612.111.22

THE MEASUREMENT OF RED CELL VOLUME.

III. Alterations of cell volume in extremely hypotonic solutions.

By ERIC PONDER and GEORGE SASLOW.

(From Washington Square College, New York University.)

1. Introduction.

We have recently studied the changes in the red cell volume of the rabbit in systems consisting largely of plasma, the tonicity of the system being modified, by the addition of small quantities of NaCl, KCl, and glucose, in such a way as to make the final tonicity of the medium surrounding the cells anything from an equivalent of 2 p.c. NaCl to 0·8 p.c. NaCl [Ponder and Saslow, 1930 *b*]. One of the principal results of these experiments was that red cells may increase in volume in hypertonic media, or may not decrease in volume as much as expected. We inferred from these findings that, contrary to general opinion but in conformity with the conclusions of Kerr [1929] and of Neuhausen and Breslin [1923], osmotically active substances such as Na and K may enter the erythrocyte from hypertonic solutions surrounding it. Owing to the nature of the methods employed in these experiments it was impossible to investigate changes in volume in solutions of tonicity less than the equivalent of 0·8 p.c. NaCl; it is important, however, that such changes should be investigated, particularly because the usual assumption that the cell can be treated as a perfect osmometer, impermeable to Na and K, is possibly at variance with the fact. In addition to this, the study of the volume changes in hypotonic solutions is closely related to the study of "osmotic" hæmolysis.

The methods available for the measurement of red cell volume in hypotonic media turn out to be severely limited, for in a hypotonic solution we have always to be prepared for the possible liberation of hæmoglobin by the less resistant cells. The colorimetric method, for example, although desirable because of its high precision [Ponder and Saslow, 1930 *a*], is unsuitable because the figure for cell volume obtained depends

on the measurement of the dilution of a hæmoglobin solution; if hæmoglobin is liberated by the red cells of the system, the whole procedure is invalid. The hæmatocrite method, which is commonly used for the purpose, is open to several objections. If cells hæmolyse, the height of the hæmatocrite column is dependent not only on the volume of the remaining cells, but also on their number; this cannot be found precisely, as one of us has already shown [Saslow, 1929]. Even if no cells hæmolyse, on the other hand, we are faced with the difficulties attendant on the packing of bodies of different shapes and volumes, and on their sedimentation in solutions of different viscosities. The use of refractometer, conductivity, and viscosity methods is precluded for similar reasons, and calculation of the cell volume is impossible because of the curious forms which the biconcave discs assume as they swell; analytical methods are also of little value, partly because the quantities of analysable substance exchanged (Na, K, etc.) are small in some of the systems to be studied, and partly because hæmolysed cells may liberate these substances into the surrounding medium.

We are accordingly compelled to use a method in which the measurements are not interfered with even by considerable amounts of hæmolysis. This method depends on the fact that red cells immersed in large quantities of saline assume a spherical form when enclosed between a slide and coverslip[1], and that the volume of this spherical form is the same as that of the cell when floating freely in saline [Ponder, 1929]. The radius of the sphere can be measured with great accuracy with a diffractometer of new design, and from the radius the volume can be calculated.

[1] The reason for the assumption of the spherical form is as yet unknown, but the spheres which result from enclosing red cells in saline between a slide and a coverslip represent an extreme form of crenation in which no change in cell volume occurs. We have already pointed out that crenation is usually independent of volume change [Ponder and Saslow, 1930 *b*]. Waller [1930] has recently suggested that the change to the spherical form is dependent on a sudden increase in external *p*H when the cells are placed in saline, and that there may be a temporary diminution in volume. It is true that the spherical form does not occur in acid phosphate buffers, but the important point is that it never occurs, be the medium acid or alkaline, so long as the cells are not covered with a coverglass. Covering them produces the spherical form at once, and in a number of suspension media of varying *p*H (*e.g.* NaCl solutions of *p*H 5·8–6·2, or in phosphate buffers of *p*H greater than 7·0). We regard the spherical form simply as an extreme case of failure of the cell to maintain its normal shape; the reason for its occurrence is no doubt similar to that for the appearance of crenation, but there is no satisfactory explanation for the occurrence of either.

2. METHODS.

(*a*) *The diffractometer and its use.*

The base of the instrument, which can be levelled by three levelling screws, is essentially an optical bench which carries the mercury arc and a focussing lens. The mercury arc has already been described, and works on 100 volts D.C. with from 100 to 500 ohms in series. The lens is an achromatic combination 1 inch in diameter and of 2 inches focal length, and parallelizes the light from the capillary of the arc. Both the focussing

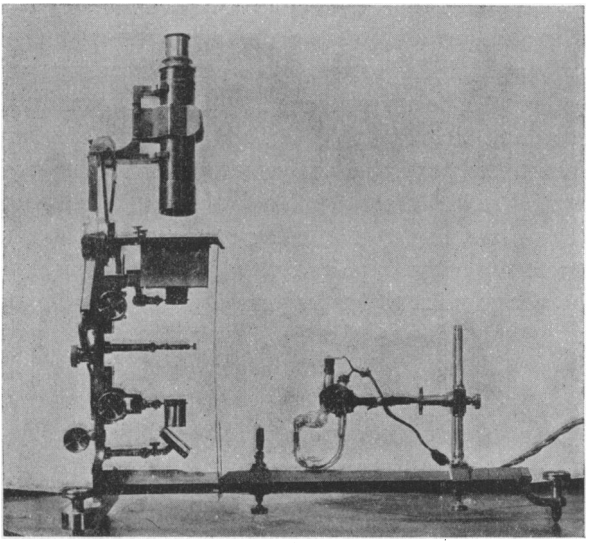

Fig. 1. The diffractometer described in the text.

lens and the arc can move to and fro on the optical bench, and both can be raised or lowered. [See Allen and Ponder, 1928.]

The parallel light falls on a surface-silvered plane mirror, attached to the upright part of the instrument, which is essentially another optical bench at right angles to that which forms the base. The mirror is movable in every direction, and, once adjusted, can be fixed in any desired position. The light from its surface now passes through a second achromatic lens, also 1 inch in diameter and of 2 inches focal length; this lens can be moved by a rack and pinion so as to focus the image of the luminous capillary of the arc on a pinhole, which can be centred on the optical axis of the instrument by means of three centring screws. At its focal

length from the pinhole is a third achromatic lens, identical with the first two; this parallelizes the light from the pinhole, producing a beam of light which passes upwards through the opening in the stage above.

The stage, 4 inches square, has a central aperture 1 inch square through which the beam passes, and carries below it a series of racks for the insertion of colour filters. The entire stage, together with the filter racks, can be moved up or down through a distance of about $\frac{1}{2}$ inch by means of a rack and pinion, so that the surface of the slide on which the cells are spread can be brought into the plane of the axis of rotation of the telescope. From the front of the stage there hangs a sheet of metal which shields the pinhole and the lenses from the direct light of the arc, and beneath the stage, surrounding the racks for the colour filters, are a series of metal plates which prevent scattered light passing up from below.

The pivoted telescope is very similar to that in Millar's [1926] instrument, and carries an achromatic object glass, $1\frac{1}{2}$ inches in diameter and 6 inches in focal length, and also an eyepiece magnifying 3 to 5 times. It swings about an axis in the same plane as that in which the film of cells is situated (the latter, in practice, being brought into the same plane as the former), and the amount of its rotation can be measured by a scale divided into degrees with a vernier reading to 0·05°; it can be moved either by hand, or by means of a fine adjustment. The eyepiece carries fine cross-hairs, which intersect on the image of the pinhole when the telescope is in the zero position.

The apparatus is very simple to use. The arc is formed by heating the capillary of the arc with a Bunsen burner; it is best to start it with about 500 ohms in series, and once it is burning steadily, to increase its intensity by reducing the resistance to about 200 ohms. The lens on the horizontal part of the instrument is set at its focal length from the capillary, and the mirror moved so that the image of the capillary covers the pinhole, which has previously been centred, by means of its centring screws, with the cross lines of the telescope when the latter is in the zero position. The image of the capillary is next focussed on the pinhole. The lens above the pinhole should now appear uniformly illuminated, and, on looking into the telescope, the image of the pinhole, with the brilliant image of the capillary of the arc crossing it, should appear on the intersections of the cross-hairs. The slide bearing the film of cells whose diffraction pattern is to be examined is placed on the stage, which is then raised or lowered until the surface of the slide lies in the axis of rotation of the telescope. The telescope is tilted until the vertical cross-hair lies in a selected position with respect to a selected diffraction band, and the

diffraction angle is read off on the scale. The mean radius r of the cells can then be calculated by means of the formula

$$r = k\lambda \operatorname{cosec} \theta, \qquad \text{.....(1)}$$

where λ is the wave-length of the diffraction band selected, and k a constant which depends on the order of the spectrum and on whether θ has been measured for a maximum or a minimum.

It has already been shown that this formula, which can be deduced from theoretical considerations, is applicable to the diffraction patterns

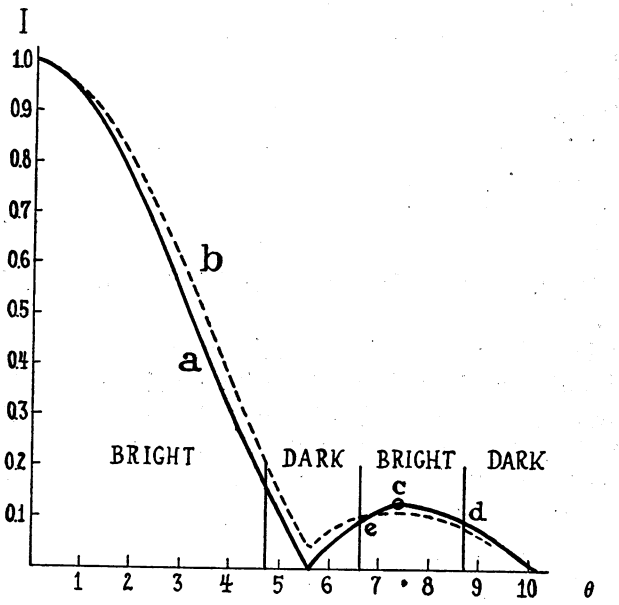

Fig. 2. Graph of illumination function in the neighbourhood of the first maximum and minimum for cells of diameter 7·8μ. Curve *a*: illumination function for cells of mean diameter 7·8μ, $\sigma = 0$. Curve *b*: illumination function for cells of diameter 7·8μ, $\sigma = 0{\cdot}2\mu$. The circle on curve *a* indicates the theoretical position of the illumination maximum; the vertical lines indicate the diffraction bands as seen by eye.

of red cells when monochromatic light is used, *i.e.* when λ is exactly known [Allen and Ponder, 1928]. At first sight one might think that it is under these very conditions that the accuracy of the diffractometer would be greatest; a little consideration will show, however, that the precision of the instrument can be increased by using it in a slightly different way.

Consider the graph of the illumination function for monochromatic light,

$$I = kNr^2 2/m J_1(m), \qquad \text{.....(2)}$$

shown in Fig. 2. Here I is the intensity of illumination in the diffraction pattern, r is the radius of the cell producing it, k is a constant depending on the wave-length λ, and m is equal to $(2\pi r \sin\theta)/\lambda$, θ being the diffraction angle. At the points where $J_1(m) = 0$ the illumination is zero and we have the various diffraction minima, while at the points where $J_1(m)/m$ is at a maximum we have the illuminated diffraction maxima. If all the cells were of the same radius ($7{\cdot}8\mu$ in this instance), the illumination function would have the appearance of curve a in the figure, and the illumination at the minima would be zero, but if the cells are of varying radii, as they always are in practice, the function assumes a different form, *e.g.* that of curve b, and the illumination at the minima becomes greater while that at the maxima becomes less. The usual way of finding the diffraction angle θ is to set the cross-hairs over either the 1st or 2nd minimum or over the 1st minimum; θ is then read off on the scale, and, in using expression (1), the appropriate value of k (0·61 for the 1st minimum, 1·12 for the 2nd minimum, and 0·82 for the 1st maximum) is employed. Inspection of the figure, however, will show that this procedure is subject to two disadvantages, although it may be satisfactory enough for ordinary purposes.

The diffraction pattern (for monochromatic light) appears to the eye as a series of dark rings alternating with a series of bright rings; these rings, however, have no real objective existence as such, for they are brought about by the fact that the eye experiences the sensation of darkness when the illumination falls below a certain value, and of brightness when the illumination exceeds this value. In practice, when the cross-lines are set on any one maximum or minimum, the eye is not guided exclusively, or even largely, by maximal or minimal illumination, but the cross-lines are set so as to lie midway between the edges of the dark band which surrounds the minimum, or of the bright band within which the illumination rises to a maximum. It is easy to see from the figure that the region of the maximum is so flat that it is impossible to pick out a sharply delimited region of maximum illumination by eye; it is, of course, equally impossible to pick out the region of minimum illumination in a band which gives to the eye the sensation of blackness only. The process of setting the cross-lines midway between the edges of the illuminated or darkened bands is permissible when a high degree of accuracy is unnecessary, but when great precision is required, it is necessary to allow for the fact that the ratio of the distances ec/ed in the graph of the illumination function may differ considerably from 0·5. The ratio is, in fact, a function of the cell radius, and may be as great as 0·47 or as

small as 0·35; it is also, although to a lesser extent, dependent on the general brilliance of the spectra.

In order to overcome this very real difficulty in determining the position of the maxima and minima by eye, we may dispense with monochromatic light altogether, and avail ourselves of the fact that when the unfiltered light of the Hg arc is used the diffraction spectra consist of a series of alternating blue and yellow bands, the margins of which are, under proper conditions, very sharp indeed. The pattern of diffractometer described above has, moreover, such a large N.A. that the second order spectra are remarkably brilliant; the cross-hairs can therefore be set on the junction between the blue and yellow band of the second order spectrum, where it is possible so to adjust the telescope that blue lies on one side of the vertical cross-hair and yellow on the other, the position of the hairs being certain to less than 0·05°. The diffraction angle is now read off, and the radius of the cell calculated from the formula

$$r = 0{\cdot}5472 \operatorname{cosec} \theta. \qquad \ldots\ldots(3)$$

The value of the constant (0·5472) was found by calibrating the apparatus, working with the unfiltered light of the arc, against the values found for the same films of cells when monochromatic light was employed[1].

(*b*) *The accuracy of the method.*

It is necessary to show that the cells are perfect spheres when in the "spherical form," and that the volume of these spheres is the same as the volume of the same cells floating as "discoidal forms" in the medium in which they are examined. It can easily be demonstrated that these assumptions are correct when the cells are suspended in an isoplethechontic[2] solution of NaCl (1·12 p.c. for rabbit's blood) by finding the volume of the individual cells by the diffraction method and comparing

[1] This calibration is very difficult. In practice, one finds the radius of cells in the spherical form, and also of cells in dried films, with monochromatic light, measuring various maxima and minima; by the use of expression (1), one thus obtains a "best value" for the cell radius in a particular preparation. The process is then repeated, using the same preparations, but determining θ with relation to the junction of the blue and yellow bands of the second order spectra. By use of expression (1), in which r and θ are now known, a value for $k\lambda$ is found; it is this which we have found to be 0·5472 in expression (3). The value of $k\lambda$, however, as found by repeated calibrations of this kind, may vary by ± 2 p.c. and such a variation may lead to a ± 8 p.c. error in the figure for cell volume. We have selected the particular value 0·5472 because, in a number of experiments, this value of $k\lambda$ gave the best agreement with colorimetric measurements.

[2] An isoplethechontic solution is one which maintains the red cell volume unchanged over a period of several hours [Ponder and Saslow, 1930 *b*].

this with the volume found colorimetrically. The following figures show the result of this procedure in five experiments:

Volume, μ^3 by diffraction	60·5	60·5	60·8	56·5	56·0
Volume, μ^3 by colorimeter	59·0	60·8	59·9	54·0	54·0

If we allow the usual error of $\pm$ 2 p.c. we can conclude from these figures that the assumption of the spherical form does not involve a change of volume, at least in isoplethechontic solutions. It is well known that the appearance of spherical forms is not influenced by hypotonicity or hypertonicity, and so we shall suppose that, even in hypotonic and hypertonic media, the spherical form has the same volume as that of the discoidal form in the same medium. Indirect evidence of this will be found below. (See section 5, Hæmatocrite determinations, in which it is found that the volume changes in hypotonic plasma (pH 7·2) are essentially the same as those in hypotonic NaCl solutions.)

It remains to show what difference in volume can be detected by the diffractometer method. Rabbit erythrocytes in 1·1 p.c. NcCl, and in the spherical form, usually give a diffractometer reading of $\theta = 13°$. It is possible to set the cross-hairs to within $\pm$ 0·05°, *i.e.* a difference between 13·1° and 13·0° is easily detectable. A reading of 13° gives a volume of $62\mu^3$, and one of 13·1° a volume of $60{\cdot}5\mu^3$; it is therefore possible to detect a difference less than $2\mu^3$, *i.e.* of $\pm$ 2 p.c. The method is accordingly as accurate as the colorimetric method, in addition to being of far greater utility when the erythrocytes are suspended in saline media.

(*c*) *Preparation of systems of various tonicities.*

The type of system with which this investigation is concerned is one in which 0·1 c.c. of rabbit's blood (prevented from clotting by the usual amount of potassium oxalate) is added to 5 c.c. of saline solutions, the tonicities of which range from an equivalent of 2 p.c. NaCl to tonicities sufficiently small to cause complete hæmolysis.

A series of solutions of pure NaCl was prepared in the following concentrations (g. NaCl per 100 g. water): 2 p.c., 1·5 p.c., 1·3 p.c., 1·1 p.c., 1·0 p.c., 0·9 p.c., ... 0·6 p.c., 0·58 p.c., 0·56 p.c., ... 0·36 p.c. In the case of the solutions of KCl, the following concentrations were used (p.c.): 2, 1·5, 1·3, 1·1, 1·0, 0·9, 0·8, 0·78, ... 0·44; here each p.c. concentration, however, is expressed in terms of a concentration of NaCl of the same depression of freezing point. The steps of the series differ from those of the NaCl series because of differences in the resistance of rabbit erythrocytes to hypotonic NaCl and KCl respectively. For glucose, the series used was the following (p.c.): 2·0, 1·5, 1·3, 1·1, 1·0, ... 0·6, 0·58, ... 0·26.

These figures again are in terms of equivalent concentrations of NaCl. The *p*H of all these solutions was between 5·8 and 6·0[1]. All solutions keep well in the refrigerator.

To 5 c.c. of each of these solutions of NaCl, KCl and glucose is added 0·1 c.c. of rabbit's blood; this will be found to be the best quantity for the production of sharply defined spectra. If too much blood is added, the concentration of plasma in the system prevents the assumption of spherical forms; if too little is added, the spectra are faint, although it should be pointed out that the brilliance of the spectra depends more on the homogeneity of the cells with respect to radius than on their absolute number. The tubes containing the solutions to which the cells have been added are allowed to stand at 25° for one hour. At the end of this time their contents are shaken up; a small sample is withdrawn on to a slide, covered with a thin coverglass, and the diffraction angle measured within a minute of the withdrawal. The cell volume is calculated from the value of θ.

3. Preliminary experiments.

We first carried out a number of experiments of a preliminary nature, in order to discover the points about which more detailed information was required. An illustrative example is given in the following table, which refers to rabbit erythrocytes.

The various entries in this table require some explanation. It will be seen that the object of the experiment is to investigate the volume of the cells at three stages of "swelling": (1) when the cells are suspended in plasma or in an approximately isoplethechontic solution of NaCl, KCl or glucose, (2) when the cells are suspended in solutions just too strong to cause lysis, and (3) when the tonicity of the suspension medium is such as to produce about 60 p.c. haemolysis.

[1] Experiments on systems made up with solutions of *p*H 5·8 to 6·0 cannot necessarily be depended on to tell us much regarding volume changes of cells under physiological conditions or in media whose *p*H is nearer that of plasma. Such systems are worthy of study nevertheless, especially as they are similar to those usually employed in experiments on osmotic changes in red cells and red cell fragility. It happens, however, that they tell us more about the behaviour of cells in plasma of various tonicities than they might be expected to, a fact which is accounted for by the effect of *p*H on red cell resistance being less important than is generally supposed (see p. 289, n. 1). For example, the red cell volume, which is closely related to red cell resistance, is practically the same in unbuffered isoplethechontic NaCl and glucose (which are of the same equivalent concentration) as in highly buffered mixtures of cells in their own plasma. It is quite possible that, if we were interested in the behaviour of cells under strictly physiological conditions, we would require to examine the influence of *p*H more closely; the results set forth in this paper, however, refer only to the systems in which they were obtained.

	Plasma	NaCl	KCl	Glucose
1. Plasma or 1·1 p.c. NaCl or equivalent:				
Volume of cells by colorimeter, μ^3	60	—	—	—
Volume of cells by hæmatocrite, μ^3	57	—	—	—
Volume of cells by diffraction, μ^3	—	62	65	61
2. Lysis zero:				
Concentration (NaCl equivalent) p.c.	—	0·56	0·58	0·36
Volume of cells by diffraction, μ^3	—	76	76	77
3. Advanced lysis:				
Concentration (NaCl equivalent) p.c.	—	0·48	0·52	0·28
Degree of lysis p.c.	—	61	60	65
Volume of cells by diffraction, μ^3	—	82	81	83
4. Lysis complete:				
Concentration (NaCl equivalent)	—	0·40	0·46	0·24

(1) When the cells are suspended in their own plasma, their volume can be found by the colorimetric method already described [Ponder and Saslow, 1930 *a*], and this can be regarded as the "normal" volume, correct to $\pm 2\mu^3$. The cell volume can also be found by the use of a hæmatocrite of the ordinary type; the hæmatocrite tubes which we use are 100 mm. long, sealed at the lower ends, and revolving at 4000 r.p.m. for 10 minutes. This method can be used only when the cells are suspended in plasma, and not when they are suspended in NaCl, KCl or glucose, for in systems in which 0·1 c.c. of blood is added to 5 c.c. of suspension medium, the percentage volume of the cells is so small that accurate measurements of the length of the column of packed cells cannot be made.

The results of a number of extensive experiments similar to that reported in the above table bring out several points of importance. (i) In general we have found a remarkably close agreement between the cell volume as found by the colorimeter and as found by the hæmatocrite. Usually the difference between the volumes yielded by the two methods has been less than the error attached to the former, although sometimes the difference has exceeded 5 p.c. Such differences are not surprising in view of the conclusions which we have already reached regarding the variability of hæmatocrite determinations [Ponder and Saslow, 1930 *a*]; our experience suggests, however, that 10 minutes' spinning at 4000 r.p.m. generally gives about the right volume for rabbit erythrocytes. (ii) The volumes found by the diffraction method for cells suspended in 1·1 p.c. NaCl, KCl and glucose, generally agree fairly closely with the volume of the cells in plasma; sometimes, however, the volumes found in the KCl solutions are slightly larger than those found in plasma, NaCl or glucose. This is the same kind of result as we obtained by the colorimetric method in a previous investigation and in systems containing a large amount of plasma [Ponder and Saslow, 1930 *b*].

(2) In attempting to find the volume of cells in solutions just too concentrated to produce hæmolysis, one dare not use the colorimetric method lest undetected quantities of hæmoglobin should be liberated from the cells and lead to erroneous results. The ordinary kind of hæmatocrite cannot be used, for reasons already stated; the volume can accordingly be estimated in absolute units only by diffraction.

Four conclusions appear from the results in the section "lysis zero." (1) The solutions of NaCl and KCl at which lysis begins have very nearly the same freezing point, but the solution of glucose at which lysis commences is much more hypotonic. This, it may be mentioned, is a very constant finding, not only with the cells of the rabbit, but also with those of many other mammals. This observation alone is sufficient to show that hæmolysis by hypotonic solutions cannot be treated as a simple osmotic effect, for, if it were, we would expect that solutions of NaCl, KCl and glucose, just too concentrated to initiate lysis, would be of the same equivalent concentration. (ii) The three substances, although in different equivalent concentrations, produce the same amount of swelling of the cells in solutions just too concentrated to initiate lysis. This is a most important result, for the classical view regarding hypotonic hæmolysis, viz. that water enters the cell, increases its volume, and stretches its surface, tacitly assumes that the same amount of stretching, and with it the same amount of swelling, takes place irrespective of the nature of the substance in hypotonic solution. Jacobs [1926–7], moreover, specifically postulates a "hæmolytic volume, V_h," at which the cell, behaving as a perfect osmometer and having taken in a definite quantity of water, hæmolyses. (iii) We may assume that cells suspended in 0·56 p.c. NaCl, 0·58 p.c. KCl, or 0·36 p.c. glucose are in osmotic equilibrium with the large volume of fluid surrounding them, for it can be shown by diffractometer measurements that the final volume of the cells ($76\mu^3$ in this case) is assumed in these systems within a minute. Treating the cell as a perfect osmometer, we can then calculate the volume of the cell at equilibrium from the amount of water which should enter, always provided that we know the quantity of "osmotically active" water which the cell contains. It will be seen from the above figures that the increase in volume just before lysis begins is 26–28 p.c.; the increase in volume which should have occurred, however, on the assumption that all the water in the cell is "free," is about 67 p.c. in the case of NaCl and KCl, and over 130 p.c. in the case of glucose. We are accordingly forced to conclude that, if we look upon the cell as a perfect osmometer, only about 50 p.c. of its contained water (and less in the case of glucose) appears to be "free."

(3) The figures under the section "advanced lysis" lead to two conclusions. (i) Approximately equal degrees of lysis may be produced by solutions of quite different equivalent concentrations; NaCl and KCl are again more similar to each other than either is to glucose. (ii) When about 60 p.c. of the cells are hæmolysed, the remaining cells present the same volume (81–83μ^3) in NaCl, KCl and glucose. Here again the swelling (36–38 p.c.) is that which would be expected in a perfect osmometer, such as the cell is generally believed to be (*e.g.* by Jacobs, Ege [1921], Hamburger [1902], Warburg and Winge [1928], Christensen and Warburg [1929], Koeppe [1900], and many others), provided only about 50 p.c. of its water is considered "free."

(4) The concentrations of NaCl, KCl and glucose which cause complete hæmolysis are again quite different, although NaCl is more like KCl than either is like glucose.

Summarizing the conclusions which can be drawn from these preliminary experiments, we see that the red cell can be treated as a perfect osmometer, impermeable to Na, K and glucose, only if we assume that about 50 p.c. of its contained water is "free" when it is immersed in NaCl or KCl, and that about 25 p.c. is "free" when it is immersed in glucose. Such assumptions are altogether untenable, particularly in view of Hill's demonstration [1930] that more than 90 p.c. of the water is "free," and also in view of our own recent results [Ponder and Saslow, 1930 *b*], which, together with the analyses of Kerr [1929], show quite clearly that the assumed impermeability of cations is non-existent, at least in solutions which are not isoplethechontic.

At the same time, one cannot overlook the fact that, in spite of this anomalous behaviour of the cells as osmometers, hæmolysis begins when a volume of 76–77μ^3 is reached (26–28 p.c. increase over the "normal" volume), and reaches 60 p.c. when the volume reaches 81–83μ^3 (36–38 p.c. increase), irrespective of the nature of the hypotonic solution, and in solutions of NaCl, KCl and glucose of quite different equivalent concentrations. The fact accordingly remains that the cells begin to hæmolyse when a "critical volume, V_h" is reached, as postulated by Jacobs. But for such a critical volume to be reached, it is by no means necessary that the cell shall exchange water with the suspending medium in the ideal way; all that is necessary is that the cell shall gain water, and so increase in volume; this it can do even if it is constantly losing cations into the surrounding hypotonic medium.

4. The relation of swelling to the tonicity of the surrounding medium.

In view of the fact that the cells increase in volume in the anomalous fashion already described, it is important to establish, with the greatest exactness possible, the relation between the amount of swelling and the tonicity of the medium in which the cells are suspended. This can be done by the diffractometer method, 0·1 c.c. of rabbit's blood being added to 5 c.c. of solutions of NaCl, KCl and glucose of various tonicities, and the volume assumed by the cells at the end of an hour being calculated from the radius of the spherical forms.

The results of one typical experiment are shown in the tabular form below. For each substance there are four columns. The first, headed tonicity, shows the tonicity of the suspension medium in terms of grams per 100 g. water and in terms of NaCl solutions of the same depression of freezing point. The second column shows the average volume of the cells, and the third the percentage of lysis present; the fourth column shows that percentage of the water contained in the cell which appears to be "free" if the cells are regarded as perfect osmometers impermeable to Na, K and glucose. It is assumed throughout that the total water contained in the cell occupies 67 p.c. of its volume[1].

NaCl				KCl				Glucose			
Tonicity	Volume μ^3	p.c. lysis	p.c. "free water"	Tonicity	Volume μ^3	p.c. lysis	p.c. "free water"	Tonicity	Volume μ^3	p.c. lysis	p.c. "free water"
1·1	62	—	—	1·1	62	—	—	1·1	58	—	—
1·0	63	—	—	1·0	62	—	—	1·0	59	—	—
0·9	—	—	—	0·9	63	—	27	0·9	61	—	—
0·8	65	—	30	0·8	67	—	42	0·8	62	—	—
0·7	73	—	59	0·78	69	—	58	0·6	63	—	36
0·6	78	—	56	0·76	70	—	61	0·5	71	—	20
0·58	78	—	51	0·74	70	—	55	0·46	75	—	26
0·56	81	—	54	0·72	—	—	—	0·36	78	0	21
0·54	82	—	53	0·70	74	—	66	0·34	—[2]	26	—
0·52	83	—	51	—	—	—	—	0·32	—	34	—
0·50	—	Trace	—	—	—	—	—	0·30	—	45	—
0·48	86	31	51	0·64	77	—	61	0·26	—	50	—
0·46	86	54	47	0·60	78	—	58	0·24	—	100	—
0·44	—	74	—	0·56	78	—	50				
0·42	—	80	—	0·52	82	Trace	52				
0·40	—	100	—	0·50	84	30	53				
				0·46	—	100	—				

[1] 67 p.c. is an average figure; the actual amount may vary in different rabbits. Such variations do not affect the general arguments in this paper, although they may affect numerical values. The same remark applies to the figure 1·12 p.c. NaCl, taken as the tonicity of the cell interior.

[2] Volumes unobtainable owing to poor spectra.

A set of representative results are plotted in Fig. 3, and, like those shown in the table, bring out two points clearly. (i) As in the preliminary experiments, the cells begin to hæmolyse when the average volume reaches

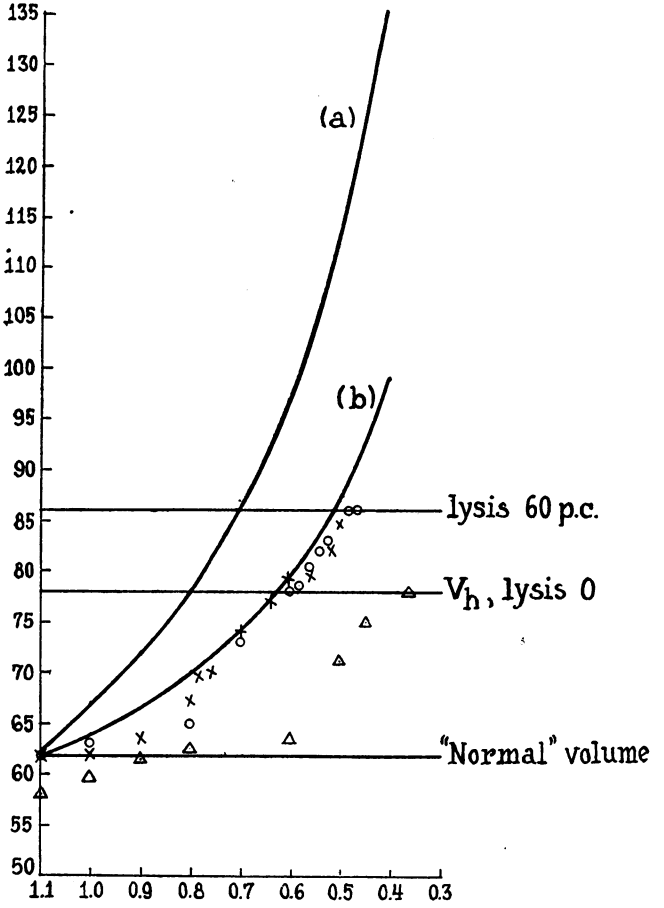

Fig. 3. Ordinate, cell volume in μ^3; abscissa, tonicity of external medium, in terms of g. NaCl per 100 g. water. The crosses show the data obtained with solutions of NaCl, the circles the data with solutions of KCl and the triangles the data with solutions of glucose. Curve *a* shows the expected volumes if all the cell water is considered to be "free," and curve *b* shows the expected volumes if 50 p.c. of the water is considered "free." The volumes attained at various degrees of lysis are indicated by the transverse lines.

about $80\mu^3$, this figure being virtually the same in NaCl, KCl and glucose, although the equivalent concentrations of the three substances are by no means the same. The "normal" volume of the cells is about $60\mu^3$;

the increase is accordingly about 33 p.c. (ii) In solutions of all three substances the volume becomes continuously greater as the tonicity diminishes, but it will be seen from the graph that the volume increases in NaCl and KCl are not greatly different from those we would expect if the cell were impermeable to Na and K, and only 50 p.c. of the water contained in the cell were "osmotically active[1]"; in the case of glucose, on the other hand, the increases are such as would be expected if only 25 p.c. of the contained water were "free." Even on these assumptions, of course, some of the observed points deviate somewhat from the expected values, but the deviations are rarely greater than the allowable experimental error.

As we cannot maintain the fiction that the cell is a perfect osmometer, impermeable to Na, K and glucose, and containing different amounts of osmotically active water, we must account for the observed volume changes in some other fashion. The simplest way of accounting for them is suggested by the experiments which show that Na, K and glucose can enter the cell from a hypertonic solution [Ponder and Saslow, 1930 *b*] and can leave the cell when the latter is suspended in a hypotonic solution [Kerr, 1929].

The problem may be stated as follows. Suppose that the cells contain water to the extent of 67 p.c. of their volume, and that nearly all of this water is osmotically active; the percentage of osmotically active water, in terms of the total volume of the cell, may then be called Q_1 (nearly 67 p.c. in this case). Suppose, however, that the cells swell in hypotonic solutions as if they contain a smaller percentage of osmotically active water, Q_2; the problem is then to find, on the assumption that the cells lose osmotically active substances, the quantity of such substances lost in order to give the observed values of Q_2.

To do this we proceed in the following way, using relations which are easily established from first principles. It is to be borne in mind that the figures used refer to the systems employed in experiment, *i.e.* in which the relative quantities of cells and of suspension medium are in the proportion of 1 litre to 100 litres.

Let the tonicity of the medium (grams p.c. in terms of NaCl), multiplied by 1000, be denoted by T, and suppose that the tonicity of the cell interior is equivalent to that of a 1·12 p.c. NaCl solution. This figure is justified both by our own determinations of the concentration of isoplethechontic solutions, and, after allowance is made for the greater

[1] This is the same type of result as was found in our earlier experiments by the colorimetric method in systems consisting largely of plasma.

depression of freezing point for rabbit's plasma, by Margaria's direct determinations [1930] on human blood. Let the percentage increase in red cell volume in any hypotonic solution, when divided by 100, be denoted by v. We can then find Q_2, the apparent quantity of free water in terms of the cell volume, from the equilibrium equation

$$\frac{11{\cdot}2Q_2}{Q_2+v} = \frac{T}{100-v}. \qquad \text{.....(4)}^1$$

For convenience, we can further express by R that percentage of the total osmotically active water which is apparently active, *i.e.*

$$R = 100Q_2/Q_1. \qquad \text{.....(5)}$$

Equation (4) represents what happens at equilibrium if the quantity of osmotically active water is Q_2, less than Q_1, but the difference between Q_1 and Q_2 is a fiction, and appears only because a quantity of osmotically active substance x is lost by the cell. The value of x can easily be shown to depend on Q_1, v and T in the following way:

$$\frac{11{\cdot}2Q_1-x}{Q_1+v} = \frac{T}{100-v}, \qquad \text{.....(6)}$$

x being a quantity in grams, lost from one litre of cells, and expressed in terms of NaCl. We can, for convenience, express x as a percentage of the original amount $11{\cdot}2Q_1$ (for the cell tonicity is assumed to be equivalent to a 1·12 p.c. NaCl solution), and call it X; plotting X against the tonicity of the suspending medium now gives a family of straight lines which differ in slope according to the value of R. Some of these lines are shown in Fig. 4, and the equation of any one of them is

$$X/a + Y/b = 1, \qquad \text{.....(7)}$$

where Y is the tonicity in grams NaCl p.c., *i.e.* $T/1000$; b has the value of the number of grams of NaCl contained in 100 c.c. of the water in the cells. The constant a has no real physical meaning, for the reasoning assumes equilibrium to occur and the cells to remain intact, which, of course, is not the case when Y becomes less than about 0·4. It, therefore, appears that

$$dX/dY = \text{const.} = f(R), \qquad \text{.....(8)}$$

and a linear function, moreover, for plotting a against R gives another straight line (marked R, a in Fig. 4) whose intercepts are $R = 1$, $a = 100$ in the example plotted.

[1] This equation is mathematically exact only if the volume of the corpuscles initially is 1/101th of the whole suspension (as in these experiments). A general equation covering other relative volumes can be developed, but it is much easier to set up individual equations such as expression (4) to apply to the particular systems under investigation.

To illustrate the meaning of the conclusion contained in (8), consider the case where $R = 0{\cdot}5$, *i.e.* where the cell swells as if only half of its contained water were osmotically active, and where the cell tonicity is taken as that of 1·0 p.c. NaCl as in Fig. 4. As we pass from one tonicity

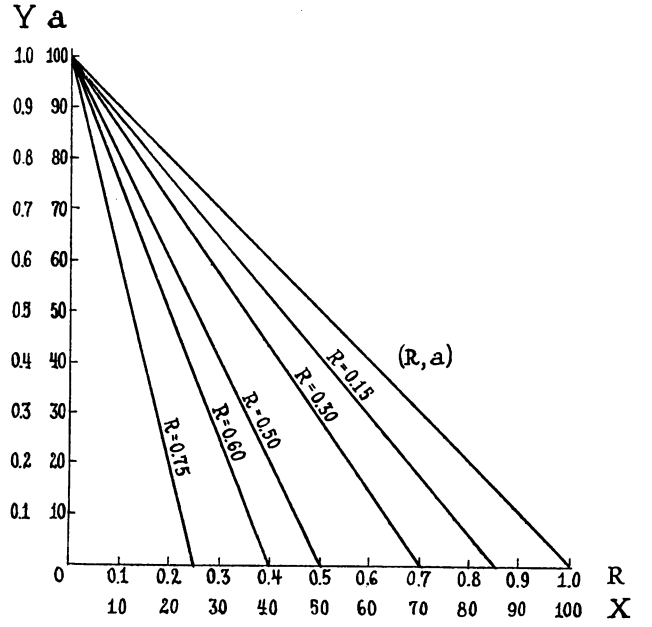

Fig. 4. Ordinates: Y, the tonicity of the external medium in terms of g. NaCl per 100 g. water, and a, the X intercept of expression (7) in the text. Abscissae: X, the percentage of the original amount of NaCl in the cell lost, and R, the apparent percentage of "free" water. The lines marked $R = 0{\cdot}15$, 0·30, etc., are plotted from expression (7). For convenience in calculation, the tonicity of the cell interior was taken as that of a 1·00 p.c. NaCl solution. The inner scales a, R refer only to the curve marked (R, a).

to another, the quantity of osmotically active substance lost by the cell increases in the following way:

Tonicity g. NaCl p.c.	1·0	0·9	0·8	0·7	0·6	0·5	0·4
X, p.c. of original NaCl lost	0	5	10	15	20	25	30

Equal steps in tonicity accordingly correspond to the loss of equal amounts of NaCl from 1 litre of cells. If the systems were prepared with different relative amounts of cells and suspension medium, the same general relations would appear, but the numerical results would be different.

Referring again to the results shown in the table at the beginning of this section, it appears to be a constant result of experiment that rabbit erythrocytes suspended in hypotonic NaCl and KCl solutions swell as if

about half their contained water is "free," *i.e.* $R = 0{\cdot}5$. A constant fraction of the osmotically active substances contained in these cells is accordingly lost as we proceed from the higher tonicities to the lower, as expressed in (8) or in the short table on p. 283. This fact is quite remarkable, particularly in that the cells appear to lose a certain definite quantity of their contained osmotically active substance, and no more.

The phenomenon, however, appears to be of quite general occurrence. Rabbit red cells suspended in glucose solutions of various tonicities, for example, behave as if R had a value of about 0·25; with each step of tonicity a constant quantity of osmotically active substance is again lost, but the quantity is nearly twice as much as before. Human erythrocytes in NaCl and KCl behave very similarly to rabbit erythrocytes, as shown by measurement either with the diffractometer or by Warburg and Winge's hæmatocrite method [Warburg and Winge, 1928]. It is also well known that muscle swells in hypotonic solutions as if part of its water were bound, and Hill's explanation of this phenomenon [1930] is very similar to the one which is advanced above, *i.e.* that the reason for the swelling's being less than expected is that the muscle loses osmotically active substances. Hill states, in fact, that the simplest and most probable explanation of "bound water" in muscle is that, after prolonged survival without a normal blood supply, the membranes of some of the muscle cells have lost their semi-permeability. In the case of muscle, moreover, the quantity of K lost to Ringer's solution can be determined, and agrees well with the expected figure[1]. The hypothesis advanced to account for the behaviour of muscle in hypotonic solutions is almost exactly the same as that which we believe to account for the behaviour of red cells under similar conditions, except that in the case of red cells the factors of slow diffusion and prolonged survival may be neglected, for the equilibrium volumes are attained within a minute and maintained for hours.

5. Hæmatocrite determinations.

In view of the fact that the diffractometer method for finding red cell volumes is indirect, it is important to compare the results with those given by a better known procedure. The only available direct method is

[1] The quantities of osmotically active substances (mainly K^+) lost from rabbit cells in accordance with the table immediately preceding are too small to be estimated sufficiently accurately, although they might be detected. The maximum quantity of K^+ expected to be lost in the systems under consideration is less than 0·1 mg. and this would be lost into 5 c.c. of a 0·5 p.c. NaCl or KCl solution.

the hæmatocrite method of Hamburger (as used by Warburg and Winge [1928]) in which 2 c.c. of a system identical with that used in the diffraction method are placed in a tube drawn out below into a narrow capillary; the tube is spun at a known speed for a measured time, and the height of the column of cells in the capillary determined. To find how much the cells swell in a given hypotonic solution, the height of the column obtained after centrifuging is compared with the height obtained when the same number of cells is suspended in the same amount of plasma; the latter figure is then denoted by 100, and the swelling expressed as a percentage of the "normal" volume. This method presents the advantage that the cells can be suspended in hypotonic plasma instead of hypotonic saline, and their swelling in the former medium studied.

This method has been extensively used by Hamburger [1902], Hedin [1891, 1895], and Koeppe [1900] for studying the changes in the volume of red cells in hypotonic solutions, and more particularly by Ege [1921], in connection with the problem of the quantities of "free" and "bound" water in the cell. By means of measurements of the depression of freezing point of corpuscle "press-juice" to which glucose was added, Ege has arrived at a conclusion not unlike Hill's, viz. that nearly all of the water in the rabbit red cell is "free." He concludes, moreover, that his studies of the volumes assumed by red cells in solutions of various tonicities indicate that the cells behave as perfect osmometers containing about 60 p.c. by volume of water, *i.e.* in which about 90 p.c. of the contained water is free. Some of his results, obtained by the Hamburger hæmatocrite (running at 3000 r.p.m. for 15 mins. or 7000 r.p.m. for 2–3 mins.) are shown in the following table:

Tonicities NaCl	Observed volume, p.c. of "normal"	Expected volume, no "bound" water
1·1	100	—
0·8	118	127
0·8	122	127
0·65	136	149
0·65	143	149
0·64	129	136
0·56	144	151
0·50	153	165
0·41	162	218

It is apparent from this table that the observed volumes are considerably smaller than the expected ones; contrary to Ege's contention, indeed, the figures indicate that a greater proportion than 10 p.c. of water is "bound," or that, if the fiction of "bound water" is abandoned,

the cell does not behave as a perfect osmometer. Ege's results, accordingly, support our own diffractometer measurements in a qualitative sense, although not quantitatively, in that his observed volumes are larger than those reported earlier in this paper. The probable explanation for this fact will be considered below.

A method identical with that of Ege has also been used by Warburg and Winge [1928], the only difference being that the latter employ human erythrocytes whereas the former used those of the rabbit. Warburg and Winge's results show clearly that the cells behave as perfect osmometers only if about half their water is considered "bound," and in this respect agree with diffractometer measurements which we have made on human cells. The results of Warburg and Winge accordingly support, not only qualitatively but also quantitatively, the observations and interpretations given in section 4 above.

When we first attempted to confirm our own diffractometer observations by the Hamburger hæmatocrite the results were most inconsistent, for not only were we unable to confirm our own findings, but we were unable to confirm either those of Ege or of Warburg and Winge. The cell volumes obtained by the hæmatocrite were usually far greater than expected, but at the same time they showed great inconsistency, and it was only after some time that we realized that the inconsistency is due to that variability in hæmatocrite determinations upon which we have already commented [Ponder and Saslow, 1930 *a*], the results obtained being very largely determined by the rate and duration of the spinning. At relatively low speeds, results rather similar to Ege's are obtained, but with considerable inconstancy; it must be remembered, however, that several factors combine to render the packing of cells in hypotonic saline less perfect than that of cells in plasma. High speed hæmatocrites are, therefore, preferable to those which run at a lower speed, and give much more consistent results. To illustrate these points, we can compare the increases in volume and the percentages of "free water" calculated therefrom in an experiment in which the cells of the same animal were suspended in plasma and in hypotonic plasma equivalent to 0·56 p.c. NaCl, and then spun in the hæmatocrite under different conditions.

Speed (r.p.m.)	Time of spinning (min.)	Volume increase, p.c. of "normal"	"Free water," p.c. of total water
3000	10	163	94
3000	40	153	79
8000	10	134	51

The percentage of water imagined to be "free" accordingly varies with

the conditions of spinning; Ege himself, indeed, finds by the hæmatocrite method that the percentage of "free water" varies from about 80 p.c. to 100 p.c. of the total water present, and sometimes gives figures which, on his assumptions, lead to the conclusion that there is more "free water" in the cell than the total water it contains. These inconsistent results are due, we suppose, to the inadequacy of the hæmatocrite method, especially at low speeds. Provided the rate of spinning is sufficiently high, however, the results obtained agree in a general way with those obtained by the diffractometer. This is shown by the figures in the following table, which refer to rabbit cells suspended in hypotonic plasma, and spun at about 8000 r.p.m. for 10 minutes.

Tonicity in terms of NaCl	Volume increase, p.c. of "normal"	"Free water," p.c. of total water
1·008	103	40
0·90	109	58
0·78	120	69
0·67	133	73
0·56	145	68

These results are similar to those obtained by diffraction, and are like those reported by Warburg and Winge using the hæmatocrite method with human cells, which also, when studied by the diffraction method, swell as if only about 50 p.c. of their contained water is "free."

The agreement between the results given by the diffractometer and those given by the high speed hæmatocrite are never very good, however; nor, indeed, can we expect them to be, considering the nature of the latter method. Duplicate determinations by the hæmatocrite, even at high speeds, may show differences amounting to 10 p.c. in volume when the cells are suspended in hypotonic saline or plasma, and such a difference may correspond to the difference between 90 p.c. and 75 p.c. "free water," or between 70 p.c. and 56 p.c.; the method cannot be expected to give consistent values for "free water," and should never have been used for this purpose.

The observation that swelling in hypotonic plasma, as determined by hæmatocrite, is substantially the same as swelling in hypotonic saline solutions, as determined by the diffractometer, indicates that the results given by the latter method are not affected in any important way by the fact that the cells are in the spherical form in saline, while in plasma they are discoidal[1]. If swelling had been different as measured by the two

[1] It is not necessary that the swelling in hypotonic saline shall be altogether the same as that in hypotonic plasma, for Kerr has shown that the quantity of Na and K which enters or leaves the cells may depend on the quantity of plasma present.

methods, it would have been necessary to consider the important possibility that the change to the spherical form confers upon the cell the property of losing osmotic substances in the way above described, but as the results of the two methods are similar, we may set this possibility aside.

6. The phenomenon of osmotic hæmolysis.

The accepted explanation for hypotonic hæmolysis, introduced originally by Hamburger, and supported by the investigations of Koeppe, Ege, Warburg and Winge, Jacobs and others, is that the cells take up water, swell, and finally burst because their membranes are stretched or under tension. During this process the volume of the cell is always supposed to be the same as that which can be calculated from simple osmotic laws when the membrane is supposed to be impermeable to cations. Some observers have postulated the existence of "bound" water within the cells, while others have treated all the water as "free"; such postulates influence the calculations somewhat, but the general conception remains the same.

This explanation is, of course, quite satisfactory in a qualitative way, for water must enter cells from hypotonic solutions, and the more water the more hypotonic the solution. When looked at quantitatively, however, the theory is unsatisfactory in at least two respects. (i) Neither the amount of swelling necessary to cause the cells to hæmolyse, nor the amount of stretching of the membrane which ensues, has yet been satisfactorily measured; the explanation is thus unsatisfactory for the reason that it is insufficiently specific. So far as our information goes (excluding that contained in this paper), either the swelling or the stretching might be of quite a wrong order. (ii) One of the principal difficulties which we encounter in using the theory in its usual form is to explain why solutions of different substances, prepared so as to be osmotically equivalent, produce lysis in such different concentrations. This fact can, of course, be accounted for by introducing subsidiary assumptions, although Moore and Roaf [1908] abandon the entire osmotic theory because of it, and believe that the swelling is not osmotic, but rather of the nature of an imbibition. Ege, for example, considers the possibility that the dissociation of osmotically active substances within the cell may change, and so give rise to different equilibrium volumes; he rightly concludes, however, that under the conditions of the usual experiments the possible changes are too small to account for the observed phenomena. Alternatively, we might adopt the hypothesis used

by physical chemists in the study of the solvation of proteins, and postulate different percentages of "free" and "bound" water in the cells as a result of their immersion in solutions of different substances; this assumption would also explain the different swelling curves obtained in NaCl, KCl and glucose. The recent demonstrations that "bound" water does not exist in this sense in sufficient quantity make such an assumption untenable. A third hypothesis is that when cells are suspended in solutions of different substances the membrane offers varying degrees of resistance to stretching, so that in NaCl, for example, the membrane is more extensible for the same difference in osmotic pressure than it is in glucose. Not unlike this hypothesis is one which depends on Brinkman's observations [1920] that the fragility of the cell depends on the lecithin-cholesterol ratio in the membrane; different substances, affecting the ratio in different ways, might conceivably cause differences in the resistance of the membrane, and so bring about lysis in different equivalent concentrations. None of these subsidiary assumptions is satisfactory, either because it is incompatible with the known facts or because it is undemonstrated or undemonstrable[1]; the classical theory, accordingly, requires to be reconsidered with respect to the two difficulties first mentioned.

(i) *Swelling and stretching.* There seems to be clear evidence that the commencement of hæmolysis corresponds to a fairly definite increase in the original cell volume, the most usual figure, for rabbit cells, being about 35–40 p.c. (for different rabbits). This figure appears to be about the same whether the cells are suspended in hypotonic saline and their volumes measured in the spherical form or whether they are suspended in hypotonic plasma and their volume measured by the hæmatocrite while they are discoidal. As has been pointed out already, this critical volume is much smaller than that expected on the classical theory which assumes the cell to be a perfect osmometer, but is of the right magnitude if we suppose the cell to lose osmotically active substances at the same time as it takes in water.

In considering the stretching of the membrane which accompanies this increase in cell volume, we have to distinguish between the stretching which takes place when the cell is measured in the spherical form and that which may occur when the discoidal form is maintained. When the cells are in the spherical form, the average increase in area before lysis

[1] One of us will show, in a paper to be published shortly, that Brinkman's original observations cannot be confirmed, and that osmotic resistance of rabbit red cells is independent of the *p*H of the washing or suspending medium within the limits 5·8–7·4.

begins is 21 p.c., *i.e.* the area increases from about $73\mu^2$ to about 88μ. This extension is considerable. When we remember that the membrane is not more than about $0{\cdot}1\mu$ in thickness, it is inconceivable that the stretching can occur within elastic limits. Considerations of this kind enable us to exclude the possibility that the smallness of the volumes observed in hypotonic solutions is due to elastic resistance of the cell membrane to further entrance of water. If we imagine an elastic resistance to exist, the osmotic pressure within the cell would be greater than that outside, but the difference can be computed from the observed volumes, and is far greater than could be maintained by any known substance spread out as a membrane of this thinness.

Unfortunately, we can obtain no measurements of the extent to which the membrane is stretched in hypotonic plasma, in which medium the discoidal form is maintained. Owing to the peculiar shape of the erythrocyte, its volume can increase about 20 p.c. without the area changing significantly (or even at all, if we imagine both the biconcavities to turn inside out simultaneously). The greatest observed volume increase in hypotonic solutions is between 30 and 40 p.c.; it therefore follows that the amount of stretching which the membrane must undergo before lysis begins cannot be very great when the cell is discoidal, and must be less than that which accompanies commencing osmotic lysis in the spherical form. We thus have the rather remarkable situation in which lysis is more dependent on the assumption of a critical volume than on a critical amount of stretching of the cell membrane, and the classical idea that the cells swell and that the membrane becomes so stretched that it leaks seems too simple to account for all the facts.

(ii) *Lysis in different equivalent concentrations.* The explanation advanced in this paper for the fact that solutions of various substances produce commencing lysis and the same "critical volume" in different equivalent concentrations is that different amounts of leakage of osmotically active substances take place in the various solutions[1]. We suppose, for example, that leakage is greater in glucose than in NaCl, and that this accounts for the "critical volume" being reached in a solution of glucose which is more hypotonic than one of NaCl.

[1] This paper contains no suggestions as to why different amounts of leakage should take place in different solutions, but is limited to a quantitative description of the phenomena observed. Presumably the different amounts of leakage are related to differences of the permeability of the red cell membrane in the different suspending media, but the nature of these differences and the factors which bring them about must be left for further investigation.

From the expressions already given, the following relation can easily be derived:

$$\frac{11{\cdot}2\,(Q_1).(R/100)}{(Q_1).(R/100)+v} = \frac{T}{100-v}, \qquad \ldots\ldots(9)$$

in which all the symbols have the same meaning as above. Given an observed increase in volume v assumed by the cells in a solution in which T is known, we can calculate Q_2, the apparent amount of "free water," from expression (4). We can then find R, from expression (5), in which Q_1 and Q_2 are now known. Several tonicities can be used to give an average value of R. Every value in expression (9) is then known except v and T, and we then have a numerically soluble equation which relates any given volume increase to the tonicity of the solution producing it, under the conditions of leakage, found by experiment, which obtain for that substance.

We can use this equation as a crucial test of the ideas which we have put forward, and can do so in the following way.

We find the "normal" cell volume, by diffraction, in 1·12 p.c. NaCl. We then find the critical volume, *i.e.* the volume of the cell in a solution just too strong to initiate lysis. From these two figures the increase in volume, v_{NaCl}, is computed as a fraction of the original volume. We next measure the volumes which the cells take up in solutions of some other substance, *e.g.* LiCl, these solutions being prepared so as to be osmotically equivalent to NaCl solutions of certain tonicities (*e.g.* 0·9, 0·8, 0·7, 0·6 g. per 100 g. water). Continuing to take the "normal" volume as that found in 1·12 p.c. NaCl, we compute the volume increases, v, observed in the LiCl solutions, and thence calculate the average value of R found in these solutions. Making the assumption that the same critical volume is attained in NaCl solutions as in LiCl solutions, we insert in expression (9) the value of v_{NaCl}; all variables are now known except T, which is solved for. In short, we are able to compute the tonicity of any substance in which the critical volume increase, v_{NaCl}, will be attained, and in which, if this critical volume increase is the same for all substances, lysis will just begin.

We have carried out this procedure in a number of cases, and the table on p. 292 will show the results obtained. All tonicities are expressed in NaCl equivalents.

Remembering that the "critical volumes," as measured for NaCl, KCl and glucose, are only critical to within about $\pm 2\mu^3$, the predicted tonicities for commencing lysis agree quite well with those observed. We interpret this agreement as constituting a striking confirmation of the suitability of the methods employed and of the validity of the several

Substance	Tonicities used for finding R	Predicted tonicity for beginning lysis	Observed tonicity for beginning lysis
NaCl	0·7, 0·6, 0·54	0·52	0·52
NaCl	0·8, 0·7, 0·6	0·63	0·56
KCl	0·78, 0·76, 0·70	0·62	0·66
KCl	0·8, 0·7, 0·64	0·64	0·58
Glucose	0·6, 0·5, 0·46	0·38	0·36
Glucose	0·7, 0·6, 0·56	0·48	0·44
LiCl	0·9, 0·7	0·67	0·63
KNo_3	0·8, 0·7, 0·6	0·59	0·59
Sucrose	0·8, 0·6	0·52	0·50

assumptions made. Such an agreement could not possibly arise by chance, nor, so far as we know, could as accurate predictions be made on any existing theory.

If the implications of this study are well founded, two consequences follow. Most of the existing difficulties in formulating an adequate theory for osmotic hæmolysis disappear, for it becomes easily understandable that swelling and osmotic hæmolysis are closely related, and yet that commencing lysis occurs in solutions of different substances of different equivalent concentrations. Further, existing methods of approaching the permeability of the red cell must be revised; indeed, if other cells show the same behaviour towards osmotically active substances as does the erythrocyte, it would appear that no adequate treatment of their osmotic behaviour can be made without a reconsideration of generally accepted assumptions.

7. Hypertonic solutions.

We have already shown [Ponder and Saslow, 1930 *b*] that the results obtained by the colorimetric method for the volume of cells immersed in hypertonic solutions are somewhat variable, and dependent on the time which is allowed to elapse before the measurements are made. One or the other of two kinds of result, however, is usually obtained; either the cells shrink in hypertonic NaCl, KCl and glucose, or they swell in such solutions (presumably after a preliminary shrinkage), so that their final volume may be 10 p.c. greater than the "normal" volume, even in 1·5 p.c. NaCl, or 2 p.c. glucose (NaCl equivalent). In those cases in which a steady shrinkage is observed, moreover, the cells decrease in volume as if they are perfect osmometers containing considerably less than 100 p.c. free water, or as if, on the present hypothesis, osmotically active substances penetrate from the external medium; in the cases in which the shrinkage is not observed, even greater penetration of such osmotically active substances must be assumed.

Measurements of the cell volume by diffraction, in systems differing

from the above, in that they contain large quantities of hypertonic NaCl, etc., and small quantities of plasma, lead to the same kind of result. The amount of shrinkage is very variable; this may be due to as yet uncontrolled factors, such as the time allowed to elapse before measurements are made, which have not yet been fully investigated. The figures in the following table will give a general idea of the nature of the observations.

Tonicity, as g. p.c. NaCl	Expected volume decrease p.c.	Observed volume decrease p.c.		
		NaCl	KCl	Glucose
2·0	29	10	14	—
1·5	16	8	6	10
1·3	9	4	1	6
1·1	0	0	0	0

The observed shrinkage is always less than that expected, and we can account for this in the same way as we have already done, viz. by assuming the penetration of osmotically active substances. Such an idea is supported by Kerr's analyses [Kerr, 1929]. A similar result has also been obtained by Ege [1921], who accounts for it on the now abandoned hypothesis of "bound" water.

If the cells were always to show shrinkage as if they contained 50 p.c. of their water "free," and also were to swell as if they contained 50 p.c. "free" water, it would follow that the simple law regulating leakage of osmotically active substances

$$dX/dY = \text{const.} = f(R),$$

would also be the law regulating penetration of osmotically active substances into the cell, the sign, of course, being reversed. We have not yet been able to show that this is quantitatively true, for shrinkage in hypertonic solutions is rather variable, but all our evidence goes to show that it is true in a general sort of way. Even the variability observed, however, is understandable, for, as the results of investigation by the colorimetric method show [Ponder and Saslow, 1930 *b*], the shrinkage becomes less and less as time goes on, *i.e.* the penetration of ions becomes greater and greater. This is precisely what Hill finds in the case of prolonged survival of muscle (in hypotonic solutions) [1930].

Summary.

1. A method is described suitable for the investigation of changes in rabbit red cell volume in hypotonic solutions. The method consists in finding the cell volume from diffractometric measurement of the radius

of the cell in the spherical form; the determinations are subject to an error of $\pm$ 2 p.c.

2. The increase in volume of cells in hypotonic media is considerably less than expected if the cells are regarded as perfect osmometers. Rejecting the idea that the cells contain "bound" water in appreciable quantities, we account for this behaviour by supposing that osmotically active substances leave the cell, to varying extents depending on the nature of the external medium.

3. Since the cells behave quantitatively as if a constant fraction of their contained water were "bound," it follows that some simple relation exists between the amount of substance lost and the tonicity of the external medium. The expression $dX/dY = \text{const.}$ is shown to be the relation required, where X is the percentage of the original amount lost and Y the tonicity.

4. Measurements by hæmatocrite of cell volume in hypotonic plasma (in which the cells are discoidal) lead to the same conclusions as those made by the diffractometer when the cells are in the spherical form in saline solutions.

5. The principal existing difficulty in osmotic theories of hypotonic haemolysis, *i.e.* that the same amount of lysis may be brought about by solutions of widely differing freezing-point depression, disappears if leakage of osmotically active substances from the cell is postulated. By measuring the amounts of such leakage we have been able to predict, with considerable accuracy, the concentrations in which a number of different substances just begin to produce lysis.

6. In the case of the rabbit red cell, hæmolysis commences when the cell volume has increased by 30–40 p.c., *i.e.* a critical volume for hæmolysis exists for nearly all the substances used. This critical volume increase is associated, in the spherical form, with a 20 p.c. stretching of the cell membrane; but in the discoidal form, the increase in area of the membrane is probably much less.

7. Diffractometric measurements of cell volume in hypertonic solutions confirm the results which we have already obtained by the colorimetric method, and lead to a similar conclusion, viz. that osmotically active substances enter the cells from hypertonic solutions.

APPENDIX.

Note on "osmotically transferable" water.

The controversy between Gough [1924], Ege [1927] and Krevisky [1930] regarding the amount of osmotically extractable water in the cell has very little to do with the question of "free" and "bound" water as considered in this paper. "Free" water is here used in Hill's sense of "solvent" water; Gough, Ege and Krevisky discuss the maximum amount of water which can be transferred by osmotic means across the cell membrane without the transference of hæmoglobin. Ege finds this to be about 80 p.c. of the contained water; Gough and Krevisky find it to be about 50 p.c. and the latter has shown that Ege's high figure is erroneous.

The validity of the results of any of these observers depends on there being, under the conditions of their experiments, a considerable excess of osmotically active substance in the fluid surrounding the cells at equilibrium. Krevisky calculates that such an excess exists, but it has not been demonstrated; since we know that osmotically active substances penetrate the cell from hypertonic solutions, we have to consider the possibility of such substances entering the cell from the strong NaCl solutions used to extract water, and so raising the tonicity of the cell interior. If this were to occur, some water would appear untransferable by osmotic means, but the real reason for its being so would not be that it was in any sense bound to the hæmoglobin, but that the osmotic pressure excess in the fluid outside the cells had been considerably reduced or even abolished.

This point may be decided by means of chemical analysis. Rabbit red cells were washed several times in 4 p.c. NaCl; this treatment results in a diminution in cell volume, the cells behaving as if about half their contained water had been osmotically extracted, as stated by Gough and Krevisky. Treatment with stronger NaCl solutions does not appear to result in the loss of more water, unless lysis occurs. Sodium and potassium analyses were then carried out on samples of (*a*) packed cells, and (*b*) washing fluid, and for these we have to thank Dr K. C. Blanchard. The results appear below (mg. per c.c.):

	1st sample		2nd sample	
	Na	K	Na	K
Cells from washing fluid	0·17	5·00	0·16	4·95
Washing fluid	18·6	Trace	18·6	Trace

A considerable difference in osmotic pressure, accordingly, exists between the cell interior and the washing fluid, even when as much water as is possible to extract osmotically has been extracted. The difference can be calculated to be almost 100 p.c. Some of the water contained in the cell must, therefore, be "osmotically untransferable," at least under conditions which permit the retention of hæmoglobin. Hill has, nevertheless, shown that most of the water is "solvent" water; we have therefore to distinguish between "solvent" water or "free" water as used in the sense of this paper, and the "osmotically transferable" water of Gough and Krevisky. The latter cannot be used as a measure of the former, but, judged from a purely experimental standpoint, "osmotically untransferable" water appears to have an actual existence nevertheless. Much of the confusion which has centred round Ege's and Gough's results is due to a difference in definition of the term "free" water (Gough using it as "osmotically transferable" water and Ege as "solvent" water), and also to the coincidence that in NaCl solutions the leakage of osmotically active substances from the cell is such as to indicate an amount of "free" water (erroneously regarded as such) which is about the same as the quantity osmotically transferable under the conditions used by Gough.

REFERENCES.

Allen, A. and Ponder, E. (1928). *J. Physiol.* **66**, 37.
Brinkman, R. and van Dam, E. (1920). *Biochem. Z.* **108**, 35.
Christensen, I. and Warburg, E. J. (1929). *Acta Med. Scand.* **70**, 286.
Ege, R. (1921). *Biochem. Z.* **115**, 88.
Ege, R. (1927). *Biochem. J.* **21**, 967.
Gough, A. (1924). *Biochem. J.* **18**, 202.
Hamburger, H. J. (1902). *Osmotische Druck und Ionen-lehre* (J. F. Bergmann, Wiesbaden). **1**, 369–70, 442–53.
Hedin, S. G. (1891). *Skand. Arch. Physiol.* **2**, 134.
Hedin, S. G. (1895). *Skand. Arch. Physiol.* **5**, 207.
Hill, A. V. (1930). *Proc. Roy. Soc.* B, **106**, 447, 477.
Jacobs, M. H. (1926–27). *The Harvey Lectures*, Series XXII, 146.
Kerr, S. E. (1929). *J. Biol. Chem.* **85**, 47.
Koeppe, H. (1900). *Physikalische Chemie in der Medizin* (A. Hölder, Wien), pp. 79–95.
Krevisky, C. (1930). *Biochem. J.* **24**, 815.
Margaria, R. (1930). *J. Physiol.* **70**, 417.
Millar, W. G. (1926). *Proc. Roy. Soc.* B, **99**, 264.
Moore, B. and Roaf, H. E. (1908). *Biochem. J.* **3**, 55.
Neuhausen, B. S. and Breslin, J. E. (1923). *Johns Hopk. Hosp. Bull.* **34**, 199.
Ponder, E. (1929). *Brit. J. Exp. Biol.* **6**, 387.
Ponder, E. and Saslow, G. (1930 *a*). *J. Physiol.* **70**, 18.
Ponder, E. and Saslow, G. (1930 *b*). *J. Physiol.* **70**, 169.
Saslow, G. (1929). *Quart. J. Exp. Physiol.* **19**, 329.
Waller, W. W. (1930). *J. Physiol.* **70**, 42 *P*.
Warburg, E. J. and Winge, K. (1928). *Acta Med. Scand. Supplem.* XXVI, 500.