

# Refractometry of Living Cells

## Part II. The Immersion Medium

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With one plate (fig. 3)

### SUMMARY

This paper continues the discussion of an immersion method of refractometry of living cells, the basic principles of which were given in Part I (Barer and Joseph, 1954). A suitable immersion medium should be non-toxic, must not penetrate the cell, and must be in osmotic equilibrium with it so that no change in volume (and hence in concentration) occurs. These requirements are best met by solutions of substances of high molecular weight. The effects observed when cells become freely permeable to such substances are described. An account of tests with various immersion media is given. The main substances tried have been peptone, proteose, protein hydrolysate, dextran, polyvinyl alcohol, polyvinylpyrrolidone, acacia gum, egg albumin, bovine gamma globulins, carboxyhaemoglobin, and bovine plasma albumin. Of these, bovine plasma albumin has proved to be most generally useful though acacia gum may be a good inexpensive substitute for some cells, particularly fungi.

The osmotic properties of the immersion medium are most important and must be carefully controlled if true determinations of solid concentration are to be made. Some of the many difficulties in defining an isotonic physiological medium are reviewed. It is suggested that the best practical definition is that such a medium should be innocuous and should not change the cell volume. A method of adjusting the salt content of the medium, based on this definition, is described, and the possibility that some cells may exhibit a degree of osmotic regulation is discussed. Finally, evidence is presented to demonstrate the harmlessness of the isotonic protein medium to many types of cells. Photomicrographs of cell division over a period of 48 hours in a protein medium are shown.

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[Quarterly Journal of Microscopical Science, Vol. 96, part 1, pp. 1-26, March 1955.]

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### BASIC REQUIREMENTS

AS already indicated the present method depends on matching the refractive index of the cytoplasm of a living cell with that of the immersion medium. The properties of the latter are very important and it is possible to lay down certain conditions which should be fulfilled by the ideal immersion fluid.

(1) Since the purpose of the investigation is to carry out measurements on normal living cells, the immersion medium must be non-toxic and must not affect the structure or function of these cells.

(2) The medium must not penetrate the cell. Should penetration occur even to a limited extent, the refractive index of the cell interior would be changed and an accurate measurement against the external medium would become impossible. The effects obtained when cells become freely permeable to the medium are further discussed below.

(3) The medium must be in osmotic equilibrium with the cell so that the latter remains unaltered in volume. Any swelling or shrinkage would inevitably change the refractive index.

(4) The refractive index of the immersion medium must be variable in small steps or preferably continuously and the range of refractive index covered must include that of the cells to be measured.

Certain conclusions can be drawn from these requirements. In the first place most of the oils and inorganic solvents which are commonly used for the refractometry of crystals and other inert objects are unsuitable for living cells. Even in those few cases where the oil is non-toxic or does not damage the cell (e.g. liquid paraffin) trouble may be caused by a thin film of water which remains in contact with it. The desirability of being able to change the refractive index continuously suggests the use of a solution of variable concentration. The only suitable solvent appears to be water; we have been unable to think of any water-soluble substance of low molecular weight which could be used. Inorganic salts (particularly iodides, which have a high refractive index in solution), urea, sugars, and glycerol were all ruled out because of either toxicity, penetration, or lack of osmotic balance. The most obvious type of substance to use is one of high molecular weight. Such substances exert

very low osmotic pressures so that they can be dissolved in a suitable saline solution in order to make the final solution isotonic with the cells studied. Another advantage of materials of high molecular weight is that their large size and low diffusibility hinder penetration into the cell interior. On the other hand, a rather concentrated solution may be needed to match the refractive index of certain cell structures. This requirement rules out many substances which are insufficiently soluble; the great viscosity of concentrated solutions of some substances of high molecular weight also makes them unsuitable for mechanical reasons. From the practical point of view, therefore, we have attempted to find inert non-toxic substances of molecular weight exceeding 10,000, freely soluble in water, and whose solutions do not exhibit excessive viscosity. Other practical considerations were that the chosen substance should, if possible, be readily available commercially and reasonably inexpensive.

#### THE PERMEABILITY OF CELLS TO THE IMMERSION MEDIUM

During the early stages of this work some experiments were carried out by Barer and Ross in an attempt to measure the refractive indices of fixed cells. For this purpose hanging drop preparations of spermatocytes of various species were subjected for a short time (generally 1-5 minutes) to the action of osmium tetroxide vapour or formaldehyde vapour. On immersing such cells in a suitable protein medium, it was found to be quite impossible to bring about reversal of contrast, no matter how high the concentration of protein used. Further investigations of a similar nature were then undertaken by the authors, using a variety of cells treated with different physical and chemical agents. As a result of this work it became clear that when cells are treated with certain agents they become freely permeable to bovine plasma albumin so that no matter what the concentration of the latter, the refractive index of the external medium can never exceed that of the cell. What presumably happens in these circumstances is that the aqueous medium within the cell is replaced by a protein solution, so that the total concentration of solids is always slightly higher than that in the external medium. This theory explains the observed fact that when viewed by positive phase-contrast such cells remain dark when immersed in protein, but become progressively paler (though never disappear) as the concentration of protein is increased.

In the case of agents such as precipitant fixatives most of the cell proteins are presumably deposited as a network of rather dense, more or less dehydrated strands, which would be expected to have a refractive index of about 1.54, corresponding to that of many dry protein films and fibres. It is not, of course, possible to immerse cells in a protein medium of such a high refractive index, but the refractive index of fixed and dehydrated cells can be measured very easily by phase-contrast microscopy with non-aqueous immersion media. The use of such media for varying the contrast of unstained tissue sections is a familiar method in phase-contrast microscopy (see Bennett and others,

1951), and Crossmon (1949) employed it to measure the refractive index of fixed sections, obtaining values in the region of 1.536. Unfortunately, a recent paper by Davies and others (1954) gives the impression that the refractive index of a fixed cell cannot be determined by phase-contrast and Crossmon's work is not mentioned; a very much more laborious and less accurate method using interference microscopy is described.

It was first thought that the loss of the permeability barrier against proteins was an inevitable accompaniment of cellular death, but further work has indicated that this is probably not the case. The action of many chemicals, particularly the common fixatives, fat solvents such as ether, alcohol, and chloroform, and many acids and alkalis in sufficient concentration, almost invariably abolishes the permeability barrier even though the gross structure of the cell may be comparatively little affected. Many cells are peculiarly susceptible to slight drying. If, for example, a thin film of mammalian blood is deposited on a slide, a wave of drying may often be seen to pass along the film. If a drop of concentrated protein solution is placed over the boundary region between the moist and drying parts of the film, it is found that most of the cells in the moist region can be reversed in contrast, whereas most of those in the drying region are dark and irreversible. This damage to the permeability properties of the cell by drying can therefore occur with extreme rapidity. The effect is not due to heat as all drying was carried out at room temperature and normally took place within a few seconds of forming a film. Nor did it appear to be related to the surface action of the glass slide, because a high proportion of cells taken from the surface of a large blood-drop exposed to the air also showed loss of the permeability barrier. Surface action at an air-cell interface cannot be ruled out. These observations cast considerable doubt on the validity of a method proposed by Mellors, Kupfer, and Hollender (1953) for measuring the mass and thickness of living cells by multiple-beam interferometry. In this method an air-bubble is manoeuvred to surround a cell and the resulting fringe system is photographed. Experiments carried out here by Dr. D. A. T. Dick with a special irrigation chamber (Dick, 1955) have shown that even the momentary passage of an air-bubble across living chick fibroblasts or snail amoebocytes leaves a track of swollen and damaged cells, some of which are already permeable to protein, and the rest soon become so. The effect is drastic and dramatic and has been described as being 'like the passage of a steam-roller'. It seems unlikely, therefore, that cells observed in an air-bubble could be regarded as viable or even normal in shape.

In contrast to these results autolytic changes were not always accompanied by rapid loss of the permeability barrier. Cells such as spermatocytes, various types of white blood corpuscles, and mouse ascites tumour cells, when kept for several days in protein solutions usually underwent considerable swelling, but still appeared to be impermeable to protein. If the original concentration of protein was sufficiently high to make these cells appear with reversed contrast, they became even more brightly reversed as time went on because

the concentration of solids and hence the refractive index fell with increasing swelling. Frequently, if the concentration of the protein was not quite high enough to cause reversal at first, such reversal did occur after a few days when the cells had swollen enough to reduce their refractive indices below that of the protein. A certain number of cells became dark in the course of time and apparently lost their permeability barrier.

A rather different type of behaviour was seen in mammalian red blood corpuscles. Suspensions of these were made up in protein solutions concentrated enough to produce reversal of contrast, and drops were left overnight between sealed slide-coverslip preparations. By the next day a certain proportion of the cells had become dark and the number of these increased progressively with the passage of time. Comparatively little swelling occurred, but eventually many of the cells haemolysed. A small proportion of the cells seemed to be highly resistant to autolytic changes and still appeared with reversed contrast even after 7 days. A more detailed report on experiments of this type will be given elsewhere. For the moment we may state that there appear to be at least two types of reaction to autolytic changes: in one the cell permeability barrier to proteins and possibly other high-molecular weight compounds is relatively unaffected, at least until a very late stage, and in the other the mechanism or structure responsible for the permeability barrier to such substances is destroyed fairly rapidly. The action of many organic solvents in making cells freely permeable to proteins suggests that lipids may be involved and it is not unlikely that some breakdown in the lipoprotein complexes believed to be present in cell surfaces may be the underlying factor.

The loss of the permeability barrier can in some cases be used as a criterion for cell death and it is therefore of interest to know how sensitive this test is as compared with other criteria. The fact that grossly swollen cells undergoing autolysis can still retain a permeability barrier suggests that the test is not necessarily a very sensitive one, because it is hard to imagine that such cells are alive in the ordinary sense, though of course it is always difficult to define the terms life and death rigorously. There is no special reason why the cell surface should not continue to be impermeable to protein long after other cellular activities generally associated with the living state have ceased. Tests were carried out in collaboration with Dr. G. N. C. Crawford in order to compare the sensitivity of the permeability method with that of vital staining. It was generally found that autolysing cells lost their ability to segregate dyes such as neutral red and became diffusely stained long before the permeability barrier to protein was lost. From this point of view, therefore, the permeability criterion for cell death does not appear to be very delicate. It may, however, be a useful method for studying the permeability of cells to large molecules—a subject about which comparatively little seems to be known. In the following section we shall describe experiments carried out with a variety of substances which were investigated in an attempt to find a suitable alternative to bovine plasma albumin. It will be seen that in many cases cells seemed to be freely permeable to these substances, particularly in high concentrations. In some

cases the presence of diffusible compounds of lower molecular weight may have been responsible for a toxic action accompanied by destruction of the permeability barrier. On the other hand, some of these substances still appeared to penetrate the cells even after they had been subjected to prolonged dialysis, so that the presence of impurities of low molecular weight became unlikely. The mechanism of penetration of such compounds is quite unknown; it is being investigated. It was also found that there were striking differences between the behaviour of the same substances with respect to different types of cells. Gum acacia, for example, did not appear to penetrate the spermatocytes and fibroblasts of several species, whereas it did penetrate the majority of bacteria tested. If a range of different non-penetrating compounds of varying molecular weight were available, it might be possible to assess varying degrees of damage to the permeability barrier. Many of the substances discussed below were investigated from this point of view, but our search has not been completely successful. As regards convenience and general applicability no substitute has been found for bovine plasma albumin. The list of compounds tested is not exhaustive, and no attempt has been made to describe properties of unsuitable substances in detail. Brief mention has been made of some of these, however, in order to assist others who might wish to investigate the properties of possible protein substitutes.

#### IMMERSION MEDIA OTHER THAN BOVINE PLASMA ALBUMIN

The concentrations of most of the substances discussed here were measured by refractometry, and for convenience they are regarded as having the same refraction increment as protein, so that concentrations are expressed in terms of the equivalent concentration of protein solution having the same refractive index.

#### *Peptone*

Many peptone preparations are available commercially. In general they are made from heart or muscle by acid or enzymic digestion, and differ in their content of insoluble material and in the molecular size-range of their components. Thus some preparations were found to contain a high proportion of substances incapable of passing through a collodion membrane, whereas others were almost completely dialysable.

The materials we have mostly used are Armour's numbers 1 and 2, and Evans's bacteriological peptone. The first two gave dark brown, cloudy solutions which had to be clarified by filtration or centrifuging. Solutions of high refractive index corresponding to that of a 50 per cent. protein solution could be prepared without difficulty and were much less viscous. Evans's bacteriological peptone was completely soluble up to at least 50 per cent. and contained virtually no insoluble residue. It appeared to be composed mainly of dialysable substances. The pH of all these solutions lay between pH 4 and pH 5.

Solutions of crude peptones in distilled water were generally unsuitable for work on most of the cells studied, and were evidently very hypertonic. *Amoeba proteus* was rapidly killed by immersion in a 20 per cent. solution, but survived for about 20 minutes in 12 per cent. Resting pseudopodia were bright (i.e. reversed in contrast when positive phase contrast was used) in 7.8 per cent. and dark in 6.3 per cent., in excellent agreement with values found in protein. Thus in low concentrations such as these, crude peptone is not hypertonic and for a time at least is moderately non-toxic. In higher concentrations, however, there is evidence of both hypertonicity and toxicity. We therefore attempted to eliminate salts and other small osmotically active molecules by dialysis. At the same time diffusible substances capable of penetrating living cells and possibly toxic compounds might be removed. Dialyses were carried out as described in Part III for bovine serum albumin and in some cases the pH was also adjusted by dialysis against suitable buffers to between 6.8 and 7.0. The dialysed peptone was frozen-dried and the resulting powder dissolved in water. This material seemed rather less toxic to Protozoa than the crude peptone. Several different types of amoebae survived for periods of up to 30 minutes in concentrations up to 10 per cent. and a few individual *Podophrya* and *Colpidium* were active for periods up to 3 hours, though many died sooner. In these cases valuable confirmation was obtained of the refractive index figures for the same organisms measured in protein solutions.

Experiments on other types of cells were for the most part unsuccessful. It was not possible to cause reversal of contrast in spermatocytes, and red blood corpuscles became grossly distorted even at concentrations considerably below those required to produce reversal in protein. Rather surprisingly it was found that bacteria could not be reversed, though the capsules of some species did become reversed. Some fungal spores, on the other hand, became reversed in very high concentrations, though fungal mycelia could not be reversed.

The unsuitability of crude peptone solutions is not surprising in view of the large proportion of relatively small osmotically active molecules and ions which they contain. The failure of dialysed peptone to cause reversal is less easily explained; it may be that some of the polypeptides present are either toxic or are capable of penetrating living cells. It would not, of course, be surprising to find that such substances are toxic, because after all several antibiotics of the polypeptide group are known, and many natural bacterial toxins are composed of protein. Only three types of peptones were used in these experiments and it may well be that other types may prove to be more suitable.

#### *Proteose*

Since trials with peptones were for the most part unsuccessful, an attempt was made to find a protein breakdown-product of higher molecular weight. Pure proteoses are not available, but the commercial preparation Bacto-Protone (Difco) is said to have a high proteose content (5.36 per cent. primary

proteose nitrogen and 7.60 per cent. secondary proteose nitrogen according to the manufacturers). This material contained much insoluble debris which had to be removed by centrifuging. The pH of the supernatant liquid was approximately 6.5. Preliminary tests on several different types of cells were uniformly unsuccessful and further detailed tests were not carried out.

#### *Protein hydrolysate*

Several commercial protein hydrolysate preparations can be obtained. Experiments were carried out with Hepamino (Evans), which is a liver hydrolysate. It contains a good deal of insoluble matter which can be removed by centrifuging, leaving a clear brown liquid of pH 6. *Amoeba radiosa* remained motile for some time in 12 per cent., but were not reversed in contrast. *Vorticella* was killed fairly rapidly in 9 per cent. No reversal of tissue cells could be obtained.

#### *Dextran*

This is a polysaccharide used for a number of purposes in haematology. It is available in different grades of molecular size. The samples tested were quite unsuitable because of limited solubility and extreme viscosity. Concentrations approaching 20 per cent. formed almost solid pastes.

#### *Polyvinyl alcohol*

This is a polymerized vinyl alcohol, again available in various ranges of molecular size. It can be dissolved in hot water to form rather viscous neutral solutions at concentrations up to 25 per cent. Amoebae became reversed and survived for periods up to 30 minutes. *Paramecium* survived for only a short time in 14 per cent. The high viscosity makes this material unsuitable for general purposes, but further work with less polymerized samples may be profitable.

#### *Polyvinylpyrrolidone*

This is a synthetic polymerized vinylpyrrolidone which was first employed by Hecht and Weese (1943) as a substitute for blood-plasma in cases of shock. It has since been extensively investigated and used for this purpose, and is said to be reasonably non-toxic. Under the name of Periston it became the standard blood substitute in the German Army and is available in this country as Plasmosan (May & Baker). The molecular weight varies from 30,000 to over 100,000, depending on the degree of polymerization. Samples were obtained from Messrs. May & Baker, Dagenham, Essex, and Messrs. Milwards Merchandise, Dacre House, Victoria Street, London, S.W. 1, and we wish to thank these firms for their generous co-operation. The material is sold as a dry powder which is readily soluble in water. The pH of different samples is said to vary somewhat, but we have usually found it to be about 5.5.

Amoebae were reversed in 15 per cent., but soon contracted and became immobile. Reversal also occurred in 10 per cent., in which the cells survived



for about 30 minutes. Values for the refractive index obtained in polyvinylpyrrolidone agreed with those obtained in dialysed peptone and protein. It was not possible to cause reversal of contrast in bacteria and fungal spores, and red blood corpuscles became grossly distorted in high concentrations. A curious effect, first pointed out by Mr. K. F. A. Ross, was observed in snail spermatoctyes. Both the cytoplasm and nucleoplasm of these cells were reversed in contrast by immersion in 20 per cent. solutions. Within a few minutes, however, the cytoplasm became progressively fainter and then dark, indicating that the permeability barrier had been damaged and that polyvinylpyrrolidone had leaked into the cytoplasm. The nucleoplasm, on the other hand, remained reversed in contrast for at least 30 minutes, suggesting that the nucleus possessed a permeability barrier of its own, and was still capable of keeping out polyvinylpyrrolidone. We have found similar effects in spermatoctyes of other species, though the time relationships were different.

Attempts to purify the material by dialysis were usually unsuccessful because very great pressure was developed in the membrane. However, one dialysed sample was prepared by using several thicknesses of protective stocking (see Part III) in order to withstand this pressure. The properties of this sample did not, however, appear to differ greatly from those of undialysed solutions. Nor were better results obtained when the pH of the solution was adjusted to neutrality.

This material is thus disappointing as an immersion medium, and its unsuitability is all the more surprising in view of the fact that it has been so widely used clinically. It is only fair to point out that the concentrations employed in clinical practice, namely, about 3.5 per cent. are very much lower than we have used for immersion refractometry, so that gross cell damage is not necessarily likely to occur. Our results do, however, suggest that polyvinylpyrrolidone is capable of leaking into cells and a number of investigations have been reported in which histological changes have been found in various organs after the injection of massive doses of this substance into animals (Ammon and Müller, 1949). Weese (1951) made the interesting observation that after a first injection only about half the amount injected could be accounted for in the blood and very little was excreted. Examination of various organs revealed the presence of polyvinylpyrrolidone in amounts sufficient to account for the quantity injected. He made the significant suggestion that the substance became bound to plasma globulins and cell-wall globulins.

Thrower and Campbell (1951), on the other hand, found evidence of only a little interaction between polyvinylpyrrolidone and some protein films, though they did not investigate globulins. They did, however, observe that the material was slightly surface-active and lowered surface tension. Any interaction between polyvinylpyrrolidone and the proteins of the cell surface might very well account for the effects already described. The low concentrations used for clinical work may be insufficient to produce permanent damage, but in the presence of high concentrations, such as are necessary for immersion

refractometry, considerable disruption of the cell surface may occur on the submicroscopic scale. It may be that the proteins at the surface of the nuclear membrane differ in character and react much more slowly with polyvinylpyrrolidone. These suggestions are highly speculative, however, and much more detailed knowledge is required concerning the possible interaction of polyvinylpyrrolidone (and other substances of high molecular weight) with proteins.

#### *Acacia gum (gum-arabic)*

This substance is of considerable historical importance as it was one of the earliest to be employed as a blood substitute (Bayliss, 1917). It is also of special interest in that it was used for immersion refractometry by Fauré-Fremiet (1929), who carried out measurements on the amoebocytes of *Lumbricus*. He did not, however, make any further use of the method, nor did he control the tonicity of the medium.

Acacia gum is a natural product whose structure has been extensively investigated (see Hirst, 1942). It is an acid polysaccharide with a molecular weight in the region of 200,000 (Oakley, 1935). It is available commercially either in granular form or as a powder; the latter is more convenient for making solutions. When added to water, the gum swells at first and then goes into solution. Concentrations up to 50 per cent. can be reached, though with rather more difficulty than in the case of bovine plasma albumin. The viscosity of concentrated solutions, though high, is not excessive for refractometry. The pH is usually about 4.0, but can easily be adjusted to neutrality by the addition of alkali.

Amoebae survived quite well in concentrations below 15 per cent., but were killed in 3–5 minutes in 30 per cent. *Paramecium* remained active for more than 1 hour in 18 per cent., but became immobile and discharged their trichocysts in 30 per cent., though their cilia continued to beat. Reversal of contrast was obtained in spermatoocytes and other types of tissue-cells and unlike what was observed with polyvinylpyrrolidone such reversal appeared to be permanent and the cells remained, so far as could be judged, in good condition for some time. Spermatozoa continued to move actively in concentrations up to about 20 per cent. The movements were slower in higher concentrations, possibly because of the greater viscosity. Excellent results were obtained with fungal spores and mycelia even in concentrations up to 50 per cent., and identical values were found for the refractive indices of such material measured both in acacia and in bovine albumin. Germination of fungal spores was observed in acacia solutions without any added nutrient substances. The mycelia from such spores did not, however, attain a great length and no reproductive stages could be seen. This suggests that acacia is non-toxic to fungi, but is incapable of supplying their nutritional requirements. In contrast to this, acacia appeared to be quite unsuitable for use with bacteria, which never became reversed at any concentration. Mammalian red blood-cells too became grossly distorted.

Further purification was attempted by the process of dialysis followed by freezing-drying. The purified material behaved in a very similar manner to the crude gum, though the viscosity seemed to be somewhat less. Spermatozoa appeared to be rather more active and survived longer, but no better results were obtained with bacteria or red blood-cells.

It is thus evident that gum acacia is a promising material for use as an immersion medium for some types of cells. We have in fact used it extensively for work on fungi, for which it does not appear to be inferior to bovine albumin. It is also suitable for many types of tissue-cells which only require comparatively low concentrations not exceeding 25 per cent., but it causes distortion of blood corpuscles and other cells in higher concentrations. So far as can be judged at present, it is not a complete substitute for protein, but it may be adequate for certain purposes. Its great advantage over bovine albumin is its very low cost. It is in fact by far the cheapest of all the substances capable of being used as immersion media. The failure of acacia gum to cause reversal of contrast in bacteria is interesting and suggests that the bacterial cell wall is very different in constitution from that of fungi and tissue cells.

#### *Egg albumin*

A small quantity of fairly pure egg albumin was obtained from Dr. A. G. Ogston. This material was found to behave rather similarly to bovine albumin, though extended trials were not possible. Commercial pure egg albumin is prohibitively expensive and has not been investigated. Crude egg albumin is, however, available commercially at a very low price in the form of dried flakes. It is commonly used in histology for attaching sections to slides. Although it seemed likely that such albumin would be grossly denatured, we nevertheless investigated its properties. The flakes go into solution in water fairly readily and concentrations up to 50 per cent. can be attained. The viscosity is lower than that of bovine albumin, but the pH (5.0) is approximately the same. The solutions have a very disagreeable odour. The crude material appears to be hypertonic and toxic. Spermatozoa were killed rather rapidly and spermatocytes became shrunk and distorted. *Paramecium* was killed almost at once in 32 per cent., but some survived for up to 30 minutes in 20 per cent.

The crude egg albumin was purified by dialysis followed by freezing-drying. The resulting material could not be dissolved in distilled water, as a slow precipitation occurred. Satisfactory clear solutions could, however, be made in sodium chloride solutions exceeding about 0.4 per cent. in concentration. Such solutions were much less yellowish than the solutions of crude material, and all trace of the disagreeable odour disappeared. Since the purified egg albumin has to be dissolved in salt solutions, it is not very suitable for work on fresh water protozoa, but it has given good results with tissue-cells, spermatozoa, fungi, and bacteria. It is also one of the very few media which has given results with red blood-cells which are in any way satisfactory. We have not employed purified crude egg albumin very extensively because

much more work is needed in order to determine how far the properties of different samples are constant. Like crude acacia gum it may prove to be a valuable alternative to bovine albumin, though again not quite so generally useful.

#### *Bovine gamma globulins*

A commercial preparation of plasma globulins (fraction II) is available from Armour Laboratories. This material has to be dissolved in dilute salt solution and appears to be highly soluble (up to at least 40 per cent.). It has given good results with tissue-cells and blood-cells. It is slightly more expensive than bovine albumin and its only advantage may be that the pH of solutions is a little on the alkaline side of neutrality. Extensive tests have not been carried out.

#### *Carboxyhaemoglobin*

As the preliminary observations which led to the development of this technique were carried out on cells immersed in haemoglobin solutions, it seems natural the latter should be used as an immersion medium. Unfortunately oxyhaemoglobin is not very stable and becomes converted fairly rapidly to methaemoglobin. Carboxyhaemoglobin is, however, more stable though less soluble. A sample of frozen-dried carboxyhaemoglobin (of sheep) was kindly supplied by Dr. A. G. Ogston. This went into solution readily, but when examined under the microscope was found to contain numerous minute particles. It is possible that the material may have been partly denatured. The presence of the particles did not greatly interfere with its use for immersion refractometry. Good results were obtained with tissue cells, amoebae, various Protozoa, and particularly with spermatozoa, which survived extremely well for long periods. An important advantage of haemoglobin over bovine albumin may be that its isoelectric point is close to pH 7; on the other hand, its deep red colour is a disadvantage and may make the determination of refractive index difficult when high concentrations are used. The main reason for not using it more extensively, however, is that no suitable preparation seems to be available commercially. Crude technical haemoglobin is available, but this was found to be quite unsatisfactory as it contained much insoluble matter.

#### *Other substances*

Several other materials were investigated, but were found to be unsuitable for various reasons. Dextrin and 'soluble' starch were insufficiently soluble. Preparations of pectin, soluble alginates, and methyl cellulose gave excessively viscous solutions. Technical grade blood albumin contained much insoluble matter. None of these materials were examined in detail.

### BOVINE PLASMA ALBUMIN

Almost the first substance used as an immersion medium was a preparation of bovine plasma albumin fraction V, manufactured in the form of a powder

by Armour & Co. (Barer and Ross, 1952). This material has been used more than any other and has been found suitable for a very wide range of cells. Indeed no type of cell has yet been found in which it has been impossible to produce reversal of contrast in at least some regions.

The Armour product is prepared according to the method of Cohn and others (1946), which involves successive precipitation of plasma at low temperatures by ethyl alcohol in the presence of acetic acid and sodium acetate. The fifth precipitate contains almost all the plasma albumin. This fraction is further purified and re-precipitated by alcohol at pH 5.2, in order to remove most of the acetic acid. The final material is an amorphous powder which according to the makers' brochure contains 3-5 per cent. globulins, less than 2 per cent. ash, and less than 6 per cent. moisture. More accurate figures were obtained from the Research Division of Armour & Co., who stated that the ash content is usually less than 1 per cent. and the moisture 2-3 per cent. There are usually less than 50 parts per million of heavy metals present. The ash consists mainly of sodium acetate and sodium chloride. Every attempt is made to keep the extraction procedure constant and only minor variations are to be expected between different batches. We have used many batches of fraction V over the last 3 years and with the exception of two batches which gave anomalous results with red blood-cells (though normal results with other types of cells) we have found its properties to be remarkably constant, at least as far as the present technique is concerned.

A crystalline preparation of bovine plasma albumin is also available from Armour & Co. This is almost free from globulins and contains less ash. It is very much more expensive than fraction V and seems to offer no special advantages for refractometry. We have also used human plasma albumin with excellent results.

Fraction V can be dissolved in water or salt solution to give a clear, faintly yellow solution. The pale yellow colour is due to a component with an absorption band at  $405\text{ m}\mu$ , possibly a flavine or flavoprotein present in very small amounts. The pH of the solution is approximately 5.2-5.5, which is a little higher than the isoelectric point of plasma albumin (5.0). Full details of the method used for making up solutions for different purposes are given in the section on technical methods in Part III. At this stage, however, it is necessary to discuss in some detail the osmotic properties of the immersion medium with particular reference to fraction V.

#### OSMOTIC PROPERTIES OF THE IMMERSION MEDIUM

If accurate quantitative measurements of concentration are to be made, it is essential that the cell volume should be identical in the immersion medium and in life. This is a condition which applies to all techniques for the determination of concentration of any constituent of living cells, and is not peculiar to refractometry alone. There is no absolute certainty that the cell volume remains unchanged when cells are removed from the body and examined

under the microscope or otherwise manipulated, and so far as can be seen there is no method available for measuring such changes if they occur. All one can do is to take the volume occupied by the cell in some accepted 'physiological' medium as a standard. This sounds simple, but in fact it raises a host of important questions which have received little consideration in the past. In the first place, what is an accepted 'physiological' medium? Many such media have been suggested, varying considerably in salt composition, pH, and osmotic properties. A valuable summary of twelve basic solutions used for mammalian tissue-culture work has been compiled recently by Stewart and Kirk (1954). It is remarkable how these media differ. The sodium chloride content, for example, ranges from 0.68 per cent. to 1.5 per cent. Stewart and Kirk have calculated the total particle concentrations of these media, i.e. the concentrations of ions and molecules after assessing the degree of dissociation of various constituents. This quantity should be proportional to the osmotic effect of the medium. The figure 0.307 M was obtained for human plasma, but although a few of the media approximated to this, the range extended from 0.294 M to 0.534 M. Even solutions advocated by the same workers showed wide variations in concentration. Thus Lewis's media extend from 0.354 M to 0.534 M. The calcium Ringer of Vogelaar and Erlichman (quoted by Stewart and Kirk) has a concentration of 0.455 M whereas that of the magnesium Ringer of the same authors is only 0.294 M. A study of the classical paper of M. R. and W. H. Lewis (1911) reveals even more dramatically what wide variations in salt content are compatible with cell growth. They cultured chick embryo tissues (liver, intestine, kidney, heart, and spleen) in hanging drop preparations, using only salt solutions of known composition. In the majority of these experiments the concentrations of calcium chloride (0.025 per cent.), potassium chloride (0.042 per cent.), and sodium bicarbonate (0.02 per cent.) were kept constant, while the sodium chloride content was varied over a wide range. At one extreme 'good' growth was obtained with 0.45 per cent. sodium chloride, at the other 'extensive' growth was obtained with 1.585 per cent. sodium chloride and 'slight' growth even occurred in 1.6975 per cent. sodium chloride. As Lewis and Lewis themselves point out, 'it is quite remarkable that cells will grow in such widely different solutions where the osmotic pressure must vary considerably'. It will be seen from these figures that chick embryo cells are capable of active growth over about a threefold range of osmotic pressure. It would be interesting to know whether cells grown in such diverse media are similar in volume and general form, but Lewis and Lewis do not discuss this. We are attempting to repeat these experiments in order to see if the solid content of the cytoplasm of such cells is affected by the osmotic pressure of the culture medium.

A study of various 'physiological' media suggested for invertebrates shows even greater variations than in mammalian media. Extensive compilations of chemical analysis of the bloods of many species have been made by Prosser and others (1950), Heilbrunn (1952), and Buck (1953). Such tables show considerable differences both in the relative proportions of constituents and

in the total osmotic concentration. For example, the depression of the freezing-point of the blood of fresh-water molluscs is generally between  $0.1^{\circ}$  and  $0.2^{\circ}$  C., whereas that for some fresh-water crustacea exceeds  $1^{\circ}$  C. Very large differences occur between various species of insects (Buck, 1953), and the salt solutions suggested do not always approximate in composition to that of the blood. Probably few investigators go to the length of making up special media for each individual species; there is a tendency to use so-called 'amphibian' Ringer or 'insect' Ringer, &c., but there is strictly no such thing as an average medium identical in composition with the blood of a wide range of species. The fact that cells survive and even thrive in such media probably only shows that they are capable of adjusting themselves to quite gross changes in composition and osmotic pressure.

#### *Blood and tissue fluids*

It seems pedantic to question whether blood is a truly physiological medium, but it must be remembered that the majority of cells are not surrounded by blood, but by tissue fluid, the composition of which is largely unknown. It is generally assumed that tissue fluid resembles lymph (Drinker and Yoffey, 1941), though clear evidence is lacking because of the difficulty in collecting tissue fluid even in large animals.

Lymph and certain other body fluids are said to be basically plasma 'ultra-filtrates' and are generally assumed to have very nearly the same composition as plasma itself, but with less protein. Even if such fluids were true ultra-filtrates, however, their constitution would inevitably differ to some extent from that of plasma because the presence of non-diffusible protein ions would lead to a Donnan equilibrium resulting in a greater concentration of negative ions ( $\text{Cl}^-$  and  $\text{HCO}_3^-$ ) and a lower concentration of positive ions ( $\text{Na}^+$ ,  $\text{K}^+$ ) in the ultra-filtrate than in the plasma itself.

The analysis of lymph is more or less that to be expected of an ultra-filtrate. The protein content of lymph for different parts of the body varies slightly and corresponding differences occur in the ionic composition. The chloride content of the thoracic duct lymph of the dog is given by Drinker and Yoffey (1941) as 396 mg. per 100 c.c. as compared with 369 mg. per 100 c.c. for serum. Much larger differences are found in the cerebrospinal fluid. In man the chloride content is 440 mg. per 100 c.c. as compared with 360 mg. per 100 c.c. in plasma. The sodium content is 324 mg. per 100 c.c. as compared with 316 mg. per 100 c.c. for plasma. The calcium content of cerebrospinal fluid, however, is only half that of plasma (Merritt and Fremont-Smith, 1937). It is generally believed that cerebrospinal fluid is not simply ultra-filtrate but in part at least a secretion. A similar opinion is now held concerning the aqueous humour (Duke-Elder and Goldsmith, 1951). The osmotic pressure of aqueous humour is actually greater than that of the blood. Until more exact figures are available for the composition of tissue fluid, the belief that blood constitutes a true physiological medium for tissue-cells must remain an approximation and it is a matter for conjecture whether

the size and behaviour of tissue-cells will necessarily be the same in blood as in life. However, for the present at any rate, blood probably remains the nearest practical approach to a true physiological medium.

#### *The osmotic behaviour of cells*

There is an extensive literature which deals with the volume changes undergone by certain types of cells in solutions of different ionic strengths. Unfortunately, nearly all such work has been carried out on only two types of cell, namely, egg-cells (usually echinoderm) and mammalian red blood-cells. Even in the case of this limited material much essential information is lacking. The mammalian red cell is very atypical both as regards its structure and its osmotic behaviour. It appears to be far more sensitive to osmotic changes in the medium than are most tissue-cells or white blood-cells. Echinoderm eggs may be more typical in these respects, but an examination of the literature shows that almost no experiments have been carried out on their volume changes in media which depart only slightly from isotonicity. As a rule volume changes have only been measured in rather grossly hypotonic or hypertonic media with the object of determining the laws connecting osmotic pressure and volume. For some cells the relationship  $P(V-b) = \text{constant}$  has been found to hold.  $P$  is the osmotic pressure of the solution.  $V$  is the cell volume, and  $b$  is a characteristic constant which can be regarded as the volume occupied by the osmotically inactive constituents of the cell.  $b$  is generally about 20–30 per cent. of the resting cell volume. By using this equation it is possible to extrapolate for osmotic pressures in the region of isotonicity; because of the factor  $b$  it will be seen that a given change in  $P$  produces a relatively smaller change in  $V$ , so that, for example, halving  $P$  would not double the cell volume. It is not at all certain, however, in view of the lack of experimental evidence, that this formula can be used for approximately isotonic media. We shall discuss the possibility below that individual cells may be capable of some degree of osmotic regulation in approximately isotonic media.

Shapiro and Parpart (1937) have measured the diameters of human and rabbit leucocytes in media of different osmotic pressures. The volume in Ringer-Locke solution was taken as unity. The results plotted in figure 1 of their paper are said to show a more or less linear relationship between  $V$  and  $1/P$ . On closer inspection, however, it will be seen that  $V$  scarcely changes between  $1/P = 1$  and  $1/P = 1.5$ , in other words, over a range in which the osmotic pressure exceeds about two-thirds of the standard pressure. These results are in fact compatible with the hypothesis that regulation of volume takes place over a fairly narrow range of concentrations, but that such regulation breaks down beyond certain limits. Investigations of this sort are not too difficult in the case of spherical cells, the diameters of which can be measured, but there are obvious difficulties in working with irregular cells. Brues and Masters (1936) estimated the volume of fibroblasts and sarcoma cells in tissue culture by regarding them as symmetrical spindles and measuring the



diameter at different points along the length of the cells. The behaviour of these cells was found to be rather variable when they were transferred from plasma to 0.9 per cent. sodium chloride. As a rule they swelled a little, but occasionally showed slight shrinkage. The mean change in volume in fibroblasts was +13.7 per cent., in sarcoma cells +4.3 per cent. The relationship  $P(V-b) = \text{constant}$  was found to hold reasonably well with  $b = 22$  per cent. for fibroblasts, and  $b = 26$  per cent. for sarcoma cells. The change in volume on transferring to a so-called isotonic salt solution is of some significance, and illustrates another of the difficulties inherent in the task of defining a 'physiological' medium. Shear and Fogg (1934) investigated the behaviour of tumour cells and normal cells from various organs when placed in saline media. They found that swelling occurred in so-called isotonic sodium chloride and 'physiological' salines. Swelling even took place in hypertonic solutions! Variation of pH between 5 and 10 did not appear to affect this swelling very much, though it was a little more marked in alkaline media. The addition of potassium, calcium, magnesium, and other salts also made no difference. The only significant finding was that swelling occurred more slowly in serum. Following up this clue, Shear (1935) found that swelling was retarded by egg albumin, gelatin, casein, serum proteins, and acacia. Some of the proteins used were even capable of causing reversal of swelling with return towards normal volumes.

All these facts point to the great difficulty of virtual impossibility of determining the true volume of the cell in life. One can attempt to measure the cell volume in blood or some other natural fluid or one can measure it in some arbitrarily selected salt solution or 'physiological' saline, always remembering that the volume in the latter may not be the same as in blood or in the body.

#### *What is an isotonic solution?*

There is much confusion in the literature and particularly in textbooks as to the definition of the term 'isotonic'. It is quite clear that what is often called an *isotonic* solution is really *isosmotic*, i.e. one having the same osmotic pressure or freezing-point depression as some standard medium such as plasma. Osmotic pressure is the physical property which can be measured without reference to the behaviour of living cells. Tonicity, on the other hand, is a biological concept which can only be referred to the properties of living cells. It would, for example, be ridiculous to call a solution of mercuric chloride or potassium cyanide 'isotonic' simply because it had the same osmotic pressure as plasma. The first essential point in discussing tonicity therefore is that the medium must not be injurious to living cells. Even in such cases, however, the special permeability properties of the cell must be taken into account. Thus, to quote a simple example given by Heilbrunn (1952, p. 127), a 0.53 M solution of sodium chloride and a 0.37 M solution of calcium chloride are both isosmotic with sea-water. Despite this, sea-urchin eggs do not change in volume when immersed in the sodium chloride solution, but shrink in the calcium chloride solution. The volume is unaltered in 0.30 M calcium

chloride. The latter solution is thus isotonic (but not isosmotic) for these particular cells, though not necessarily for others. Considerations of this sort lead to a simple comprehensive definition of isotonicity. *Two solutions are said to be isotonic for a given type of cell if (a) they are compatible with life and (b) the cell volume is the same in each.* This definition is independent of variables such as differences in composition, relative proportions of ions, and pH, and it does not depend on the total osmotic pressure of the solutions. This is the only satisfactory practical definition for work on immersion refractometry in which the really important factor is the constancy of cell volume. Osmotic pressures either calculated theoretically or determined by freezing-point measurements may not be entirely satisfactory and we have usually adopted the empirical method of adjusting the salt content of our media until the mean cell volume is the same as in a standard medium. Results obtained in this way have, as a matter of fact, agreed rather well with experimental freezing-point determinations.

#### *Adjustment of tonicity of immersion media*

The method used is essentially that developed by Ross (1953) in his study of the volume changes of cells during fixation. We are indebted to Mr. Ross for most of the results quoted in this paragraph. The basis of the method is to determine frequency-polygons for cell size in different media. Cell diameters were determined by means of an eyepiece micrometer. As a rule measurements were carried out on not less than 50 cells in each medium. The first cells tested were the primary spermatocytes of *Helix aspersa*. Attempts to measure the distribution of cell diameter in snail-blood gave variable results, probably because of contamination with mucus and digestive fluids. Hédon-Fleig's solution was then used and gave virtually identical results with those obtained in a simpler medium containing only 0.7 per cent. sodium chloride and 0.02 per cent. calcium chloride. Histograms were then derived for cells in a range of sodium chloride concentrations varying from 0.3 per cent. to 1.0 per cent. A remarkable finding was that the general shape and modal value of the histogram was virtually unaltered over a surprisingly wide range of salt concentration; in fact, between 0.5 per cent. and 0.8 per cent. Significant swelling occurred in 0.4 per cent. and shrinkage in 0.9 per cent. Nuclear diameters were also measured with very similar results. The next stage was to make up a 20 per cent. protein solution of bovine plasma albumin fraction V in distilled water and to determine frequency distribution curves of the same type of cells in this medium. The cells were found to be considerably swollen with a modal value of  $24\mu$  for the diameter as compared with  $19\mu$  in Hédon-Fleig or 0.7 per cent. saline. This frequency distribution corresponded quite well to those obtained in 0.2 per cent. and 0.15 per cent. sodium chloride (fig. 1). On this evidence, therefore, it appeared that a 10 per cent. fraction V solution in distilled water would be equivalent in tonicity to a sodium chloride concentration of between 0.075 per cent. and 0.1 per cent. Taking the latter value as a convenient round figure, a 20 per cent. protein solution

would require the addition of 0.5 per cent. of sodium chloride in order to make it isotonic with 0.7 per cent. salt solution. Histograms for spermatoocytes in 20 per cent. protein in 0.5 per cent. sodium chloride and also in 20 per cent. protein in 0.6 per cent. sodium chloride are shown in fig. 1.

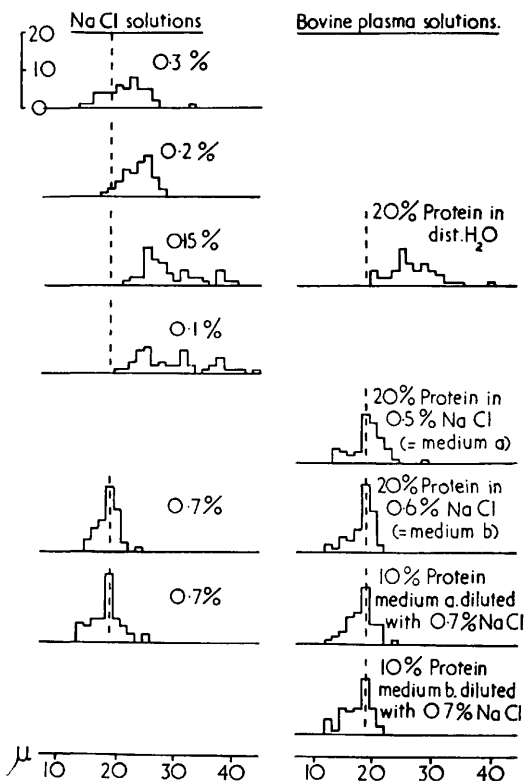


FIG. 1. Histograms showing diameters of snail spermatoocytes in various media. For explanation see text. Ordinates, number of cells; abscissae, cell diameters.

These are very similar to those obtained in 0.7 per cent. protein-free sodium chloride. Each of these media was then diluted with an equal volume of 0.7 per cent. sodium chloride, giving 10 per cent. protein solutions. The histograms obtained in these media were again closely similar to those in 0.7 per cent. sodium chloride. Thus within the limits of accuracy of the method the simple rule that a 10 per cent. protein concentration is approximately

equivalent to a 0.1 per cent. sodium chloride concentration appears to be justified. A 20 per cent. protein solution is sufficiently concentrated to cause reversal of contrast in the cytoplasm of most tissue-cells. In special cases it may be necessary to use much more concentrated solutions, and here it is possible that slightly greater errors may be introduced. If, for example, a 10 per cent. solution of protein were equivalent in tonicity to 0.08 per cent. sodium chloride instead of 0.1 per cent. the error in working with a 20 per cent. protein solution dissolved in 0.5 per cent. sodium chloride would only be 0.04 per cent. of sodium chloride, assuming 0.7 per cent. sodium chloride to be isotonic. If, on the other hand, a 40 per cent. protein solution were used, dissolved in 0.3 per cent. sodium chloride, the error would be equivalent to 0.08 per cent. sodium chloride. In view of the fact that snail spermatocytes appear to maintain the same size over quite a wide range of salt concentrations, neither of these errors is likely to be of much importance with these particular cells. It may be, however, that other cells are more sensitive to salt concentration, and if in addition they require the use of concentrated protein solutions, the errors may be significant. This may be the case with mammalian red blood-cells, which are very sensitive to small changes in salt concentration and also have high refractive indices, requiring the use of protein solutions of up to 40 per cent. concentration. It is generally stated that 0.9 per cent. sodium chloride is isotonic for mammalian cells, though Ponder (1948) takes 1 per cent. as isotonic for red cells. According to the simple rule given above a solution of 40 per cent. bovine plasma albumin in 0.5 per cent. sodium chloride should be isotonic with 0.9 per cent. sodium chloride, but the values for mean corpuscular haemoglobin concentration obtained by cell refractometry with such a solution are 10–20 per cent. lower than the accepted clinical values. If a 40 per cent. protein solution dissolved in 0.6 per cent. sodium chloride is used, the results agree with the lower normal clinical values, while if 0.7 per cent. sodium chloride is used instead of 0.6 per cent., the results are in excellent agreement with clinical values. Recent freezing-point determinations by Dr. D. A. T. Dick have shown that a 10 per cent. bovine plasma albumin solution in distilled water is actually equivalent to 0.08 per cent. sodium chloride. On this basis therefore a 40 per cent. protein solution should be made up in 0.6 per cent. sodium chloride, if 0.9 per cent. sodium chloride is taken as isotonic; or in 0.7 per cent. sodium chloride, if Ponder is correct in taking 1 per cent. sodium chloride as isotonic. With the majority of cells, apart from red blood-cells, these differences are unimportant and the simple rule given above can be used.

*Are individual cells capable of osmotic regulation?*

Much work has been done on the osmotic regulation and water balance of whole organisms, both vertebrate and invertebrate. On the other hand, almost nothing seems to be known about the osmotic regulation of individual tissue-cells. The possibility that such cells may be capable of regulating their volume and water content to some extent at least cannot be ignored. If the

concept of the constancy of the internal environment has any general validity, it might be expected that it would be advantageous for the cell to maintain a more or less constant volume and water content, thus ensuring that the concentrations of enzymes, nucleic acids, and other substances essential for normal metabolism, growth, and cell-division should be kept within fairly well defined limits. It may be argued that in mammals at least the external environment of the cell is kept fairly constant by various mechanisms (e.g. respiratory exchanges and renal excretion), which regulate the composition of the blood. Some of these mechanisms, such as changes in renal excretion, only act slowly, however, and cells may very well be exposed to quite large temporary fluctuations in the composition of tissue fluid. Thus Hill, Long, and Lupton (1924) showed that enormous quantities of lactic acid may be liberated in severe exercise, and blood concentrations of over 100 mg. per 100 c.c. may be attained. The local changes in the immediate vicinity of the tissues may perhaps be much greater. That too much faith should not be placed in the constancy of blood composition in an individual has been shown by Schreider (1953). He carried out a statistical investigation on the reliability of a number of physiological and biochemical characteristics and showed that some of these were so variable that in order to determine a modal value, 15-20 measurements on different days might be needed for one individual. Schreider reached the disturbing conclusion that 'the postulate of fixity must be rejected on biometrical grounds'. This does not necessarily mean that the internal composition of cells varies greatly or that there is any immediate correlation between intracellular conditions and fluctuations in blood composition. Schreider himself has pointed out that living organisms contain many internal environments separated by barriers which may serve to damp out fluctuations. In terms of the present problem such barriers might constitute a regulating mechanism whereby the cell volume is maintained relatively constant in the face of quite large variations in composition of the external medium. It must be admitted that direct evidence for osmotic regulation in single cells is very scanty. The observations of Ross (1953) on snail spermatozoocytes and of Shapiro and Parpart (1937) on mammalian leucocytes suggest that some degree of regulation does occur over a surprisingly large range of salt concentrations. It must be remembered, however, that these workers estimated cell volumes by measuring the cell diameters with a micrometer eyepiece. It is doubtful if such measurements of diameter can be made with an accuracy greater than about 2.5 per cent., corresponding to  $\pm 0.5\mu$  in a diameter of  $20\mu$ . The error in cell volume is therefore three times this amount or 7.5 per cent. Many more measurements of this type on a variety of cells would be desirable. Much evidence which may have an important bearing on this question has been assembled in a valuable review by Robinson (1953) on the active transport of water in living cells. Robinson propounds the view that the cell is in a state of dynamic equilibrium and that metabolic processes involving energy changes are necessary for maintaining a constant cell volume. The cell contents appear to have a higher osmotic pressure than

plasma and are not in thermodynamic equilibrium with it. There is an impressive body of facts to show the correlation between cell volume and respiratory processes; if the latter are depressed by the action of poisons, cellular swelling occurs in individual cells, and in whole animals the extracellular fluids become more concentrated. Many effects of this type are reversible. Further evidence in support of these views has been provided by

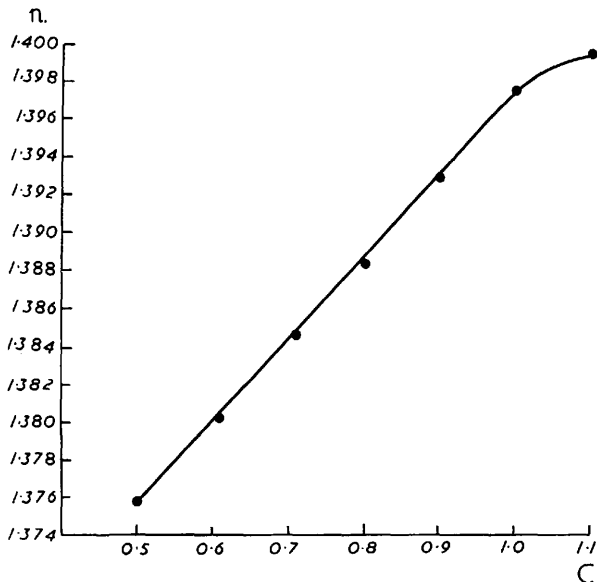


FIG. 2. Relationship between mean refractive index ( $n$ ) of human red blood-cells and equivalent salt concentration of protein medium expressed in terms of grams per cent. of sodium chloride (C).

the work of Krebs and his school (for references see Bartley, Davies, and Krebs, 1954). This concept certainly fits in with observations made during the course of the present work, for example the swelling of cells found in the first stages of autolysis in sealed slide-coverslip preparations. From the point of view of immersion refractometry the existence of some degree of osmotic regulation would be extremely useful, as it would allow for some margin of error in making the immersion exactly isotonic. The presence of protein in the medium may itself be a stabilizing factor in view of Shear's (1935) finding that swelling in salt media was retarded or reversed by several proteins.

The refractometric method can itself be used to investigate this problem. As a result of swelling or shrinkage, the concentration of solids and hence

the refractive index will change; the reaction of the cell to changes in salt concentration can therefore be studied by measuring its refractive index in bovine plasma albumin solutions to which different amounts of salt have been added. Fig. 2 shows some preliminary results obtained by Miss F. M. Gaffney on human red blood-cells. The ordinates are the mean refractive indices of cells of one individual, the abscissae being the salt content of the medium expressed in terms of equivalent sodium chloride concentration. The relationship is virtually linear between 0.5 and 1.0 per cent. of salt, but there is considerable deviation from linearity above 1.0 per cent. This is only to be expected, because the cell contents can obviously only be packed down to a limited extent. Thus over the range studied there appears to be no indication of any special regulatory ability on the part of the cell. This is in keeping with our knowledge of the behaviour of red blood-cells. We have, however, carried out a few experiments on lymphocytes and have found evidence of a plateau in the region between salt concentration 0.8 per cent. and 1.0 per cent. This may suggest some degree of regulation. Further work on the problem is in progress.

#### EVIDENCE FOR NON-TOXICITY OF THE MEDIUM

It is extremely difficult to provide clear evidence that a medium is completely harmless to all types of cells. In the case of cells which are non-motile and which do not ordinarily undergo division, the evidence must rest mainly on the general appearance of the cell before and after immersion and on the reproducibility of refractive index measurements. Provided that mechanical damage and compression which often lead to swelling of the cell are avoided, repeated estimations of refractive index should give very reproducible results. Recently Dick (1955), working in this laboratory, has developed a simple perfusion chamber which enables cells to be irrigated with many changes of fluids of different composition. Even after more than ten changes of protein or gum acacia, cultures of chick fibroblasts or snail amoebocytes often remained in excellent condition and gave reproducible values for refractive indices.

The assessment of damage is perhaps easier in the case of motile or dividing cells. The evidence may be summarized as follows:

##### *Protozoa*

Most motile protozoa appear to tolerate bovine plasma albumin dissolved in distilled water quite well, particularly at concentrations below about 20 per cent. At higher concentrations motility decreases or may be abolished altogether. It is not always possible to decide whether this is due to a toxic action or simply to the resistance to motion offered by the viscous medium. In some cases normal motility is recovered when the organisms are transferred from a very concentrated solution to water. One cannot, however, exclude the possibility that concentrated protein solutions may exert some sort of surface action or may hinder the diffusion of gases or nutrient materials

into or out of the cell. Occasionally one comes across organisms which do not appear to tolerate the acidity (pH 5.2–5.5) of ordinary albumin solutions. Better results may be obtained in such cases by using dialysed pH-adjusted albumin.

In general, amoebae appear to be more sensitive to protein than most other Protozoa studied, but even so they remain in good condition in concentrations below about 15 per cent. for about 30 minutes. Since most amoebae are reversed in contrast in such concentrations, it is not usually necessary to use higher ones. Slightly longer survival can be obtained with pH-adjusted protein. *Euglena*, *Stylonychia*, and *Rhabdostyla* all appeared to tolerate concentrations up to about 30 per cent. of ordinary albumin and 40 per cent. or more of pH-adjusted albumin. *Chylodon* and *Podophrya* appeared in good condition in up to 40 per cent. of ordinary albumin. The parasitic forms *Trichomonas muris* and *Giardia* were strongly reversed and in good condition in 30 per cent. ordinary albumin. *Vorticella*, on the other hand, though it tolerated fairly low concentrations, usually underwent a strong contraction in high concentrations of even pH-adjusted albumin.

#### *Ciliated epithelial cells*

Scrapings from the frog respiratory passages contain ciliated cells which continued to exhibit vigorous ciliary movement for long periods in concentrations of ordinary albumin which bring about strong reversal of the cytoplasm.

#### *Spermatozoa*

The spermatozoa of a very large number of species ranging widely over the animal kingdom have been examined. In concentrations of ordinary albumin not exceeding about 10 per cent. there is scarcely any noticeable effect on sperm motility, and cells survive in apparently good condition for long periods. Human spermatozoa, for example, will survive up to about 3 days in a 5 per cent. albumin solution. In higher concentrations motility is reduced, probably because of increasing viscosity. The structural components of most sperm-heads are very dense and require high concentrations (generally between 35 and 55 per cent.) of albumin in order to bring about reversal. For this reason sperms which are reversed in contrast are usually immotile or very feebly motile. Of the various media tested, only bovine plasma albumin and dialysed egg albumin gave satisfactory results with spermatozoa.

#### *Amoebocytes*

The amoebocytes and blood-cells of several species of invertebrates have been transferred to the protein medium either directly from the blood or body fluids or from tissue cultures. Photomicrographs of an earthworm cell undergoing active movements in a protein solution sufficiently concentrated to cause strong reversal of contrast were shown in fig. 1 of the first paper of this



series (Barer and Joseph, 1954). In general, such cells appear to tolerate both ordinary and pH-adjusted albumin quite well and promising results have recently been obtained with acacia gum.

#### *Mammalian leucocytes*

Good motility has been observed in mammalian white cells kept for quite long periods in albumin solutions. A remarkable example of their hardiness was found by chance in a preparation of rat leucocytes which had been examined for several hours in a protein concentration sufficient to bring about reversal of contrast. The sealed slide-coverslip preparation was left overnight on the microscope stage at room temperature. The next morning, on glancing casually down the microscope we were astonished to find many leucocytes still undergoing active movement across the field of view.

#### *Growth of fungal spores*

Excellent germination and development of the spores of many different types of fungi has been observed even in high concentrations of albumin. The complete life-cycle from germination through the sexual and asexual phases to the formation of new spores has been followed in slide-coverslip preparations (Barer and Joseph, 1955). No nutrient substances were added to the protein medium. The ability to support the growth of fungi is in some respects a disadvantage because stock protein solutions frequently become contaminated with yeasts or moulds after about 5 days even if kept in a refrigerator. Spore germination has also been observed in acacia gum solutions, but further growth and development are slower and do not usually reach so complete a stage as in protein.

#### *Cell division*

Perhaps the strongest evidence which suggests the comparative harmlessness of bovine plasma albumin is the fact that normal cell division can be observed in it. Occasional divisions have been seen in the germ cells of the locust (Ross, 1954) and we have also observed the complete cycle in the protozoon *Cothurnia*. Such divisions are, however, comparatively infrequent and sporadic. Much more successful results have been obtained with grasshopper spermatocytes, and one can be reasonably certain of finding active division in several cells of every single preparation. Typical photographs of this process are shown in fig. 3. In some cases divisions have been followed in the same preparation for as long as 3 days. All our experiments have been carried out so far with ordinary albumin at pH 5.5 and without the addition of nutrient substances. Experiments to determine whether the rate of division can be influenced by such substances are in progress.

Regarded as a whole, the evidence quoted above, though not as complete as one could wish, nevertheless suggests that at least for a very large number of different types of cells the albumin medium and in some cases acacia gum, are comparatively innocuous.

We again wish to thank the Rockefeller Foundation, Royal Society, and Medical Research Council for making this work possible. We also wish to acknowledge the help of many colleagues, particularly Dr. D. A. T. Dick, Miss F. M. Gaffney, and Mr. K. F. A. Ross. Dr. A. G. Ogston has kindly supplied several protein samples and Dr. W. E. van Heyningen and Dr. E. Bidwell provided facilities for freezing-drying.

## REFERENCES

- AMMON, R., and MÜLLER, W., 1949. *Dtsch. med. Wschr.*, **74**, 465.  
 BARER, R., and JOSEPH, S., 1954. *Quart. J. micr. Sci.*, **95**, 399.  
 ——— 1955. In course of publication.  
 ——— and ROSS, K. F. A., 1952. *J. Physiol.*, **118**, 38P.  
 BARTLEY, W., DAVIES, R. E., and KREBS, H. A., 1954. *Proc. Roy. Soc. B*, **142**, 187.  
 BAYLISS, W. M., 1917. *Ibid.*, **89**, 380.  
 BENNETT, A. H., JUPNIK, H., OSTERBERG, H., and RICHARDS, O. W., 1951. *Phase microscopy*. New York (John Wiley).  
 BRUES, A. M., and MASTERS, C. M., 1936. *Amer. J. Cancer*, **28**, 314.  
 BUCK, J. B., 1953. In *Insect physiology*. Ed. Roeder, K. D. New York (Wiley).  
 COHN, E. J., STRONG, L. E., HUGHES, W. L., MULFORD, D. J., ASHWORTH, J. N., MELIN, M., and TAYLOR, H. L., 1946. *J. Amer. Chem. Soc.*, **68**, 459.  
 CROSSMON, J. C., 1949. *Stain Techn.*, **24**, 244.  
 DAVIES, H. G., WILKINS, M. H. F., CHAYEN, J., and LA COUR, L. F., 1954. *Quart. J. micr. Sci.*, **95**, 271.  
 DICK, D. A. T., 1955. *Quart. J. micr. Sci.* (in the press).

FIG. 3 (plate). Photomicrographs showing first meiotic division of primary spermatocytes of the grasshopper *Chortippus parallelus*. Division is taking place in a 12 per cent. solution of bovine plasma albumin (equivalent tonicity 1.1 per cent. sodium chloride). A, diakinesis (late stage). B, diakinesis, shortly before disappearance of nuclear membrane. Note bright nucleoplasm (25 min.). C, early disruption of nuclear membrane (1 hour 25 min.). D, complete disruption of nuclear membrane. Chromosomes have moved to periphery of cell (1 hour 27 min.). E, prometaphase. Chromosomes beginning to line up on equatorial plate. Some faint spindle-fibres can be seen (2 hours 27 min.). F, prometaphase. Two dark centrosomes are visible (2 hours 40 min.). G, late prometaphase. Thick spindle-fibres visible (4 hours 30 min.). H, metaphase. Distinct spindle-fibres. Chromosomes and spindle are displaced by a crescentic mass of mitochondria (7 hours 6 min.). I, early anaphase. Chromosomes beginning to separate. Mitochondria are now clustered on each side of the spindle. Note large bright zone around chromosomes. The outline of the cell shows irregular bulging. (25 hours 47 min.). J, anaphase. The centrosome and spindle-fibres can be seen. Note also prominent polar bulging and central bright zone (26 hours 5 min.). K, advanced anaphase. Mitochondria are beginning to form 'sheaves'. Vigorous polar bulging with elongation of cell (26 hours 15 min.). L, telophase. Incomplete cleavage. Note mitochondrial sheaves, from the ends of which granules appear to be forming. Bulging in equatorial and polar axes (26 hours 40 min.). M, cleavage almost complete. Cells still linked by mitochondrial sheaves and remains of spindle. Granules at ends of sheaves more distinct (26 hours 51 min.). N, separation of daughter-cells which are still linked by a mitochondrial bridge (28 hours 13 min.). O, division complete. Daughter-cells transformed into secondary spermatocytes (48 hours 35 min.).

All photographs were taken with a Zeiss Winkel phase-contrast microscope with 4 mm. fluorite objective of N.A. 0.75. Length of scale, 25  $\mu$ .

Times from the beginning of observations are given in brackets. It should be stressed that these cells are spherical and in no way compressed. With the microscope used, similar cells in a saline medium give very poor images in which almost no internal detail can be made out. A discussion of this effect and further examples will be found in Barer, R., *Naturwiss.*, **41**, 206 (1954).

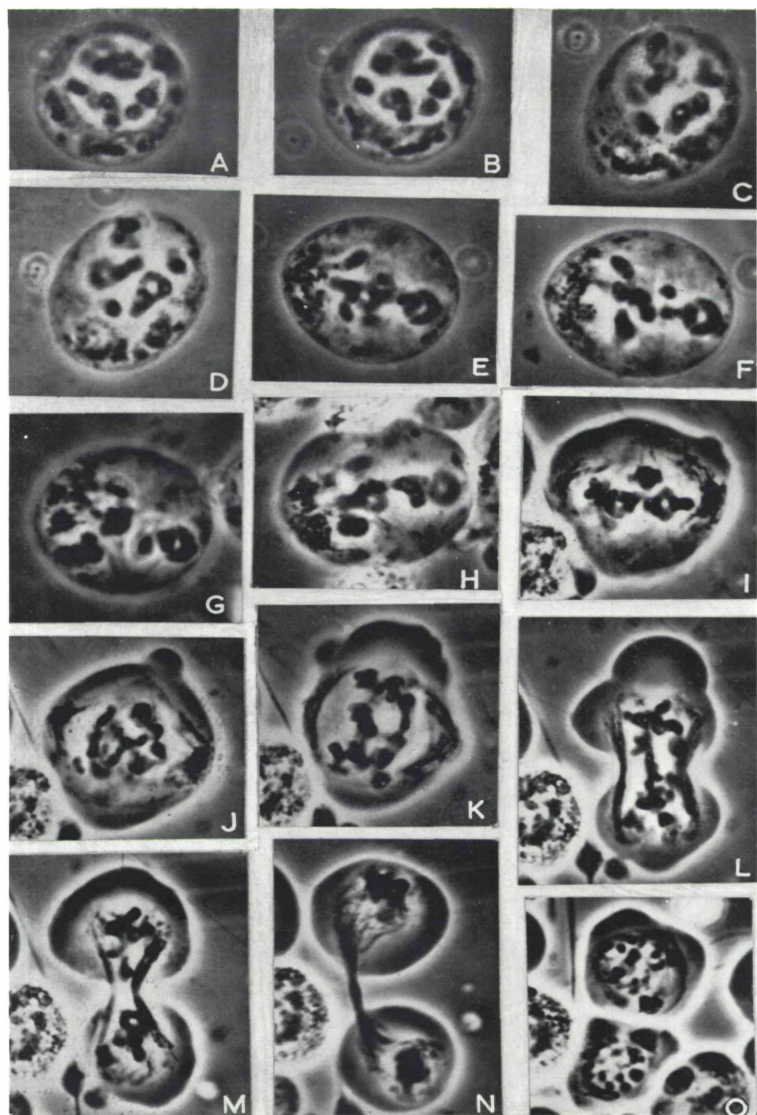


FIG. 3  
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- DRINKER, C. K., and YOFFEY, J. M., 1941. *Lymphatics, lymph and lymphoid tissue*. (Harvard University Press).
- DUKE-ELDER, S., and GOLDSMITH, A. J. B., 1951. *Recent advances in ophthalmology*. 4th ed. London (Churchill).
- FAURÉ-FREMIET, E., 1929. *Protoplasma*, **6**, 521.
- HECHT, G., and WEESE, H., 1943. *Münch. med. Wschr.*, **90**, 11.
- HEILBRUNN, L. W., 1952. *Outline of general physiology*. Philadelphia (Saunders).
- HILL, A. V., LONG, C. N. H., and LUPTON, H., 1924. *Proc. Roy. Soc. B*, **96**, 438.
- HIRST, E. L., 1942. *J. Chem. Soc.*, 70.
- LEWIS, M. R., and LEWIS, W. H., 1911. *Anat. Rec.* **5**, 277.
- MELLORS, R. C., KUPFER, A., and HOLLENDER, A., 1953. *Cancer*, **6**, 372.
- MERRITT, H. H., and FREMONT-SMITH, F., 1937. *The cerebrospinal fluid*. Philadelphia (Saunders).
- OAKLEY, H. B., 1935. *Trans. Faraday Soc.*, **31**, 136.
- PONDER, E., 1948. *Hemolysis and related phenomena*. London (Churchill).
- PROSSER, C. L., BISHOP, D. W., BROWN, F. A., JAHN, T. L., WULFF, V. G., 1950. *Comparative animal physiology*. Philadelphia (Saunders).
- ROBINSON, J. R., 1953. *Biol. Revs.*, **28**, 158.
- ROSS, K. F. A., 1953. *Quart. J. micr. Sci.*, **94**, 125.
- 1954. *Ibid.*, **95**, 425.
- SCHREIDER, E., 1953. *Nature, Lond.*, **171**, 339.
- SHAPIRO, H., and PARPART, A. K., 1937. *J. cell. comp. Physiol.*, **10**, 147.
- SHEAR, M. J., 1935. *Amer. J. Cancer*, **23**, 771.
- and FOGG, L. C., 1934. *U.S. Public Health Repts.*, **49**, 225.
- STEWART, D. C., and KIRK, P. L., 1954. *Biol. Revs.*, **29**, 119.
- THROWER, W. R., and CAMPBELL, H., 1951. *Lancet*, **1**, 1096.
- WEESE, H., 1951. *Wien. med. Wschr.*, **101**, 263.