined succinctly by Barer and Joseph (1955a) in the following manner: “Two solutions are said to be isotonic for a given type of cell if (i) they are compatible with life, and (ii) the cell volume is the same in each solution.” The concentrations of saline solutions generally accepted as being isotonic with the tissue fluids of various animals are usually based on determinations of the ionic content of the animal’s blood or lymph, but it is important to point out that for many animals this is entirely unknown, and it is wrong to assume that the tonicity of the fluids of closely related phylogenetic groups of animals are necessarily the same. If the tonicity of the body fluids of a particular animal is unknown, it is best, if it can be done, to compare the size of spherical cells from the animal in question in that animal’s blood or the tissue fluid in immediate contact with the cells, and in salt solutions of varying concentrations until one is found in which no alteration of cell size is apparent.

This method was used by the present writer in 1952 to determine the concentration of saline necessary as a solvent for bovine plasma albumin to produce solutions isotonic for various tissue fluids; and as it is a method that can be recommended for adjusting the tonicity of any new immersion medium that may be tried, it will be described here in detail.

The spherical primary spermatocytes from the ovo-testis of the snail, *Helix aspersa*, were used, although other spherical cells that show little size variation in the population, such as spermatocytes from the testis of *Locusta*, would be equally suitable. The tonicity of the blood of *Helix aspersa* is generally stated to be equivalent to that of a 0·7% solution of
sodium chloride, and the sizes of the cells in this and in the uncontaminated blood of the snail are the same (Ross, 1953). The diameters of 50 primary spermatocytes (with the cover-slip supported so as to ensure that no cells were compressed), were measured mounted in this 0·7% NaCl solution, and also in lower concentrations down to 0·1% NaCl; and

![Diagram](image)

**Fig. 2.** Histograms showing size distributions of living primary spermatocytes of *Helix aspersa* in hypotonic, isotonic and hypertonic sodium chloride solutions, and in hypotonic and isotonic saline/protein solutions of different concentrations. Ordinates: number of cells. Abscissae: cell diameters. Fifty cells measured in each preparation.

the size distributions of the cells measured are shown as histograms in Fig. 2. The vertical dotted line in each histogram represents the modal value for the diameter of these cells in isotonic 0·7% NaCl (just under 19 μ). It will be seen that considerable swelling occurs in NaCl solutions of 0·2% and below. Similar cells were then mounted in a 20% solution

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**Bovine plasma solutions**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Solution Description</th>
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<tbody>
<tr>
<td>0·1%</td>
<td>20% protein in Dist. H₂O</td>
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<tr>
<td>0·15%</td>
<td>20% Protein in 0·5% NaCl (medium a)</td>
</tr>
<tr>
<td>0·2%</td>
<td>20% Protein in 0·6% NaCl (medium b)</td>
</tr>
<tr>
<td>0·3%</td>
<td>10% Protein medium a diluted with 0·7% NaCl</td>
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<tr>
<td>0·4%</td>
<td>10% Protein medium b diluted with 0·7% NaCl</td>
</tr>
<tr>
<td>0·7% (isotonic)</td>
<td>10% Protein medium a diluted with 0·7% NaCl</td>
</tr>
<tr>
<td>0·9%</td>
<td>10% Protein medium b diluted with 0·7% NaCl</td>
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**NaCl solutions**

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<td>0·1%</td>
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<tr>
<td>0·9%</td>
<td>10% Protein medium b diluted with 0·7% NaCl</td>
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**Dist. H₂O**

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<tr>
<td>0·9%</td>
<td>10% Protein medium b diluted with 0·7% NaCl</td>
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**SOL.**

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(w/v) bovine plasma albumin fraction V powder dissolved in distilled water, and as the dry powder contains only a little free salt (between 0.1% and 1% according to the maker's specifications) such a solution might be expected to be hypotonic for the cells in question. The diameter of the cells in this solution were measured as before, and their size distributions compared with those in the hypotonic solutions already measured.

It will be seen from Fig. 2 that the amount of swelling of the cell population indicated that a 20% solution of the bovine plasma albumin in distilled water had a tonicity between that of a 0.2% and a 0.1% NaCl solution, and almost exactly equivalent to a 0.15% NaCl solution. This meant that, in order to make up a 20% solution of the bovine plasma albumin isotonic with the cells in question, it was necessary to dissolve the powder in a NaCl solution with a concentration of between 0.5% and 0.6%. Figure 2 also shows that the size distributions of the cells in 20% solutions of bovine plasma albumin, fraction V dissolved in 0.5% and 0.6% NaCl approximated extremely closely to those in (isotonic) 0.7% NaCl, and these solutions can therefore all be regarded as isotonic. As might be expected, the size distributions of the cells in 10% solutions of the above saline/protein media diluted with 0.7% NaCl were the same again and these solutions also were isotonic (see Fig. 2).

The salt content of the Armours bovine plasma albumin, fraction V, varies very little in individual batches of the product, and the foregoing experiments provide the data necessary for making up solutions of any required tonicity. One simply needs to assume, for the purposes of tonicity adjustment, that the dry powder contains approximately 0.75% of salt. Thus, to make up a 20% solution of the powder isotonic with mammalian blood and body fluids, usually assumed to be equivalent to that of a 0.9% NaCl solution, one dissolves the powder in 0.7% NaCl; and to make a 40% solution of the same tonicity one dissolves the powder in 0.5% NaCl. Isotonic dilutions of these media to any required refractive index, can then be made by adding (in this case) 0.9% NaCl. It is of interest to find that the estimate of 0.75% of salt in the dry powder based on the experiments described above, is in good agreement with the measurement of depression of freezing point made by Dick (1954) (quoted by Barer and Joseph 1955a), which showed that a 10% solution of the powder in distilled water has a tonicity equivalent to that of a 0.08% sodium chloride solution.

Cell measurements of the kind described above are strongly to be recommended for adjusting the tonicity of any new immersion media that may be tried for the refractometry of living cells, as the technique of measuring 50–100 cell diameters with a micrometer eyepiece is not as lengthy or tedious as might appear. It is usually only necessary to make
such measurements on 3 to 5 such suspensions in order to determine the
equivalent tonicity of the substances investigated.

Most cells stay alive and apparently unaffected in the simple solution
of bovine plasma albumin and sodium chloride described above, except
that it is highly advisable to add a trace amount of calcium ions to the salt
solutions, since their presence seems to be essential for the proper meta­
bolism of the cell membrane. 0.02 c.c. of a 10% CaCl$_2$
solution added to
100 c.c of NaCl solution is adequate for the purpose; and this has been
done in all the experiments described here.

2. Adjustment of the pH of the Immersion Media

Solutions of Armour's bovine plasma albumin in distilled water and
in the simple saline solutions described above are all markedly acid,
having a pH of about 5; and while a large number of cells seem to be
unaffected by this acidity, it is often desirable to adjust the pH of the
medium to approximate more closely to that of the body fluid of the
animal from which the cells have been taken. This is necessary, for
example, for the refractometry of mammalian muscle fibres which in acid
media usually go into a state of tonic super-contraction (Ross and
Casselman, 1960). This can be done by dialysing the protein solutions
against a suitable saline containing a phosphate buffer (Barer and Joseph,
1955a), but a simpler and no less effective way, if high concentrations of
protein are not required, is to use isotonic sodium bicarbonate as a dilu­
ting medium. For example, one can make up a 40% solution of bovine
plasma albumin fraction V suitable for mammalian material by dis­
solving the powder in 0.5% NaCl (plus a trace of CaCl$_2$) in the manner
described above, and dilute it with 1.3% NaHCO$_3$; solutions of 25% and
below have a pH of between 6.8 and 7.2, the protein itself acting in some
measure as a buffer. The pH of the dilution required for refractometry can
be measured by a meter.

3. Practical Details of Making and Storing the Solutions

Solutions of Armour's bovine plasma albumin are best made by add­
ing the powder in small quantities to the water or saline in a small beaker
or flat-bottomed specimen tube and stirring at each addition with a glass
rod; and it is easiest to use a refractometer to determine when the re­
quired concentration is attained. Solutions of concentrations higher than
30% w/v are very viscous and froth considerably as the powder goes into
solution and, although this may result in some of the protein becoming
denatured, this does not appear to have any adverse effect on the solu­
tion as immersion media. It does mean, however, that these concentrated
solutions need to stand for an hour or more before becoming free of air
bubbles; and if they are required immediately it is advisable to centrifuge them. The solutions can be stored for more than a week in small corked specimen tubes, if they are placed in a refrigerator at 0° to 5°C when not in use to retard the growth of any contaminating organisms. It is advisable to check their refractive indices if they have not been used for several days, as evaporation and condensation on the side of the specimen tube sometimes occurs. These protein solutions are, of course, ideal culture media for some fungi and bacteria, and the chances of accidental contamination by spores of these organisms vary greatly with laboratory conditions. However carefully the glassware itself may be cleaned, spores are always liable to fall into the solution from the air while it is being dissolved; and although the presence of small amounts of fungal mycelia and bacteria in the media often appears to leave other cells mounted in them unaffected, contaminated solutions should not be used for refractometry. It is, therefore, advisable not to make up more solution at one time that one needs for a few days experimental work and, if this is stored in a refrigerator, special sterilization of glassware is not necessary. In some air-conditioned laboratories solutions so stored and opened only occasionally will remain clear and free of organisms for many weeks, but this is unusual. Detergents such as "teepol" should never be used for cleaning slides or glassware since even traces of these have a powerful lytic action on living cells and can give rise to very misleading results.

4. The Refractometry of the Immersion Media

An ordinary Abbé refractometer is very suitable for measuring the refractive indices of the immersion media, and is capable of measuring liquids with a wide range of different refractive indices very accurately. A small "pocket" refractometer working on the same principle but covering a more restricted range (1.333 to 1.420) is manufactured by Messrs. Bellingham & Stanley of Hornsey Rise, London. This instrument is relatively inexpensive (about £15), and is quite accurate enough for biological purposes since it measures refractive indices accurately to the nearest 0.0005. It can be obtained either directly calibrated in refractive indices, or (more usually) in per cent sucrose (g per 100 g of solution), with a conversion table into refractive indices which can conveniently be plotted on a graph similar to that in Fig. 1 (page 4). They are also capable of measuring the refractive indices of very small drops of fluid: about 0.001 c.c. or less.

All commonly used refractometers have built-in yellow filters with a transmission spectrum equivalent to the mean of the two sodium lines (589 mµ) and are calibrated for this wavelength. As phase change meas-
urements with the interference microscope are usually made in mercury green light with a wavelength of c. 540 mμ, it has been suggested that this could constitute a source of error. Bennett et al., (1958), however, have recently investigated this, and have concluded that for bovine plasma albumin solutions with refractive indices between 1.334 and 1.420, the error in refractive index measurement will not exceed 0.001 even in the highest concentrations. Consequently for practical purposes of immersion refractometry of living cells this error can normally be ignored; but it may have to be taken into account if other immersion media with higher refractive indices and different dispersions are used.

The temperature in most laboratories in temperate climates seldom fluctuates by more than ±5° from 20°C, and the fluctuation will not affect refractive index measurements by more than 0.001 when solutions with refractive indices lower than 1.420 are being measured. Consequently it is seldom necessary to correct for this, unless a warm stage is being used.

The present writer has found that a drop of a fairly dilute suspension of cells in a bovine plasma albumin solution, sufficient to include up to about ten separate cells in a single microscope field, when a 2 mm objective and a ×10 eyepiece are used, can be placed in a refractometer, and will give a refractive index reading that is indistinguishable from that given by the mounting medium alone. This is extremely useful because it means that two drops of the suspension can be taken from a pipette in quick succession and placed one in the refractometer and one on a slide, and this prevents any errors due to mixing or evaporation.

5. Preparation of Specimens

If a drop of cell suspension in a solution of low refractive index (e.g. saline) is added to a protein solution of higher refractive index, the refractive index of the mixture will be slightly lower than that of the original protein solutions; and Barer and Joseph (1955a), have discussed this dilution error in some detail. Normally, however, it is convenient to add only a very small drop of the suspension to an excess of the protein, and if the volume of the added suspension is only 1% of that of the protein solution, or less, the error is negligible. Even when very concentrated protein solutions, e.g. 40%, are used, 0.01 c.c. of a cell suspension containing, say, 90% by volume of fluid of a refractive index equal to water, added to 1 c.c. of the protein solution will lower the refractive index of the resulting mixture by less than 0.0005; and the error will be less than this if lower concentrations are used.

The length of time that cells may stay alive in these protein media is conditioned more frequently by the way in which the preparation is mounted than on the presence of the mounting medium itself. The most
usual way to make preparations for examination is to cover them with a cover-slip, supporting it if necessary to prevent large cells from being squashed, although the presence of tissue debris is usually sufficient to prevent this. The protein right at the edge of the cover-slip in contact with the air soon dries to form a very thin crust, and this prevents any further evaporation of fluid for many hours and supports the edges of the cover-slip so as to prevent further squashing. It also, unfortunately, acts as an effective barrier to the diffusion of oxygen and CO₂, so that after about an hour the cells often deteriorate. If, however, the preparation is ringed round with some immiscible liquid, such as liquid paraffin, immediately after it is made, and while the protein at the edge of the cover-slip is still wet, oxygen and CO₂ can readily diffuse through the two liquids. Joseph (1954) has observed cells dividing in protein media for as long as 3 days when mounted in this manner.

6. Evidence for the Viability of the Immersed Cells

The evidence for the continued viability of cells mounted in the saline/protein media described above has been discussed at some length by Barer and Joseph (1955a). Briefly, apart from the fact that the cells remain the same size and show no obvious morphological changes, this is based on the continued mobility of motile cells such as amoebocytes, spermatozoa, ciliated epithelia and of motile protozoa in these media (although often at a decreased rate in the more viscous high concentrations of proteins), the continued growth and division of cells of bacteria and fungi, and the fact that animal tissue cells also may be observed undergoing normal divisions in these media. The latter, which provides the most striking evidence that the cells are not adversely affected, was observed by the present writer in 1952 in the course of his study of the changes of refractive index of the cytoplasm of the dividing spermatoocytes of Locusta migratoria (Ross, 1954b).

7. The Practical Limitations of Bovine Plasma Albumin Immersion Media

Although Armour’s bovine plasma albumin fraction V is by far the most useful mounting medium so far found for immersion refractometry, there are some cells for which it may not be suitable, particularly those that have cell membranes with peculiar permeability properties, such as cells that imbibe proteins by pinocytosis; and this has not as yet been sufficiently investigated. Allen, in the course of his recent studies of amoeboid movement, by interference microscopy (Allen, 1958), attempted to use bovine plasma albumin as an immersion medium but found it unsatisfactory as it was taken in by pinocytosis (and the present writer
observed, and sketched, but failed to recognize, the funnels found during the same activity in *Amoeba proteus* mounted in a 15% bovine plasma albumin in 1953). It is obvious that protein solutions cannot be used for the refractometry of cells that are capable of actively and rapidly assimilating protein through their membranes if this process is at all a rapid one. The possibility of the protein having serological lytic effects on the cell membranes of certain types of cells is also one that cannot be entirely disregarded.

C. IMMERSION MEDIA OTHER THAN BOVINE PLASMA ALBUMIN

FRACTION V

1. Proteins

The present writer’s experience with media other than Armour’s bovine plasma albumin fraction V is rather limited, but several other workers have used other substances dissolved in saline and found them satisfactory for many kinds of living cells. Of proteins, Dr. Barer and his colleagues found that human plasma albumin, dialysed commercial egg albumin, carboxyhaemoglobin and Armour’s bovine plasma globulin fraction II were satisfactory for all the cell material on which they were tried. The latter forms solutions with a pH close to 7·0, and so no pH adjustment should be necessary in making up its solutions.

Armour’s highly purified microcrystalline bovine plasma albumin is much more expensive than fraction V, and its solutions, which also have a pH of about 5·0, appear to have practically no advantages over the latter as immersion media. A cell measurement test of the kind described above (p. 8) does, however, indicate that it has only half the salt content of fraction V, and this may be useful if fairly concentrated solutions are required for the refractometry of some fresh water Protozoa sensitive to hypertonicity.

2. Non-Proteins

Of non-proteins Barer and Joseph have found that solutions of acacia gum (or “gum arabic”), a polysaccharide with a *MW* of about 200,000, either in its commercially available form or when further purified, were excellent immersion media for many cells; although, very surprisingly, it appeared to penetrate the cell walls of all bacteria, and this could not have been simply an effect of the high concentrations necessary for their refractometry since fungal mycelia in similar concentrations appeared normal. Red blood corpuscles in concentrated solutions also appeared grossly distorted but this might have been due to incorrect adjustment of the tonicity. The solutions have a markedly acid pH, c. 4·0, unless this is adjusted.
Allen in 1958 used a polyglucose product manufactured by Du Pont Nemours, of Wilmington, U.S.A., which seemed to show considerable promise for refractometry, since, in addition to the properties shared by bovine plasma albumin, it appeared not to be taken into Amoebae by pinocytosis. Unfortunately the manufacture of this product has, temporarily at least, been discontinued, and a rather similar polyglucose product; “Fycoll” manufactured by Aktieselskabet Pharmacia of Copenhagen, Denmark, recently investigated by the writer, seems to have a toxic action on living cells and is unsatisfactory.

To sum up, one can say that there is a considerable need for more work to be done in investigating new media that might be useful for the refractometry of certain kinds of living cells, and in understanding why, unaccountably, some substances, that would appear to be suitable, do not work. The rest of this article, however, will be concerned with a very wide variety of cases in which the media now known can be used entirely successfully.

IV. IMMERSION REFRACTOMETRY WITH PHASE CONTRAST MICROSCOPY

A. DESCRIPTION OF THE INTENSITY-MATCHING METHOD FOR MEASURING REFRACTIVE INDICES

1. The Appearance of the Image

The matching method of using a phase-contrast microscope to measure the refractive indices of living cells immersed in media of the same refractive index, was developed in all its essentials by Dr. Barer, the present writer and Mr. S. Joseph (né Tkaczyk) in 1952 (Barer and Ross, 1952; Barer, Ross and Tkaczyk, 1953). It can be used for the refractometry of the cytoplasm of living cells when this is optically homogeneous and relatively free from large granular inclusions, and for peripherally placed organelles of specialized cells such as sperm tails, cilia and pseudopodia. It can also be used for measuring the refractive indices of whole cells that are themselves optically homogeneous, such as enucleate red blood corpuscles and many species of bacteria. It cannot be used for the refractometry of cytoplasmic inclusions, or of other bodies located deeply within cells, unless they happen to have the same refractive index as the surrounding cytoplasm.

It is dependent on the fact that when any of these homogeneous regions of living cells are surrounded by a medium with a refractive index equal to their own, there is no optical path difference, or phase
change, in the light passing through them and the adjacent medium, and, under a phase contrast microscope, they will exactly match the background field in relative brightness, or intensity, and will therefore be practically invisible. When the refractive indices of the medium and object are only a little different, however, the latter will appear appreciably brighter or darker than the background.

Most commercially marketed phase contrast objectives have 90° positive phase plates, which means they are constructed so that the diffracted light is retarded one-quarter of a wavelength behind the directly transmitted light, and, if these are used, a homogeneous object will appear darker than the background if its refractive index is slightly greater than the mounting medium, and brighter than the background, or “reversed”, if its refractive index is slightly less than the background. Negative phase plates, however, in which the diffracted light is advanced relative to the direct light, are also sometimes used; and with these the opposite is true. Thus, if one knows the characteristics of the phase plate in the objective one is using, one can usually tell at a glance whether the refractive index of the mounting medium is higher or lower than the object being measured.

With ordinary +ve phase contrast objectives, an object which causes a retardation of phase in the light passing through it relative to that passing through the background, through having a higher refractive index than the mounting medium, will appear darker; and one that causes an acceleration in phase, as a result of having a lower refractive index than the mounting medium, will appear bright: although for reasons that will be explained below (p. 22) this is only true when the phase differences involved are smaller than about a third of a wavelength in most cases, or half a wavelength at the most. Fortunately the phase changes produced in the peripheral region of living cells mounted in saline or protein media, are usually appreciably smaller than this.

2. The Accuracy of the Method

All ordinary phase contrast objectives are capable of showing in this way phase differences of as little as 7° or about a fiftieth of a wavelength quite clearly (Oettlé, 1950). As the phase change in light passing through an object, compared to that passing through an adjacent region of the mounting medium, is proportional to the product of the difference between the refractive index of the object and mounting medium and the object’s thickness, and the thickness of homogeneous regions of living cells that are measured by immersion refractometry are seldom less than 5 μ thick, this means that refractive index differences of 0.0018 (equivalent to 1% of cell solids) can be detected without difficulty. Consequently
it is normally true to say that when the cytoplasm of a cell mounted in a suitable medium appears to match the background when examined under a phase contrast microscope, its refractive index must be within 0.0018 of that of the mounting medium. It may be considerably nearer to it than this, although the refractive indices of thinner objects such as bacteria on flagella cannot be measured so accurately. Taking 0.0018 as the mean

Fig. 3.
refraction increment of cell solids, this means that the solid content of the cytoplasm of living cells can usually be measured to the nearest 1%, or more accurately than this if a thicker region is measured.

3. The Method Applied to Single Cells

Figure 3A shows an example of the method applied to the refractometry of cytoplasm. It is a photomicrograph, taken with a 4 mm phase contrast objective, with a 90°, 25% absorbing, positive phase plate, of some spermatocytes, developing spermatids and spermatozoa of *Locusta migratoria*, mounted in an isotonic saline/protein with a refractive index of 1.353 (≈ 10.5% protein). The cytoplasm of some of the spermatocytes (about 15 μ diameter) exactly matches the background field in brightness, which indicates that the refractive index is equal to that of the mounting medium of within 0.001 of this value. Others have bright or "reversed" cytoplasm, indicating that its refractive index is slightly lower than the cytoplasm and the light passing through it is advanced in phase. The sperm tails and spermatids appear dark indicating that they have higher refractive indices than the mounting medium and the light passing through them is retarded.

4. The Method Applied to Cell Populations

The cytoplasmic refractive index of non-dividing tissue cells usually shows only small individual variations in cells of the same kind, and in these cases it is possible to find a medium in which the cytoplasm of the majority of the cells appears matched. This, however, is certainly not
true of all cells; the individual refractive indices of a sample of normal mammalian red blood corpuscles, for example, seldom vary by less than $0.010$, and the maximum variation in the refractive indices of bacterial populations can be greater than this. In these cases, in all mounting media with refractive indices between the limits of that of the cell population, both bright and dark cells will be visible in addition to those that appear matched. This is shown, for instance, in the sample of human red blood corpuscles (from a patient suffering from a mild iron deficiency anaemia), shown in Fig. 3B, which are suspended in an isotonic saline/protein solution with a refractive index of $1.381$ ($= 25\% \text{Hb}$).

In these cases it is of interest to know the upper and lower limits of the refractive indices of the cell population, the mean refractive index of the population, and whether or not the variations of refractive index of the population approximates to that of a statistician's "normal distribution". As the matched cells in any one medium will be relatively few in number and sometimes hard to see at all, this is best done by making up a series of solutions of closely spaced refractive indices covering the likely limits of variation of the population, and making counts of the relative numbers of bright and dark cells in each. The upper and lower refractive indices of the population can then be defined within narrow limits, and the mean refractive index of the population will be the one in which $50\%$ of dark and bright cells occur. The percentage of bright or dark cells in the media of different refractive indices can be plotted graphically as "integrated distribution curves" and these will be symmetrically S-shaped, like that in Fig. 4A, if the refractive index distribution is such that the proportion of matched cells similarly plotted would take the form of a "normal" bell-shaped curve (see Barer and Joseph, 1955b).

The presence of two discontinuous populations is indicated when the percentage of dark and bright cells remains unchanged over an appreciable range of refractive indices. A striking instance of this was found by the present writer and Mr. Joseph in a specimen of blood supplied by Dr. J. B. Howie from the Radcliffe Infirmary, Oxford, in 1952. This was from a patient with pernicious anaemia who had been treated for 7 days with vitamin $B_{12}$, and the distribution of bright and dark cells visible in media of various refractive indices is shown in Fig. 4B. Approximately half the population had refractive indices between $1.383$ and $1.396$, and the remainder had much lower refractive indices all between $1.374$ and $1.376$. In all solutions with refractive indices between $1.376$ and $1.383$, the proportion of bright and dark cells remained unchanged; in media with refractive indices in the middle of this range the less dense, bright, cells of lower refractive indices tended to float upwards to the plane of the cover-slip, leaving the denser dark cells in the plane of the slide.
It is quite possible that the population with the lower refractive index, all of which had a haemoglobin content lower than normal, were reticulocytes formed under the stimulus of the vitamin $B_{12}$ and newly introduced into the circulation.

**Fig. 4.** Curves showing the distribution of the corpuscular refractive indices in two samples of human red blood cells. Ordinates: percentages of "positive" corpuscles (showing up dark under ordinary positive phase contrast objectives) that have lower refractive indices than the mounting medium. Abscissae: refractive indices of the mounting media, and the equivalent w/v concentration of Hb, assuming a refraction increment ($\alpha$) of 0.0019. A. A normal blood sample, showing a single continuous population with a "normal" distribution of corpuscular refractive indices. B. A case of pernicious anaemia after seven days treatment with vitamin $B_{12}$, showing evidence of two separate populations. (The extreme limits of the refractive indices of these populations are represented by arrowheads joined by horizontal dotted lines.)

The counts necessary for investigations of this kind are not as tedious as many kinds of routine haematological counting techniques, and are easily performed by one person with the aid of a differential cell counter. The work necessary to obtain the data for plotting curves of the kind shown in Fig. 4 can be done, with practice, in considerably less than an hour.
B. The Interpretation of the Phase Contrast Image

1. The Relationship between Intensity and Phase Change

The great advantage of phase contrast microscopy for immersion refractometry lies in the way in which small phase changes in the light passing through objects, caused by small departures of their refractive indices from that of the immersion medium, show up so strikingly as differences of brightness or intensity: but for objects giving relatively large phase changes, these differences of intensity may be very misleading if not interpreted correctly.

For any phase contrast objective the relationship between the phase change caused by an object and its intensity relative to that of the background illumination is not a linear one, but in the form of a curve, the steepness of which is dependent on the nature and absorption of the phase plate. Figure 5 shows three such curves for ordinary 90° positive phase plates with absorptions of zero, 25% and 75% respectively. The ordinate represents the brightness of an object as multiples of that of the background illumination, which is taken as unity, and the abscissa the phase change given by the object (in degrees).

Fig. 5. Curves showing the relationship between the phase-change and intensity of objects seen with ordinary 90° positive phase contrast objectives with phase plates having absorptions of zero, 25% and 75%. Ordinate: intensity of object relative to that of the background. Abscissa: phase-change given by the object (in degrees).
phase change, positive or negative, in the light passing through them. It will be seen that for all these phase plates, an object giving a zero phase change will match the background in intensity, and although the full extent of the curve is not shown here (see Fig. 5 in Barer, 1952b), all phase-advancing objects (giving negative phase changes of up to three-quarters of a wavelength) will appear brighter than the background.

Objects giving positive phase changes, however, only appear dark over a much lower range of phase changes. When a non-absorbing phase plate is used, a phase retarding object will only appear darker than the background when it gives a phase change of below quarter of a wavelength (90°), and will be maximally dark if it gives a phase change of one-eighth of a wavelength (45°). If it gives a phase retardation of exactly 90°, it will match the background intensity in a manner similar to that of an object giving a zero phase change; but the “false match point” can be distinguished from the match of a zero phase change by the fact that, if the phase change is further increased by mounting the object in a medium of lower refractive index, it will appear brighter than the background, and conversely it will appear darker in a medium of higher refractive index. The corresponding values for the “false match point” with the 25% and 75% absorbing phase plate are 81° 48' and 53° 12' respectively; and the phase retardations giving a maximally dark appearance will have half these values (40° 54' and 26° 36') in each case. The phase change, \( \phi \), indicated by the “false match point” for 90° + ve phase plates of any absorption is given by the formula:

\[
\phi = 2 \times \tan^{-1} \left( \frac{1}{\sqrt{\frac{1}{100 - A}^{100}}} \right)
\]

Where \( A \) equals the per cent absorption of the phase plate.

Most commercially marketed phase plates have absorptions of about 75%, and some are made with still higher absorptions. Because the higher absorbing phase plates give steeper curves they are slightly superior for the critical determination of a refractive index match, but in practice there is seldom any need to measure refractive indices more critically than to the nearest 0.001 as the biological variation of the material is almost always greater than this, and a low absorption phase plate such as the 25% absorbing plate made by Messrs. Watson is perfectly adequate for this.

If interference microscopes had not been invented, these curves would have attracted more attention since they provide data by which phase retardations through objects could be estimated fairly accurately by densitometric techniques, providing one knows on what part of the
curve an observed intensity level lies. This can easily be determined by slightly altering the refractive index of the mounting medium. As it is, they provide an approximate indication of phase change that may at times be most valuable. Figure 6B shows the spores *Bacillus cereus* mounted in water under a 2 mm, 25% absorbing, 90° positive phase con-

![Image](image_url)

**Fig. 6.** A. Vegetative cells of *Bacillus cereus* mounted in dist. H₂O, under a 2 mm phase contrast objective with a 90°, positive, 25% absorbing phase plate. The bacilli appear dark, indicating that the phase change in the light passing through them is appreciably less than 82°. B. Spores of the same organism, similarly mounted, under the same phase contrast objective. The centres of the spores show up bright, indicating that the phase change through this region is appreciably more than 82°. As the diameter of the spores is less than that of the vegetative cells, this means that their refractive indices must be considerably higher.

The phase contrast image can also be misinterpreted owing to the optical artifacts introduced by the incomplete separation of the direct

2. The "Halo" and "Shading Off" Optical Artifacts

The phase contrast image can also be misinterpreted owing to the optical artifacts introduced by the incomplete separation of the direct
and diffracted light, and this also can be of importance in refractometry. The incomplete separation is inherent in the phase contrast system and Fig. 7A shows how this is brought about. The direct light (shown by solid lines) from the annular light form is focused by the condenser to pass through the plane of the specimens and then pass only through the special annular region of the phase plate, which, in the case of the positive phase plate shown here, is slightly thinner than the rest, so that it is accelerated. If a specimen is now placed in the field, some of the light is diffracted and this (shown by dotted lines) is scattered in all directions.

Fig. 7. A. Diagram of a typical phase contrast system showing how the light passing directly through the specimen and the light diffracted by the specimen is incompletely separated. Some of the diffracted light (represented by heavy dotted lines) passes through the phase ring in the phase plate along with the direct light (represented by hard lines). B. The image of a retarding object of uniform refractive index and thickness (such as that shown in cross section in A) as it would appear if the direct and diffracted light could be completely separated: and as it does, in fact, appear under most kinds of interference objectives. C. The actual appearance of the image under the phase contrast system, showing the "halo" and "shading off" optical artifacts induced by the incomplete separation of the direct and diffracted light.

Nearly all of this light entering the objective passes through the thicker non-annular portion of the phase plate, but inevitably some diffracted light passes through the annular regions too along with the direct light (heavy dotted lines). This produces an unresolved reversed image of the object superimposed on its main image; this is responsible for the two well-known optical artifacts of phase contrast images—the "halo effect" and what might be described as the "shading-off effect". (For details see Zernike, 1942.)

If they were absent the image of an object of uniform thickness and refractive index, such as that illustrated in section in Fig. 7A, would
appear as in Fig. 7B, with no surrounding halo and with a uniform intensity over its whole area. Actually it appears very much as in Fig. 7C, with a strong surrounding halo and with the intensity of the inner regions shading off to become the same as that of the background. This is one reason why, in refractometry, the cytoplasm near the centre of a cell must never be assumed to have the same refractive index as the mounting medium when it matches the background if it is surrounded by non-matching areas nearer the edge.

The "halo effect" can also lead to serious confusion in cells that contain numerous highly refractile granules or other bodies giving rise to abrupt phase gradients in the cytoplasm. The halo in the immediate vicinity of these will not only mask the match of the cytoplasm if it is of the same refractive index as the mounting medium, but will also completely obscure adjacent morphological details. For the same reason, phase-contrast microscopy cannot be used for the immersion refractometry of striated muscle fibres, as the spacing of the striations is such that the halo from the A bands obscures the match of the I bands and vice versa (Ross and Casselman, 1960).

3. The Advantages of Using Low-absorbing Phase Plates

The "halo" and "shading off" optical artifacts are undoubtedly reduced by making the annular regions of the phase plate and the corresponding regions of the light form as narrow as possible, but there is a limit to what we can do here without making the system excessively difficult to line up. They can, however, also be reduced to a surprising degree by using lower absorption phase plates than is customary, such as the 25% absorbing plates manufactured by Messrs. Watson; the resulting improvement in the resolution of morphological detail, to those unfamiliar with such objectives, is often quite striking. This was found empirically by Baker, Kempson, Thomas and Brunet (Kempson et al., 1948; Baker et al., 1949) when they made very careful and comprehensive tests for the most suitable sizes and absorptions of phase plates in the course of the development of their excellent phase contrast system, now manufactured commercially by Messrs. W. Watson & Sons, Barnet, England.

The overall contrast of images with such low-absorbing objectives is appreciably lower than for those with higher absorbing phase plates, but this is unimportant compared with the fact that the images are a truer representation of what is there. It is very much to be hoped that more manufacturers may be persuaded to make low-absorbing phase objectives.
V. IMMERSION REFRACTOMETRY BY INTERFERENCE MICROSCOPY

A. THE ADVANTAGES OF INTERFERENCE MICROSCOPY FOR IMMERSION REFRACTOMETRY OF LIVING CELLS

Interference microscopes have two main advantages over phase contrast microscopes which greatly extends their scope for making refractive index measurements on living cells; they reduce the optical artifacts and phase changes can be measured.

1. The Reduction of Optical Artifacts

Firstly, the optical artifacts just described, the "halo" and "shading-off" effects that are inevitable with a phase contrast system, are absent, and the image of an object of uniform thickness and refractive index approximates closely to that in Fig. 7B. With some interference systems, for example, the "double focus" objectives used in the Smith interference microscope (manufactured by Messrs. Charles Baker of Holborn, London), there is a "halo" hardly noticeable with small objects, caused by the incomplete separation of the object and reference beams (see Appendix, p. 56), but this is completely eliminated in the "shearing" objectives in the Smith microscope, and is not found with the Dyson interference microscope (manufactured by Messrs. Cooke, Troughton & Simms of York).

It is therefore true to say that, with most cytological material, the intensity of individual cell details in the image give a truer impression of the phase changes in the light through them than with any phase contrast microscope; although the relationship between phase change and intensity in monochromatic light is only linear over a range of about one-quarter of a wavelength (Mitchison, Passano and Smith, 1956), and consequently the more refractile regions of nearly all living cells appear with diminished contrast or even reversed.

It means, however, that phase changes in the vicinity of highly refractile granules and other sharp boundaries are truly indicated to an extent that is never possible with phase contrast. In the immediate vicinity of such boundaries it is true one gets a gradient due to diffraction; but Huxley and Hanson (1957) have shown, from a densitometer trace across the image of a myoglobin crystal, that with a corrected 2 mm objective this diffraction gradient is not more than 0·6 μ wide. This probably means that phase measurements on regions more than 0·3 μ outside the apparent boundary of an object, are not subject to error.
2. The Measurement of Phase Change and Deductions therefrom

The second great advantage of interference microscopes is that they enable the phase changes through the different regions of an object to be measured directly, by passing (either broad or narrow) fringes of the orders of the Newtonian series of interference colours across the field, and observing the displacement in colour of the fringe in the object compared to that of the field in its immediate vicinity. If monochromatic light is used the displacement of the minimum intensity (maximum darkness) of a fringe can be similarly estimated, rather more accurately. Phase changes can be measured to the nearest $\frac{1}{600}$ of a wavelength in this way, and frequently to a greater accuracy than this.

This enables the refractive indices of many regions of living cells mounted in non-matching media, i.e. media with refractive indices different to the cell or region in question, to be measured in a variety of different ways, and also enables their thicknesses to be calculated very critically.

a. Measurement of the Refractive Index and Thickness of Living Cells in Non-matching Media, from Single Phase-change Measurements

Since the phase change in the light passing through an object is proportional to the product of the refractive index of the object relative to that of its surrounding medium and its thickness, this means that, if its thickness is known, the refractive index of an object surrounded by a medium of a different refractive index can be measured. This is given by the formula:

$$n_0 = \left(\frac{\phi \times \lambda}{t}\right) + n_m \quad (4)$$

where $n_0 =$ the refractive index of the object, $n_m =$ the refractive index of the mounting medium, $\phi =$ the phase change (positive or negative) in the light passing through the object, expressed as a fraction of a wavelength, $\lambda =$ the wavelength of light used (most conveniently expressed in $\mu$), and $t =$ the thickness of the object in the direction of the optical axis of the microscope (in $\mu$). (It is frequently more convenient to express $\phi$ as an angle, since the Smith interference microscope reads this directly, and in this case $\phi/360$ should be used in this and the subsequent formulae (5)–(8).

This means that objects with refractive indices too high for them to be measured easily by immersion refractometry, e.g. higher than the most concentrated protein solution obtainable, and also objects that cannot be surrounded by a medium of the same refractive index as its own, such as cytoplasmic inclusions in living cells, can be measured by those means.
Similarly the thickness, $t$, of an object of known refractive index can be measured by applying the formula:

$$t = \phi \times \frac{\lambda}{n_0 - n_m}$$

(5)

the symbols being the same as in formula (4).

b. Barer's "Double Immersion" Method of Measuring the Refractive Index and Thickness of Living Cells

If an object is capable of being immersed successively in two media of different refractive indices and neither its thickness nor its refractive index is known, both can be obtained by an ingenious "double immersion" method devised by Dr. Barer and used by him for measuring the refractive indices and thicknesses of human mouth epithelial cells in 1953. The object is immersed in two media of known refractive index and the phase change in the light passing through it is measured in each case. The refractive index of the object $n_0$ can then be calculated from the formula:

$$n_0 = \frac{\phi_1 n_{m_1} - \phi_2 n_{m_2}}{\phi_1 - \phi_2}$$

(6)

where $n_{m_1}$ = the refractive index of the mounting medium with the lower refractive index, $n_{m_2}$ = the refractive index of the mounting medium with the higher refractive index, $\phi_1$ = the phase change (in wavelengths) given by the object mounted in the medium of lower refractive index ($n_{m_1}$), and $\phi_2$ = the phase change (in wavelengths) given by the object mounted in the medium of higher refractive index ($n_{m_2}$).

The thickness of the object, $t$, is given by the formula:

$$t = \frac{\phi_1 - \phi_2}{n_{m_2} - n_{m_1}} \times \lambda$$

(7)

where $\lambda$ = the wavelength of light used, and the other symbols are as in formula (6).

In practice, it is often extremely difficult to observe the same cell successively in two different mounting media, as it is usually washed out of the field when the medium is being replaced. Even cells such as amoeboocytes, than can adhere to glass surfaces, often undergo local changes in thickness while this is happening (Dick, 1954), so that the method is usually impracticable for single cells unless they are slightly compressed to a constant thickness in a cell compressor.

The method can, however, be applied to cell-populations when the cells themselves are sufficiently optically homogeneous, (e.g. many bacteria
and bacterial spores), by measuring the phase changes in a selected number of different cells in each medium and obtaining values for mean phase change in each case. Provided that the individual variations of cell thickness and refractive index are not very great, and that there is no marked correlation between the two, the values for the mean thickness and mean refractive index of the population obtained in this way can be both accurate and meaningful (Ross and Billing, 1957; Ross, 1957).

c. Ambrose's "Bubble" Method for Measuring the Refractive Index and Thickness of Living Cells

Finally, both the refractive indices and thicknesses of compressed cells can be arrived at by an elegant method first used by Ambrose (1957), when an air bubble, large enough to be in contact with both slide and cover-slip, is introduced in the preparation adjacent to the cells being measured. The phase changes, positive and negative, through the compressed part of the cells and the air bubble (with a refractive index of 1) are measured and their thicknesses, $t$, that are assumed to be equal, are given by the formula:

$$t = \phi_a \times \frac{\lambda}{1 - n_m}$$

where $\phi_a$ = the phase change measured through the air bubble (a negative value), $n_m$ = the refractive index of the mounting medium and $\lambda$ = the wavelength of light used.

The refractive index of the compressed part of a cell adjacent to the bubble, $n_c$, can be calculated from the formula:

$$n_c = \frac{n_m(\phi_a - \phi_c) + \phi_c}{\phi_a}$$

where $\phi_c$ is the phase change measured in the compressed region of the cell and the other symbols are as in formula (8).

This, of course, can only be done on compressed regions of a cell that happen to be optically homogeneous, and care must be taken to see that the air bubble is not actually in contact with the cell, as this can have very deleterious effects (Dick, 1954). An elaborate cell compressor is unnecessary since the mounting medium is not replaced, and this can be of any convenient refractive index. This method was used by Davies (1959) and by Davies and Thornburg (1959) for measuring the refractive indices of crystalline proteins in their important work in determining their specific refraction increment (see p. 5).
3. The Accuracy of the Interference Microscope for Immersion Refractometry with Matching Media

When a white light source is used, the accuracy with which it is possible to detect small phase changes with the interference microscope as recognizable changes in colour depends on the order and actual colours of the Newtonian series of fringes that are selected for the background colour (or reference area) when the instrument is adjusted to give broad fringes, since the eye is much more sensitive to some colour changes than to others. When colours at the upper end of the first order and the lower end of the second order are selected, phase changes of \( \frac{1}{6} \) of a wavelength can be detected without difficulty: and in direct immersion refractometry it is necessary only to detect these phase changes and not actually to measure them. This means that, even with a white light source, an interference microscope is as accurate as a standard phase contrast microscope for direct immersion refractometry of the kind already described in Section IV, and the refractive indices of homogeneous cell regions more than 5 \( \mu \) thick can be measured accurately to the nearest 0.0018 on approximately 1% of solid. If the interference microscope is used with a monochromatic light source, this accuracy is somewhat increased, since even smaller phase changes will show up as detectable differences in intensity. The accuracy obtainable is comparable to that of the more heavily absorbing 90° positive phase plates in the phase contrast objectives already described, and is more than adequate for most biological purposes.

B. Special Applications of Immersion Refractometry with Interference Microscopy

From the preceding section, it will be seen that the interference microscope is an extremely versatile instrument; it is not only capable of being used in the same way as a phase contrast microscope for direct immersion refractometry, but can also be used for a wide variety of special quantitative cytological studies dependent on immersion refractometry that cannot be done with a phase contrast microscope. Some of these special applications will now be described.

1. The Refractometry of Cytoplasmic Inclusions in Living Cells

The refractive indices of inclusions contained in, and surrounded by, living cytoplasm cannot be measured by direct immersion refractometry either by phase contrast or interference microscopy, since, if the region of such an inclusion should give a zero phase change with respect to the
mounting medium, and thus appear of the same colour or intensity as the background field, the apparent zero phase change will not be due to the inclusion alone but to the inclusion together with the overlying and underlying cytoplasm. The commonest case of this happening is illustrated diagrammatically in Fig. 8A which represents a cell mounted

![Diagram](image)

**Fig. 8.** A. Diagramatic representation of a cell, containing an inclusion with a higher refractive index than that of its cytoplasm, mounted in a medium with a refractive index higher than that of the cytoplasm but lower than that of the inclusion. The light passing through the cell from left to right is advanced in phase while traversing the cytoplasm, and retarded in phase by an equal amount while traversing the inclusion, so that it emerges from the cell in phase with the light which has passed through the mounting medium alone. B. Diagramatic representation of the same cell as in A mounted in a medium with a lower refractive index than the cytoplasm or inclusion (e.g. physiological saline). The light passing through the cell is retarded in phase by amounts proportional to the refractive indices and thicknesses of the structures it traverses. C. Diagramatic representation of the same cell as in A and B mounted in a medium with a refractive index equal to that of the cytoplasm. Only the light passing through the inclusion is retarded in phase. The light traversing the cytoplasm is in phase with the light passing through the mounting medium alone.

in a medium with a higher refractive index than the cytoplasm, containing a phase retarding cell inclusion surrounded by a phase advancing cytoplasm. The phase changes due to cytoplasm and inclusion are equal and of opposite sign, so that the inclusion (but not the cytoplasm) appears matched.
It is not possible, either, to measure the phase change due to a cytoplasmic inclusion with an interference microscope when the refractive index of the surrounding medium is lower than that of the cytoplasm; again because this is modified by the phase change in the cytoplasm. A typical instance of this is shown in Fig. 8B which shows the same cell as in Fig. 8A mounted in a medium of lower refractive index than the cytoplasm on its inclusion (e.g. saline). It shows a phase change of one-quarter of a wavelength in the light traversing the cytoplasm and of one-half a wavelength in the light traversing the inclusion and some of the cytoplasm as well. It is impossible here to determine how much of this phase change is due to the inclusion alone unless both the refractive index and total thickness of the intervening cytoplasm is known. The latter is normally impossible to estimate.

If, however, the cell is immersed in a medium of the same refractive index as the cytoplasm, you get the state of affairs illustrated in Fig. 8C. There no phase change in the light traversing the cytoplasm, and the phase change in the light passing through the inclusion alone can be measured.

Since the thickness of the inclusion in the direction of propagation of the light waves (i.e. in the direction of the optical axis of the microscope) can be assessed fairly accurately if its shape is simple, its refractive index can be calculated from formula (4) above. Under these circumstances only the inclusions are clearly visible under the interference microscope (see Fig. 9B and C), and immersion in a matching medium has been described as a sort of “optical dissection” of the living cell.

This method was first tried out by the present writer in 1953 on a relatively large homogeneous cytoplasmic inclusion ; the spherical nebenkern in the developing spermatid of Locusta migratoria. This is an aggre- gation of all the mitochondria at a certain stage of spermatogenesis, to form a body which ultimately becomes cigar-shaped, but which begins by being a sphere about 5 μ in diameter lying in almost completely clear cytoplasm (Ross, 1954a). At this stage its width, which can be assumed to be equal to its depth in the direction of the optical axis of the microscope, can be measured to the nearest ± 0·2 μ by an eyepiece micrometer scale, and the refractive index calculated for this and the phase retardation measured through the centre (formula (4), p. 28).

As the mitochondria comprising the nebenkern consist predominantly of protein and lipoprotein, a fairly close approximation of the dry solid content can be arrived at by assuming a refraction increment (α) of 0·0018. It was found that the cytoplasm of all the spermatids had a refractive index of 1·354, equivalent to approximately 11% of cell solids, and, in an isotonic saline protein solution of this refractive index, only
The phase-retardation of light, of wavelength 542 μ, passing through the centres of the spherical *nebenkerns* in twenty developing spermatids of *Locusta migratoria* mounted in an isotonic protein medium with a refractive index equal to that of their cytoplasm (= 1.354); the measured diameters of these bodies, and their refractive index and solid content calculated from these measurements.

The nuclei (which were very heterogeneous) and the *nebenkerns* were visible (Fig. 9B and C). Table I (A) shows the refractive indices and percentage solid content based on phase retardation and diameter measurements on the *nebenkerns* in twenty different spermatids so mounted.

One interesting thing that can be seen is that the refractive indices, and hence the solid concentrations, in the *nebenkerns* seem quite clearly to be inversely related to their measured diameter. This, however, is entirely to be expected with a body formed by the coming together of all the cytoplasmic mitochondria, since such a body would be expected to become denser as its diameter decreases.

Since the phase changes can be measured accurately to \( \frac{1}{50} \) of wavelength or more accurately, the accuracy with which refractive indices can

---

**TABLE I (A)**

<table>
<thead>
<tr>
<th>Diameter of <em>Nebenkern</em> in μ</th>
<th>Phase-change retardation (in wavelengths)</th>
<th>Refractive index</th>
<th>Approx. per cent solid (assuming ( a = 0.0018 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t )</td>
<td>( \phi )</td>
<td>( n )</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>0.184</td>
<td>1.3771</td>
<td>24.0</td>
</tr>
<tr>
<td>4.2</td>
<td>0.216</td>
<td>1.3814</td>
<td>26.0</td>
</tr>
<tr>
<td>4.2</td>
<td>0.210</td>
<td>1.3807</td>
<td>26.0</td>
</tr>
<tr>
<td>4.2</td>
<td>0.167</td>
<td>1.3750</td>
<td>22.5</td>
</tr>
<tr>
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<td>0.189</td>
<td>1.3778</td>
<td>24.0</td>
</tr>
<tr>
<td>4.7</td>
<td>0.189</td>
<td>1.3751</td>
<td>22.5</td>
</tr>
<tr>
<td>4.7</td>
<td>0.200</td>
<td>1.3764</td>
<td>23.5</td>
</tr>
<tr>
<td>4.7</td>
<td>0.200</td>
<td>1.3764</td>
<td>23.5</td>
</tr>
<tr>
<td>4.7</td>
<td>0.205</td>
<td>1.3770</td>
<td>24.0</td>
</tr>
<tr>
<td>4.7</td>
<td>0.184</td>
<td>1.3745</td>
<td>22.0</td>
</tr>
<tr>
<td>4.7</td>
<td>0.216</td>
<td>1.3783</td>
<td>24.5</td>
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<tr>
<td>4.7</td>
<td>0.161</td>
<td>1.3720</td>
<td>21.0</td>
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<tr>
<td>4.7</td>
<td>0.178</td>
<td>1.3739</td>
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<td>5.3</td>
<td>0.189</td>
<td>1.3730</td>
<td>21.5</td>
</tr>
<tr>
<td>5.3</td>
<td>0.194</td>
<td>1.3736</td>
<td>22.0</td>
</tr>
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<td>5.3</td>
<td>0.210</td>
<td>1.3753</td>
<td>23.0</td>
</tr>
<tr>
<td>5.3</td>
<td>0.178</td>
<td>1.3719</td>
<td>21.0</td>
</tr>
<tr>
<td>5.3</td>
<td>0.200</td>
<td>1.3742</td>
<td>22.0</td>
</tr>
</tbody>
</table>
be measured in this way is limited not by the accuracy of phase change measurements, but by the accuracy with which the thickness of the object can be measured. With an object as large as these *nebenkerns* the measurement of their refractive index is unlikely to have been wrong by more than $\pm 0.004$ or about $2\%$ of solid concentration, but with the smaller cytoplasmic inclusions more commonly found in living cells the accuracy will be less.

The photomicrographs in Fig. 9C and D illustrate the technique. They show a group of the spermatids mounted in an isotonic saline protein medium with a refractive index of $1.354$ at two different background intensities. Only the nuclei and *nebenkerns* show a phase retardation relative to the background medium. This state of affairs is the same as is illustrated diagrammatically in Fig. 8C and in Fig. 10B. Fig. 9A (comparable to Fig. 8B and Fig. 10A) shows similar cells mounted in saline. The phase retardation through cytoplasm and inclusions are relatively big, and this tends to obscure cell detail. The *nebenkerns*, however, are visible. Fig. 9B is of a group of spermatids mounted in a medium with a slightly higher refractive index than cytoplasm. Under these conditions cytoplasm and *nebenkerns* show up with maximum contrast, because the light through the cytoplasm is advanced in phase and the light through the cytoplasm and *nebenkern* is retarded. Consequently for morphological studies with the interference microscope this is the best kind of mounting medium: and this is usually true with phase contrast also (see Fig. 3A).

In certain special circumstances, it may not be necessary to mount the cells in a medium with the same refractive index as the cytoplasm in order to measure the refractive index of these inclusions. This is when a number of similar cells, with abundant clear cytoplasm, are in contact with one another, and are slightly squashed so that the mounting medium is virtually excluded, as in Fig. 10C. From Fig. 10 it will be clear that the phase change through the inclusion relative to the cytoplasm in a preparation of this kind will be equal to the phase change through the inclusion relative to the cytoplasm and mounting medium in an isolated cell mounted in a medium with refractive indices the same as the cytoplasm (Fig. 10B). Consequently if the refractive index of the cytoplasm is known (and this could conveniently be arrived at by Ambrose's "bubble" method if there is an air bubble at the edge of a squashed group of cells), the refractive index of an inclusion can be found. The photomicrographs in Fig. 9E and F (which are taken with an interference microscope at almost exactly the same background settings as Fig. 9C and D) show the state of affairs in a group of squashed *Locusta* spermatids containing *nebenkerns*; Table I (B) shows phase change and thickness measurements.
Fig. 9.
through five nichtenkerns in a similar saline-mounted squashed preparation of spermatids, and these refractive indices and solid concentrations

<table>
<thead>
<tr>
<th>Diameter of Nebenkern in μ</th>
<th>Phase-change retardation (in wavelengths)</th>
<th>Refractive index</th>
<th>Approx. per cent solid (assuming a = 0.0018)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>0.147</td>
<td>1.3738</td>
<td>22.0</td>
</tr>
<tr>
<td>4.3</td>
<td>0.178</td>
<td>1.3781</td>
<td>24.5</td>
</tr>
<tr>
<td>4.7</td>
<td>0.139</td>
<td>1.3703</td>
<td>20.0</td>
</tr>
<tr>
<td>4.7</td>
<td>0.158</td>
<td>1.3728</td>
<td>21.0</td>
</tr>
<tr>
<td>4.7</td>
<td>0.200</td>
<td>1.3780</td>
<td>24.5</td>
</tr>
</tbody>
</table>

The phase-retardation of light, of wavelength 542 mμ, passing through the centres of spherical nichtenkerns in five developing spermatids of *Locusta migratoria* mounted in saline that were closely packed together and slightly compressed; the measured diameters of these bodies, and their refractive indices and solid content calculated from these measurements, assuming the refractive index of the cytoplasm to be 1.354.

Fig. 9. Photomicrographs, taken in mercury-green light with a Smith interference microscope with a 2 mm "double focus" objective, of the living spermatids of *Locusta migratoria*, containing nichtenkerns. A. Two isolated spermatids mounted in 0.9% NaCl; with the analyser goniometer set at 110°. Internal cell-structures are obscured. B. A group of similar isolated spermatids mounted in isotonic bovine plasma albumin with a refractive index (1.366) rather higher than that of their cytoplasm; with the analyser set at 112°. The cytoplasm looks paler than the background, indicating (at this analyser setting) an acceleration in phase. The internal cell structures show up plainly, and are dark, indicating a retardation in phase. C. An isolated group of spermatids mounted in an isotonic bovine plasma albumin solution of the same refractive index (1.354) as the cytoplasm; with the analyser goniometer set at 149° to give a maximally dark field. Only the cell inclusions are visible, and the nichtenkerns (n) show up bright. D. The same group of spermatids as in C with the analyser goniometer set at 111°. At this setting, the centres of some of the keinerns appear maximally dark. The rotation of the analyser from the position shown in C represents a phase-change of 76°. E. A compact group of spermatids mounted in 0.9% NaCl, under slight compression: with the analyser goniometer set at 149°, to give a maximally dark (cytoplasm-filled) field. F. The same preparation as in E with the analyser goniometer set at 112°, making the centres of some of the nichtenkerns appear maximally dark. The rotation of the analyser from the position shown in E represents a phase-change of 74°.
FIG. 10. Diagrams showing the phase-changes in light passing through Locust spermatids containing spherical *nebenkerns* in living preparations mounted in different media. The arrows represent the direction of the light passing through the specimens, and the vertical distance between the arrow-heads represent the phase-changes due to cell structures. A. A cell mounted in saline, showing phase retardations caused by the cytoplasm and *nebenkern*. B. A similar cell mounted in a protein medium of the same refractive index as the cytoplasm; showing the phase-change due to the *nebenkern* alone. C. Similar cells mounted in saline, closely packed together and slightly flattened. The adjacent regions of cytoplasm can be used as a reference field with which to measure the phase-changes due to the *nebenkerns* alone.

calculated on the assumption that the spermatid cytoplasm had a refractive index of 1.354. It will be seen that the values obtained are in every way comparable with those in Table I (A).

This method could be very useful for measuring the refractive indices of the cytoplasmic inclusions in cells that occur in sheets only a few cells thick which are difficult to separate into individual cells without damage. The cytoplasmic refractive indices of the individual cells would, however, need to be equal, and it would be necessary that there should be some undamaged cells at the edge of the group where this can be measured, either by direct immersion refractometry or by Ambrose’s “bubble” method.

2. *Refractometry as an Indication of Submicroscopic Morphology*

As already mentioned (p. 6) the proper interpretation of refractive index measurements made on cytoplasmic inclusions is dependent on knowing as much as possible about their chemical composition; so that these quantitative measurements ought usually to be accompanied by a histochemical investigation.
An instance in which this was done, and in which the refractive index measurements on some cytoplasmic inclusions provided an indication of their submicroscopic morphology, will now be briefly described. All stages of this investigation were undertaken at the suggestion of Dr. J. R. Baker to whom belongs the credit for the underlying idea.

Chou in 1956 showed that there were two kinds of lipid droplet in the neurones of *Helix aspersa* that apparently consisted of pure lipid; some small highly refractile colourless globules that appeared to consist of pure triglyceride (T in Fig. 11) that occurred in the axon and axon hillock; and some, seemingly less refractile, rather larger globules (P in Fig. 11) in the periphery of the cell body, that appeared to contain only phospholipid. Table II shows refractive index measurements made on these two
TABLE II

<table>
<thead>
<tr>
<th>Measured phase retardation through globule (wavelengths)</th>
<th>Diameter of globule to the nearest 0.2 μ (measured by eyepiece micrometer)</th>
<th>Refractive index of globule from ϕ and t</th>
<th>Range of refractive index of globule; assuming a maximum error in diameter measurement:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ϕ</td>
<td>t</td>
<td>n</td>
<td>(t) = ±0.2 μ</td>
</tr>
<tr>
<td>0.306</td>
<td>1.2</td>
<td>1.496</td>
<td>1.477–1.526</td>
</tr>
<tr>
<td>0.277</td>
<td>1.2</td>
<td>1.484</td>
<td>1.466–1.510</td>
</tr>
<tr>
<td>0.234</td>
<td>1.0</td>
<td>1.485</td>
<td>1.464–1.517</td>
</tr>
<tr>
<td>0.272</td>
<td>1.0</td>
<td>1.506</td>
<td>1.480–1.544</td>
</tr>
<tr>
<td>0.228</td>
<td>1.0</td>
<td>1.482</td>
<td>1.461–1.514</td>
</tr>
</tbody>
</table>

Mean refractive index of triglyceride globules, 1.491

Triglyceride globules in the axon in a matching medium of r.i. 1.3585:

Phospholipid globules in the cell body in a matching medium of r.i. 1.3615:

*Mean refractive index of phospholipid globules, 1.416*

The mean retardation of light of wavelength 542 mμ passing through the centres of lipid droplets in neurones from the dorsal ganglia of *Helix aspersa* mounted in isotonic saline/protein media with refractive indices equal to those of the cytoplasm, the diameters of the lipid droplets and their refractive indices calculated from these values.

kinds of globule by the present writer and Chou (1957) by immersion refractometry and phase change measurements in the manner just described. It will be seen that, even allowing for the quite considerable possible error due to the difficulty of measuring their diameters accurately (column 4), the refractive indices of the phospholipid-containing globules are considerably lower than the triglyceride globules. The latter have refractive indices comparable with many pure lipid substances and are compatible with their consisting of pure triglyceride. The refractive indices of the phospholipid-containing globules, on the other hand, had lower refractive indices than any pure lipid substance including pure phospholipid.

The only conclusion one could draw from this was that the phospholipid-containing globules must also contain water in intimate association. Now, the phosphoric acid/choline radicle of a phospholipid molecule is
hydrophilic, and the molecules tend to orientate themselves with this part towards an aqueous phase (\(V\) in Fig. 12A). Schmidt in 1939 discussed the stable configurations of such molecules and suggested that a phospholipid droplet in cytoplasm might have the form indicated as \(P\) in Fig. 12A, consisting of alternate concentric shells of phospholipid and water. For triglyceride, on the other hand, he suggested the unhydrated and relatively unorientated form \(T\) in Fig. 12A.

We suggested that the refractive index measurements we had made were quite compatible with this suggestion (Ross and Chou, 1957). Later, again at the suggestion of Dr. J. R. Baker, Chou and Meek (1958) undertook an electron microscope investigation of the same material and, after some trouble, identified the two kinds of globules with certainty. They found the triglyceride globules had the amorphous form shown in Fig. 12B, and the phospholipid-containing globule, had the concentric structure shown in Fig. 12C.

3. Critical Thickness Measurements with the Aid of Immersion
Refractometry

Because interference microscopes are capable of measuring phase changes accurately to the nearest \(\frac{1}{6}\) of a wavelength, or more accurately, and the refractive indices of homogeneous objects can usually be measured to the nearest 0.002 by immersion refractometry, it is often possible to get a very accurate estimate of the thickness of a homogeneous object from their measurements by means of formula (5) above (p. 29). With relatively large objects this is less likely to be valuable since they can usually be measured, proportionally more accurately, by direct means; but for small objects of the order of size of many living bacteria, it enables an estimate of their thickness to be made that is more accurate than is possible by direct measurement by eyepiece micrometer or from photomicrographs.

The accuracy of any direct measurement is limited by the numerical aperture of the optical system and the wavelength of light used, so that in the visible spectrum (using, say blue-violet light with a wavelength of 470 m\(\mu\), and an objective with a numerical aperture of 1.4) it is not possible to determine the dimensions of a microscopic object more accurately than to the nearest 0.4 \(\mu\). Furthermore, from a consideration of the shape of the diffraction patterns at the edges of microscopic objects, it seems highly probable that the commonest method of attempting to assess the real dimensions of an object, i.e. by measuring from the mid-point of the blur or intensity gradient due to diffraction at the edge of an image, will result in a definite underestimate of the width of spherical or cylindrical objects such as living bacteria and many other biological objects (Ross, 1957);
and the writer has recently obtained experimental evidence, in collabora-
tion with Dr. O. W. Richards at the laboratories of the American
Optical Co., Southbridge, Mass., that appears to confirm this. Provided
the objects are large enough to be fully resolved, however, phase change
measurements made through their centre or mid-line will not be affected
by these considerations, and will be directly proportional to their true
thickness.

One method of measuring phase changes in the light passing through
the middle of living bacteria is illustrated in Fig. 14 and discussed in
the Appendix, p. 49; and many bacteria are sufficiently homogeneous
and of the right size for the method to be applied for measuring their
mean thickness. Table III shows the mean phase retardation, based on
ten measurements on individual bacilli, measured in this way on four-
teen different cultures and subcultures of Lactobacillus bulgaricus
mounted in 0.25% NaCl. The mean refractive index of each culture was
also measured by immersion refractometry, and none of these differed
appreciably from 1.404; that is to say when each culture was mounted
in a protein medium with the refractive index, approximately equal
numbers of phase retarding and phase advancing bacilli were visible
under phase contrast or interference microscopes. From these data the
mean thickness of each culture was calculated, and it can be seem (in
the final column of Table III) that the values for the mean thickness so
obtained differed by a maximum of 0.1μ.

An alternative method of measuring the mean thickness of bacteria is
to use Barer’s “double immersion” method with formula (7) (p. 29); this
has the advantage that it also enables one to get values for their

Fig. 12. A. An interpretation of the submicroscopic structure of protoplasm
by Schmidt (1939).

protein; — phospholipids and related substances;

E triglycerides; O water molecules; • ions.

(V) a vacuole with aqueous contents surrounded by a bimolecular phos-
pholipid lamella. (P) a phospholipid droplet. (T) a triglyceride dro-
plet. Between these droplets is a protein framework which holds in its
meshes water and other substances.

B. Electron micrograph of a row of triglyceride droplets in a neurone of
Helix aspersa.

C. Electron micrograph of a phospholipid droplet in a neurone of Helix aspersa,
showing the concentric structure which had been inferred from refractive index
measurements. (Fig. 12, B and C, is reproduced with permission, from J. T. Y.
TABLE III

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Date of culture and subcultures</th>
<th>Date of examination</th>
<th>Mean retardation (φ) from 10 measurements on different bacilli (wavelengths)</th>
<th>Mean thickness (t) (μ)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>11/7</td>
<td>20/7</td>
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<td>2</td>
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<td>0.147</td>
<td>1.14</td>
</tr>
<tr>
<td>5</td>
<td>25/7</td>
<td>29/7</td>
<td>0.148</td>
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<td>30/7</td>
<td>0.145</td>
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<tr>
<td>10</td>
<td>25/7, sub. 27/7</td>
<td>30/7</td>
<td>0.148</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Measurements with the 2 mm double-focus objective of the Smith microscope:

<table>
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<tr>
<th>Culture no.</th>
<th>Date of culture and subcultures</th>
<th>Date of examination</th>
<th>Mean retardation (φ) from 10 measurements on different bacilli (wavelengths)</th>
<th>Mean thickness (t) (μ)</th>
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<tbody>
<tr>
<td>11</td>
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<td>21/7</td>
<td>0.151</td>
<td>1.19</td>
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<tr>
<td>12</td>
<td>11/7, sub. 16/7, 24/7</td>
<td>27/7</td>
<td>0.158</td>
<td>1.23</td>
</tr>
<tr>
<td>13</td>
<td>25/7</td>
<td>28/7</td>
<td>0.146</td>
<td>1.14</td>
</tr>
<tr>
<td>14</td>
<td>25/7</td>
<td>29/7</td>
<td>0.151</td>
<td>1.18</td>
</tr>
</tbody>
</table>

Measurements with the 2 mm shearing objective of the Smith microscope:

The mean retardations of light, of wavelength 254 nm, through living Lactobacillus bulgaricus from fourteen different cultures mounted in 0.25% NaCl, and their mean thicknesses calculated from these and the mean refractive index of the culture (which was 1.404 in every case).

The mean refractive index, with formula (6) (p. 29), which can be compared with those obtained by direct immersion refractometry, and provide a check on the accuracy of the method. Table IV shows mean phase retardation measurements made on nine cultures of Lactobacillus bulgaricus mounted both in 0.25% saline and in non-matching protein media with various refractive indices between that of the saline and the bacilli themselves, and the values for their mean thickness and refractive index derived therefrom. The values for the mean thickness differ by a maximum of 0.11 μ, and the values for their mean refractive index differ from the value of 1.404 already obtained by direct immersion refractometry by a maximum of 0.009. This variation represents a maximum difference in thickness of 0.14 μ reckoned on the basis of the phase change measurements in saline: and it seems very unlikely that the values for the
### Table IV

<table>
<thead>
<tr>
<th>Culture no.</th>
<th>Mean retardation in saline ($\phi_1$)</th>
<th>Mean retardation in protein ($\phi_2$)</th>
<th>Refractive index of protein solution ($n_m$)</th>
<th>Mean thickness ($t$) ($\mu$)</th>
<th>Mean refractive index of bacilli ($n$) calculated from $t$, $\phi_1$ and $n_m$.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.155</td>
<td>0.091</td>
<td>1.3655</td>
<td>1.12</td>
<td>1.410</td>
</tr>
<tr>
<td>2</td>
<td>0.157</td>
<td>0.072</td>
<td>1.3760</td>
<td>1.13</td>
<td>1.410</td>
</tr>
<tr>
<td>3</td>
<td>0.146</td>
<td>0.065</td>
<td>1.3745</td>
<td>1.11</td>
<td>1.407</td>
</tr>
<tr>
<td>4</td>
<td>0.147</td>
<td>0.069</td>
<td>1.3755</td>
<td>1.11</td>
<td>1.407</td>
</tr>
<tr>
<td>5</td>
<td>0.148</td>
<td>0.069</td>
<td>1.3735</td>
<td>1.11</td>
<td>1.403</td>
</tr>
<tr>
<td>7</td>
<td>0.149</td>
<td>0.068</td>
<td>1.3730</td>
<td>1.14</td>
<td>1.406</td>
</tr>
<tr>
<td>8</td>
<td>0.143</td>
<td>0.065</td>
<td>1.3760</td>
<td>1.03</td>
<td>1.411</td>
</tr>
<tr>
<td>9</td>
<td>0.145</td>
<td>0.079</td>
<td>1.3700</td>
<td>1.02</td>
<td>1.413</td>
</tr>
<tr>
<td>10</td>
<td>0.148</td>
<td>0.086</td>
<td>1.3800</td>
<td>1.02</td>
<td>1.414</td>
</tr>
</tbody>
</table>

Mean refractive index of Lactobacillus bulgaricus from immersion refractometry = 1.404

The mean retardations of light, wavelength 542 m$\mu$, through living Lactobacillus bulgaricus from ten different cultures and sub-cultures mounted in 0.25% NaCl and in protein solutions of various refractive indices; and the values for their mean thickness and mean refractive index calculated from these measurements.

Mean thickness of the living bacilli measured in this way were ever wrong by more than this amount.

These methods can be used for any optically homogeneous isolated biological object of regular shape, large enough to be well resolved, and capable of being immersed successively in the mounting media, and for populations of such objects that show little individual variation. It cannot, however, be used for cytoplasmic inclusions in living cells, nor for objects smaller than about 1 $\mu$ in diameter, since these will be insufficiently well resolved under a 2 mm objective for the phase changes measured through these centre points to be truly indicative of their thickness.

#### 4. Immersion Refractometry of Cells of High Refractive Index in Non-matching Media

Some small cells and organisms, notably the spores of many fungi and bacteria, have refractive indices too high to be measured by direct immersion refractometry, with the immersion media usually employed.
Bovine plasma albumins, for example, will not dissolve to form solutions of a higher concentration than about 55% w/v with a refractive index of 1.433; concentrations as high as this are made only with difficulty.

Consequently spores of this kind always give retardations in phase when placed in any available concentration of the mounting medium, and the only accurate way in which it is possible to find their refractive indices is by Barer’s “double immersion” method, using equation (6). Table V shows the results of measuring the mean refractive indices of the

### TABLE V

<table>
<thead>
<tr>
<th>Material</th>
<th>Mean solid content (in g per 100 ml)</th>
<th>Mean water content (in g per 100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vegetative cells:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>1.386</td>
<td>29.0</td>
</tr>
<tr>
<td><em>B. cereus</em> var. <em>mycoides</em></td>
<td>1.400</td>
<td>36.5</td>
</tr>
<tr>
<td><em>B. megaterium</em></td>
<td>1.388</td>
<td>30.0</td>
</tr>
<tr>
<td><strong>Spores:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. cereus</em>, Experiment 1</td>
<td>1.512</td>
<td>99.0</td>
</tr>
<tr>
<td>&quot; 2&quot;</td>
<td>1.513</td>
<td>99.5</td>
</tr>
<tr>
<td><em>B. cereus</em> var. <em>mycoides</em>, Experiment 1</td>
<td>1.528</td>
<td>108.0</td>
</tr>
<tr>
<td>&quot; 2&quot;</td>
<td>1.519</td>
<td>102.5</td>
</tr>
<tr>
<td><em>B. megaterium</em>, Experiment 1</td>
<td>1.527</td>
<td>113.0</td>
</tr>
<tr>
<td>&quot; 2&quot;</td>
<td>1.540</td>
<td>114.5</td>
</tr>
<tr>
<td>Leather</td>
<td>1.530*</td>
<td>109.0</td>
</tr>
<tr>
<td>Wool</td>
<td>1.540*</td>
<td>114.5</td>
</tr>
<tr>
<td>Dried casein</td>
<td>1.540*</td>
<td>114.5</td>
</tr>
</tbody>
</table>

Values for the mean solid and water content of bacterial vegetative cells, spores and other materials calculated from refractive index measurements.

* Chamot and Mason (1938).

spores of *Bacillus cereus*, *B. cereus* var. *mycoides* and *B. megaterium*, measured by this method, compared to the refractive indices of the vegetative cells of the same organisms obtained by direct immersion refractometry; and their respective solid contents and water contents derived from formulae (1) and (2) assuming that they consist predominantly of proteins with a refraction increment of 0.0018, and with a specific volume of 0.75 ml/g (see p. 5). It is immediately clear that the water content of the spores is very much less than that of the vegetative cells, and is comparable to that found in seemingly dried protein products such as leather, wool and dried casein. This is of particular interest in the light
of Davies (1959) and Davies and Thornburg's (1959) recent confirmation that the refraction increment of highly concentrated crystalline proteins is the same as that of dilute solutions, 0.0018, for it means that both the spores and the dried protein products tabulated here must contain appreciable amounts of "bound water".

VI. APPENDIX: SOME PRACTICAL ASPECTS OF MEASURING PHASE-CHANGE WITH INTERFERENCE MICROSCOPES

Most of the following remarks apply equally to the two interference microscopes that are at present\(^1\) commercially available. These are:

(a) The Smith Interference Microscope, manufactured in England by Messrs. Charles Baker of Croydon, and under licence in the United States by the American Optical Company of Buffalo (where it is sold under the name of the "A. O. Baker" microscope).

(b) The Dyson interference microscope manufactured in England by Messrs. Cooke, Troughton & Simms of York.

The performances of these two instruments have been objectively and comprehensively compared by Davies (1958). Both instruments are capable of measuring phase-changes to almost exactly the same degree of accuracy, and each has particular merits for certain kinds of studies. For measurements on living material of a rapidly perishable nature, however, most workers with extensive experience of both instruments are agreed that the Smith interference microscope possesses a distinct advantage in being simpler and quicker to adjust and manipulate, mainly because the slide and cover-slip does not have to be mounted between large surfaces of a viscous immersion fluid as in the case of the Dyson microscope. The two English companies manufacturing the Smith and Dyson microscopes (Messrs. Charles Baker and Messrs. Cooke, Troughton & Simms) have recently amalgamated (November 1958); so that it may reasonably be expected that certain of the accessories that could with advantage be used with both instruments (such as the Payne photometer-eyepiece discussed below, p. 53), will be made interchangeable.

1. Phase-change Measurements by the Extinction Point Method

The most usual method of making accurate phase-change measurements with an interference microscope is by the extinction point method, using monochromatic or nearly monochromatic light. The instrument is first adjusted to give broad fringes so that, with a white light source, the background field appears all of one interference colour. These fringes are

\(^1\) Since this article was written, a third interference microscope has been placed on the market by Messrs. Ernst Leitz, Wetzlar W. Germany.
then caused to pass across the field (by rotating an analyser in the Smith microscope, or by moving a wedge with a micrometer screw in the Dyson microscope), and the adjustment in which the background appears maximally dark in monochromatic light is determined. The analyser, or screw, is then rotated in the direction appropriate for measuring a phase retardation or a phase advance (depending on the refractive index of the object being measured relative to that of the medium in its immediate vicinity), until the object being measured itself appears maximally dark, and the rotation in each case is proportional to the phase-change. Figure 9 C–F shows this method being applied to the centres of the nebenkerns of the spermatids of *Locusta migratoria* with the Smith interference microscope.

This is quite a satisfactory method for making measurements on objects that show fairly large phase-changes in aqueous media, such as the relatively thick parts of tissue cells or highly refractile bacterial spores, because when the object being measured appears maximally dark, the rotation of the analyser, or screw, has been big enough to make the background field to appear quite bright. Against a bright field, the exact setting of the instrument at which the object being measured appears maximally dark can be judged by eye quite critically; and, under these conditions, phase-changes can be measured to an accuracy of \( \frac{1}{50} \) of a wavelength or even more accurately.

The method is less satisfactory, however, for objects giving phase-changes of less than about one-fifth of a wavelength, such as, for example, thin pseudopodia and most living bacterial vegetative cells mounted in water. It then becomes difficult for the eye to discern the exact instrumental setting at which the object appears maximally dark against a background field that is itself not very bright. Under these conditions there is a tendency for an observer to turn the analyser, or screw, too far, which makes the object being measured appear in higher contrast to the background but with less absolute depth of intensity, and thus introduces a systematic high error in the phase-change measurement. Therefore, without resorting to photography and densitometric equipment not universally available, it is not possible to measure small phase-changes in this way with any very great precision.

2. *Eyepiece Devices for Increasing the Precision of Phase-change Measurements*

Three devices are now commercially available that effectively increase the accuracy of phase-change measurements of all magnitudes made with the respective interference microscopes for which they are designed.
Each is in the form of an eyepiece accessory that introduces a special area into the field for comparing and matching different depths of intensity in the background and object.

(a) *The Smith half-shade eyepiece* is designed for use with the Smith interference microscope manufactured by Messrs. Charles Baker in England. Its optics and working principles have been described, from rather different standpoints, by Smith (1954), Ross (1957) and Davies (1958), and need not concern us here in any detail. When it is in use the field appears traversed by a narrow horizontal strip illustrated in Fig. 13A and B. This is actually the image of a strip of metallic aluminium laid down on the surface of a prism in the eyepiece from which light from all parts of the field is internally reflected; but, because light internally reflected from the glass and metal surfaces are polarized differently, the
image of the strip is permanently out of phase with the image of the rest of the field by a fixed amount, actually by 120° in the device as at present designed. This means that, with a white light source, the strip will always appear of a different interference colour to the rest of the field. In monochromatic, or nearly monochromatic light, the relative intensities of the two regions will vary in intensity with the setting of the analyser so that the strip can be used as a comparison area.

Figure 13A and B shows how this device is used for making phase-change measurements. It is first necessary to move the microscope stage, so that the image of the object being measured lies partly inside and partly outside the image of the strip. The analyser is then rotated so that the intensity of the background in the strip region matches that of the background in the rest of the field, as in Fig. 13A. At this setting the relative intensities of the object being measured, inside and outside the strip, are markedly different. The analyser is then rotated until the intensity of the image of the part of the object in the strip region matches that of the object lying outside the strip, as in Fig. 13B. The rotation of the analyser between these two settings gives a direct measurement of the phase-change given by the object; and as these two settings can be obtained with great precision, phase-change measurements accurate to \( \frac{\lambda}{25} \) of a wavelength can frequently be obtained if the area of the object being measured is fairly large.

However, with small objects a systematic error is introduced in a rather curious manner; because when the images of such objects are less wide or not very much wider than the strip itself, the very different intensity of the adjacent regions of the strip will mislead the eye in its assessment of the match of the object at second position of the analyser described above. This is clearly illustrated in the photomicrographs in Fig. 14. Figure 14A shows the first position of the analyser when the phase-change through a bacillus of Lactobacillus bulgaricus is being measured. The image of the strip is almost invisible except where it is crossed by the bacillus, and this setting can be gauged very accurately. Figure 14B shows the second position of the analyser where the intensity of the image of the bacillus inside the strip region appears to match the intensity of the image of the bacillus outside the strip. This, however, is an optical illusion as can be at once appreciated from Fig. 14C which is the same photomicrograph as in Fig. 14B with the adjacent image of the adjacent regions of the strip blocked out: the image of the bacillus in the strip region is appreciably darker than the rest. The adjacent regions of the strip have been making the image of the bacillus inside the strip look lighter than it really is. This means that with small objects, of the order of size of living bacteria seen with a 2 mm objective, there is a
FIG. 14. Photomicrographs illustrating the matching error made in using the Smith half-shade eyepiece, as at present designed, for measuring phase-changes in very small microscopic objects such as Bacteria. In all the photographs a bacillus of Lactobacillus bulgaricus lies obliquely across the horizontal image of the metallized strip in the middle of the microscope field. In A (taken at the first position of the analyser) the intensity of the background field within the image of the strip accurately matches that of the rest of the background field. In B (taken at the second analyser position for making a measurement) the intensity of the image of the bacillus within the strip appears to match that of the image of the bacillus lying outside this region. C is the same photograph as B, but with the adjacent regions of the image of the background around the bacillus cut out. It can now be seen that the image of the bacillus within the strip is actually appreciably darker than that in the background; and that, in B, the eye had been misled by the adjacent dark regions of the strip into judging this region of the bacillus to be lighter than it really was. A true match in intensity was not, therefore, obtained.
tendency to turn the analyser too far, so that when the object appears matched inside and outside the strip, the part inside is actually darker. This means that a systematic high error in phase-change measurement is being made. The device is therefore not satisfactory for measuring phase-changes on small objects as it is at present designed; but the remedy is quite simple. The aluminized surface can be carefully removed at one end so that the image of the strip does not cross the whole field. The end of the strip will then form a much more satisfactory comparison area. If a few small "islands" of aluminized surface are left behind in a region from which the rest of the strip has been removed these will probably be even more satisfactory as comparison areas for small objects. The width of the strip seem in the field is almost exactly \( \frac{1}{4} \) of the diameter of the field, and if its length were made the same as its width it would be excellent for almost all purposes. The writer has recently tested a Smith half-shade eyepiece modified in this way, and has found it as satisfactory as the Payne photometer eyepiece (described below). In general it can be said that this subjective error is reduced by having the comparison area as small as possible, and such small areas are just as good for measuring the phase-changes of the larger objects.

(b) The Koester half-shade eyepiece designed for use with the Smith interference microscope manufactured in the United States by the American Optical Company (the "A. O. Baker" interference microscope) has been fully described by its inventor (Koester, 1959). It is a simple and ingenious device in the form of a biquartz plate made of two sections of right-handed and left-handed quartz cut perpendicular to the optical axis and butted together, so that each occupies half of the microscope field when mounted in the image plane of the microscope between the quarter wave plate and analyser. The plate is of such a thickness that the image in the two halves of the field illustrated in Fig. 13C and D are permanently 20° out of phase with each other. The method of operation illustrated in Fig. 13C and D, is as for the Smith half-shade eyepiece. Its great disadvantage will be at once appreciated. The comparison area is enormous, since it consists of half the field, so that the difficulty of obtaining a true match when measuring small objects, just discussed in the case of the Smith half-shade eyepiece, is present to a marked degree. Indeed, even with quite large objects, it may be difficult to avoid being misled by the differing intensities of the adjacent background regions; in Fig. 13D the object being measured is actually of the same intensity in both halves of the field; but the half in the left-hand section appears brighter than the half in the right-hand section because of the surrounding dark background field. The remedy is to use an eyepiece diaphragm
to reduce the size of the field and that of the object when the second analyser setting is being determined, but this is not very practicable for very small objects. The Smith and the Koester eyepieces are only suitable for use with the Smith interference microscopes, but both can be adapted to fit the British or American instruments.

(c) The Payne eyepiece-photometer was designed for use with the Dyson interference microscope but could easily be adapted for use with any interference microscope, and is described and discussed more fully by Davies (1958). In the image plane there is an inclined glass surface on which is a small semicircular fully reflecting area which is illuminated separately by a system of mirrors from the light source which can be varied in intensity by means of two polaroids. This is the comparison area which is set at a suitable fixed intensity for making a measurement, and matched successively to the background and the object as before. This comparison area is the smallest in any of the devices so far described, being only \( \frac{1}{15} \) of the diameter of the field in length and \( \frac{1}{30} \) in width, which means that the matching of small objects is much less liable to systematic errors of the kind described above. The variability of its intensity also enables random errors of measurement to be reduced to a minimum. Ten successive measurements recently made by the present writer showed a maximum variation of \( \frac{1}{40} \) of a wavelength, and in nine of these the variation was less than \( \frac{1}{50} \) of a wavelength. This compares favourably with similar successive measurements made on bacilli with the Smith half-shade eyepiece, which showed a maximum variation of \( \frac{1}{15} \) of a wavelength (Ross, 1957). For these reasons the present writer regards the Payne eyepiece-photometer as the best of the three devices to increase the accuracy of phase-change measurements, as they are at present designed, in spite of being rather troublesome to set up and align initially. It also could easily be modified as a colour-matching device by the insertion of a compensator between the two polaroids (Smith, 1959), although in its present form it can only be used with monochromatic or nearly monochromatic light.

3. The Refractometry of Birefringent Objects

Both the Smith microscopes and the Dyson microscope can be used for measuring both the refractive indices of birefringent objects if the planes of vibration (or electric vectors) of the ordinary and extraordinary rays in the object being measured are known. With the Dyson microscope, which does not use a polarizing system to produce interference, this can be done quite simply by using a rotating polaroid below the condenser which can be turned so as to occlude each set of rays in turn. The phase-
change due to each set of rays can then be successively measured or suitable immersion media can be used to produce zero phase-changes for each set of rays. The Smith microscopes rely on a polarizing system to produce interference but their objectives and condensers are so orientated that, for all objectives other than the 2 mm “double focus” objective, the plane of vibration (electric vector) of the “ordinary” object beam is in the “north-south” direction in the microscope field as viewed by an observer in the normal position behind the instrument (Smith, 1958). Consequently it is necessary to orientate the object in the field so that the plane of vibration of its ordinary ray is also in this direction for the refractive index due to this to be measured, and at right angles to this to determine its other refractive index. (The reverse is true for the 2 mm “double focus” objective where the plane of vibrations of the “ordinary” object beam is E.-W. in the field.) Since nearly all objects of biological origin are positively birefringent, the refractive index due to the ordinary ray will be the lower of the two in almost all cases.

In practice it is seldom necessary to take into account the birefringence of parts of living cells when estimating their solid content from refractive index measurements, since this birefringence is usually rather weak, and the difference between the two refractive indices is less than the experimental error of the technique. The birefringence of the A band regions of living muscle fibres, however, is approximately 0·004, so that their w/v solid content will be about 1% higher than that found with the ordinary ray and 1% lower than that found with the extraordinary ray. The birefringent inclusions in living cells, such as chromosomes and certain phospholipid droplets, all have lower birefringences than 0·004, and their refractive indices can seldom be estimated to this accuracy. With the shearing objectives on the Smith microscope, an elongated object such as a muscle fibre can, of course, only be orientated with its long axis in the “north-south” direction in the field because when it is orientated “east-west” two images will overlap.

A special difficulty is involved in the refractometry of most living striated muscle fibres, due to the small sarcomere interval and the very small distance between their individual bands. This frequently results in the colour, or intensity, of one set of bands being affected by diffraction from the phase boundaries of adjacent bands of different refractive index (see p. 27), so that an apparent match of one set of bands may not always indicate that they have exactly the same refractive index as the mounting medium. This effect is fully discussed by Huxley and Hanson (1957), and by Ross and Casselman (1960). In practice, it is sometimes possible to get a close approximation of the true refractive indices of the
I band regions when the muscles are stretched and the interval between the adjacent phase boundaries separated; but the refractive indices of the A band regions cannot be obtained directly, because, in mammalian fibres, they are hardly ever wider than $0.5 \mu$, even when fully contracted.

4. Distinguishing Phase-advancing and Phase-retarding objects under Interference Microscopes

With a phase-contrast microscope, an object giving a small retardation in phase can be immediately distinguished from one giving an advance in phase by whether it is darker or brighter than the background (p. 22). When immersion refractometry is used with an interference microscope, a simple means of distinction is often desirable but not quite so straightforward. When monochromatic or nearly monochromatic light is used one can adjust the instrument to give a maximally dark background, and then turn the analyser or screw in the direction appropriate for measuring a retardation. If the object darkens it is a retarding object with a higher refractive index than its surrounding medium, and if it gets brighter the contrary is true, provided that the phase changes are small.

Such objects can, however, be immediately distinguished by their colour when a white light source is used, provided that the microscope is first adjusted so that this can be correctly interpreted. Both the Smith and the Dyson microscopes are capable of being adjusted so that, when the analyser or screw is turned in a certain direction the background field may change colour, either up the Newtonian series of interference colours, or down it. The colour of any phase object in the field will change in the same direction, but, at any one setting, it will exhibit a different colour to the background field shifted either up or down the Newtonian series depending on whether it is itself phase-advancing or phase-retarding. It is consequently nearly always possible to select and adjust the fringe system of the microscope so that when, for example, the background is red of the 1st order, an object known to give a phase retardation (such as human oral epithelium cell mounted in water) will appear of a colour higher in the Newtonian series such as violet or blue. If the microscope is now left in this adjustment and another object is examined which appears yellow or of some colour lower in the Newtonian series, it will be a phase-advancing object.

The above applies only in the case of objects giving phase-changes of less than about half a wavelength. Objects giving phase-changes greater than this are almost always recognizable by having fringes of a different colour at their edges. In monochromatic light, dark fringes move
inwards towards the centre of the object when the analyser or screw is
turned in the appropriate direction for measuring a retardation if the
object is a phase-retarding one. They will move outwards if it is phase
advancing.

5. Phase-change Measurement with the “Double-focus” Objectives
   in the Smith Interference Microscopes

Because the reference and the object beams are incompletely separ­
ated in the Smith “double focus” objectives, their capabilities for making
accurate phase-change measurements have been questioned, and they
have come in for a certain amount of unmerited adverse criticism (e.g.
by Hale, 1958).

They are, in fact, entirely satisfactory for measuring phase-changes
in objects of relatively small area, and it is important that their limita­
tions should be defined as precisely as possible. From Fig. 15A it will be
seen that, because the foci of the reference and object beams are super­
imposed, some of the reference beam is always intercepted by the object.
This has the effect of giving a low error in phase-change measurements of
certain values. The maximum amount of this error depends on the rela­
tive areas of the object and reference area, and two examples of this
are illustrated in Fig. 15B and C for the 2 mm “double-focus” objec­
tive, which has a reference area approximately 20 μ in diameter. It can be
seen that an object 5 μ in diameter will only intercept 6% of the light
of the reference beam, so that, at the most, phase-change measurements
on such an object will be 6% low; an amount that can be frequently
neglected. A larger object, however, say 14 μ in diameter as illustrated in
Fig. 15C, will give a maximum low error of 50%, and the remedy here
is to use a lower power “double-focus” objective with a larger reference
area. These errors, however, are maximum errors that only occur when
the phase-change due to the object is 1/4, 3/4, 1 1/4 wavelengths or multiples
of this. Objects giving phase-changes of 1/2, 1, 1 1/2 etc. wavelengths are
correctly measured regardless of the size of the object (Smith, 1959).

This has been confirmed experimentally by the present writer using
the 2 mm Smith “double-focus” and “shearing” objectives to make
measurements on identical bacilli of Lactobacillus bulgaricus approxi­
mately 1 μ in diameter, and the nebenkerns in the spermatids of Locusta
migratoria 4.5 μ in diameter. The very small differences found between
the measurements with the two kinds of objectives were no greater than
the random errors found with similar repetitive measurements made with
the same objective (see Table III, p. 44).
Fig. 15. Diagram illustrating how the maximum low errors in making phase-change measurements with the "double focus" objectives of the Smith interference microscope are affected by the size of the object relative to that of the reference field. A, a diagrammatic representation of the paths of the "reference" and "object" beams in the "double focus" condenser and 2 mm objective. B and D, enlarged diagrams of the parts of the ray paths surrounded by a circle in A. In B, with a circular object 5 μ in diameter, only 6% of the light of the reference beam is intercepted, and the error introduced can usually be ignored. In D, with a circular object 14 μ in diameter, nearly half the light of the reference area is intercepted, which can cause a very considerable error in phase-change measurement. C and E are plan views of B and D respectively in the plane of the objects. The dotted line represents the limit of the reference area, which, with the 2 mm objective, is approximately 20 μ in diameter.
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REFERENCES

This list refers only to publications specifically mentioned in this article, and is not intended as a complete bibliography. A very extensive list of references to work done in this field has been compiled by Dr. O. W. Richards, and is included in the Reference Manual of the A.O. Baker Interference Microscope, published by the American Optical Co., Instrument Division, Buffalo 15, N.Y., U.S.A.

THE IMMERSION REFRACTOMETRY OF LIVING CELLS


