THE OSMOTIC BEHAVIOR OF CRENATED RED CELLS

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A survey of the results of the experiments which have been done on the volume changes of mammalian red cells in solutions of various tonicities shows that the red cell sometimes behaves as a perfect osmometer, while at other times it does not (Ponder, 1940). More specifically, if a red cell, the initial volume of which is denoted by 100, is immersed in an infinite volume of a medium of tonicity T, the new volume which the cell ought to take up by the exchange of water alone is

$$V = W(1/T - 1) + 100 \qquad (1)$$

where W is the percentage of water in the cell by volume, and where T is the depression of freezing point of the suspension medium divided by the depression of freezing point of the undiluted plasma. When V is measured experimentally and compared with V in expression (1) it is often found that there is a considerable discrepancy, the swelling of the cell being described by the expression

$$V = RW(1/T - 1) + 100 \qquad (2)$$

where R is a "correction factor introduced to reconcile observation with theory."

Three suggestions have been put forward as to the meaning of the constant R, and two of these have already had to be abandoned, at least as complete explanations. The first is that R measures the fraction of cell water which is free, the remainder being bound. To account for the variations in the value of R, this explanation requires that the amount of free water varies, in an unexplained way, from about 50 per cent of the total cell water to about 100 per cent, quite apart from its being very doubtful that the red cell contains any appreciable quantity of bound water at all (Hill, 1930; Macleod and Ponder, 1936). The second explanation is that the cell reaches osmotic equilibrium by losing or gaining osmotically active substances as well as by gaining or losing water; under these circumstances the percentage of osmotically active substance lost or gained would be 100 $(1 - R)$ $(1 - T)$. The quantity of salt lost from red cells into hypotonic solutions, however, is much smaller than this explanation demands (Ponder and Robinson, 1934; Davson, 1934; Ponder, 1940). The third explanation is one to which I called attention in 1940, and is that the entry of water from hypotonic solutions, and the loss of water to

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hypertonic solutions, is opposed by elastic forces of which R is an indirect measure. It is with this possibility that this paper is concerned.[1]

I. Shape Changes and Elastic Forces

It is customary to describe the swelling of red cells in hypotonic solutions as taking place by the biconcavities turning inside out, the short axis of the cell increasing while the diameter decreases, and the cell utimately hemolyzing either as a spheroid of low eccentricity or as a sphere. This picture of what happens is all very well for the purposes of mathematical (Ponder, 1924) or illustrative (Haden, 1934) treatment, but in the case of the individual red cell it does not represent the facts. So far as individual cells are concerned, the shapes assumed during swelling are very variable and cannot be accounted for on any supposition other than that they have rigidity of form.

(*a*) If the red cells of man or of rabbit are examined in hypotonic media in which the extent of the swelling of the average cell is known (*e.g.*, in hypotonic plasma of $T = 0.7$, in which the volume is about 130 per cent of the volume in undiluted plasma), the swelling will be found to be accompanied by different shape changes in the case of different cells. Some cells retain their biconcave form, apparently increasing in volume by increasing their thickness uniformly or by becoming a little thicker at the ends. Others become cup-shaped, one biconcavity being obliterated. Still others are spheroidal and convex, and a few are spheres. If the cells show crenation in the isotonic medium, the crenation may persist in the hypotonic medium, and swollen cells may present it to a varying degree. Neighboring cells do not necessarily swell to the same extent; and a crenated thorn-apple form may be seen side by side with a spheroidal or a cup-shaped cell which is obviously swollen. The most superficial observation shows that the individual cell does not always behave as if it were a balloon surrounded by a uniform non-elastic membrane, for the radii of curvature for different points on the surface assume such a variety of values that it is necessary to suppose either that the tensions vary enormously, *i.e.*, that there is non-uniformity of structure and rigidity of form, or that the cell is sometimes not a balloon-like body, but a gel.

(*b*) As swelling proceeds, the diameter of the average cell decreases about 8 per cent as the thickness increases. This observation (Ponder, 1924, 1933) has been confirmed by Teitel-Bernard (1932) and by Haden (1934). The

[1] A fourth explanation, that the values of R which are significantly less than 0.95 (this would allow 5 per cent of the total cell water to be "bound" as water of hydration or hemoglobin) are due to experimental error, is quite untenable. Even when one makes every allowance for the errors which may occur in the measurement of red cell volume, one cannot explain away the frequency with which low values of R have been found by different observers, working independently, and using a variety of methods.

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decrease in diameter is what would be expected of a spheroidal balloon-like body surrounded by a membrane and containing fluid contents, but the proof that a decrease in diameter would occur in such a body during swelling (Ponder, 1924) depends absolutely on the membrane having elastic constants.

(*c*) If we trace the changes in shape and area which occur as swelling proceeds, we get a diagram which is incompatible with anything except a body with rigidity of form. Let us represent increase in volume on the abscissa of Fig. 1,

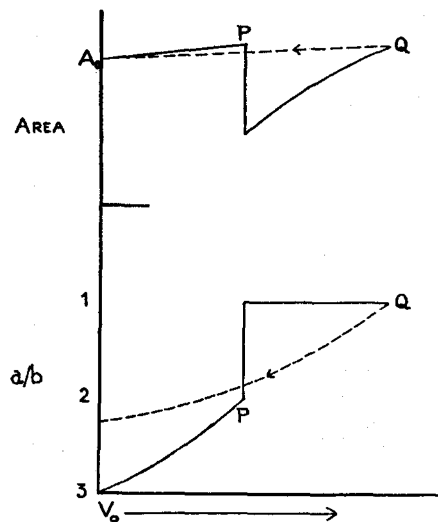

FIG. 1. Upper ordinate, red cell area, with initial area A_0; lower ordinate, length/thickness ratio a/b. Abscissa, red cell volume, with initial volume V_0. Solid lines, course of events as cell swells; dotted lines, probable course of events after lysis. P, yield point; Q, critical volume. The cell is represented as returning to its critical volume but not to its original a/b ratio because it does not usually return completely to the original biconcave discoidal form (see Furchgott, 1940).

starting with the initial volume V_0, and let us also represent the cell area, starting with the initial area A_0, and the length/thickness ratio a/b as a rough measure of shape,[2] on ordinates. (1) As swelling proceeds, the area of the cell increases only slightly, and the increasing volume is accommodated by a change in shape, so that the length/thickness ratio becomes smaller. The constancy of the area must mean that the extensibility of the membrane is not very great. (2) As the swelling continues, a point is reached (the point P in the diagram) at which some structure appears to give way and at which the cell becomes a

[2] The use of the length/thickness ratio as a measure of shape again involves the introduction of an abstraction which may have little meaning in the case of an individual cell (see (*a*) above).

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sphere. With this sudden change in shape the a/b ratio becomes 1.0 and the area decreases. The yield point P may correspond to smaller volume increases in the case of some cells than in the case of others. (3) With further increases in volume, the area of the sphere increases until hemolysis occurs when the critical volume is reached at the point Q, the ratio a/b remaining unity. The critical volume may be smaller under some circumstances than under others, *e.g.*, when the cells are suspended in saline instead of in plasma.

This diagram clearly applies to a body which has rigidity of form. If we replace the units of volume on the abscissa with units of force, it becomes a stress-strain diagram, and the line V_0P represents the behavior of the body within the elastic limits of its materials. We can certainly make this change in the units on the abscissa in cases in which the constant R of expression (2) is found experimentally to be less than unity, because under such circumstances we have experimental justification for thinking that an elastic force opposes osmotic swelling. It will be shown below that R is less than unity under circumstances which are associated with crenation and gelation, when the elastic resistance to osmotic swelling is presumably great. When R is found experimentally to be unity, on the other hand, the elastic resistance offered by the cell material must be too small to measure.[3]

(*d*) When the critical volume is reached the cell hemolyzes and the stretching forces disappear. The hemolyzed ghost then returns more or less completely to the shape of the cell from which it was derived (dotted lines in Fig. 1), and this in itself is evidence that its materials possess elastic properties, particularly as the return to the discoidal, or at least spheroidal, form takes only "a fraction of a second" (Teitel-Bernard, 1932).

II. Crenation and Gelation

The changes in red cell shape during swelling suggest that its materials possess rigidity, but do not decide the question as to whether the rigidity is sufficient to resist osmotic swelling to a measurable extent. About fifteen years ago, when the tendency was to look upon the red cell as a body with fluid contents and possessing no structure except for a membrane about 100 Å thick, it was difficult to think of it as a structure sufficiently rigid to oppose

[3] It should be noticed in this connection that the R values of experiment are nearly always less than 1.0, and rarely greater. This makes it likely that the small downward deviations of R have a real significance, and are not due simply to experimental error. We often, for example, find an R value of 0.9, and, adding up all the possibilities of experimental error, we have to admit that an error of 0.1 is possible. But we rarely find an R value of 1.1. The fact that so many of the high values turn out to be a little less than 1.0 can be accounted for by supposing that only some 95 per cent, instead of all, of the cell water is "free," but until we know exactly what the figure for the "free" water is, there is the possibility that even values of R in excess of 0.9 are indirect measures of a small elastic resistance.

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osmotic forces of the order of 1 atmosphere. We now know more about red cell structure and about the influence which the interior exerts on shape transformations (Ponder, 1942), and if we consider that under some circumstances the red cell interior may actually be a gel, the existence of elastic forces sufficient to resist osmotic swelling becomes not only possible but probable. Since it is in the case of crenated cells that the evidence for rigidity and the existence of forces opposing osmotic swelling is the strongest (section I, *a*, and section III), we shall advance the argument considerably if we are able to show that crenation and gelation go hand in hand.

The likelihood of their doing so will appear greater if we consider how little water has to be abstracted from the cell in order to convert its interior into a gel. Although it can now be regarded as settled (Waller, 1935; Ponder and Saslow, 1930*b*) that crenation does not necessarily correspond to a diminution in cell volume, it nevertheless tends to occur under conditions in which water is abstracted, *e.g.*, in hypertonic media and after the addition of oxalates. Since the molecular weight of the "wet" hemoglobin crystal (hemoglobin + water) is 132,000 and that of the hemoglobin molecule alone is 66,700, each 66,700 parts of hemoglobin contains 65,300 parts of water. The "wet" crystal therefore contains about 49 per cent of water; the red cell itself, however, contains about 60 to 65 per cent of water, and so by the abstraction of a comparatively small quantity, such as certainly could be removed either by hypertonic solutions or by the addition of an excess of oxalate, its interior will be brought into the state of the wet crystal, which is a rigid structure.

Teitel-Bernard (1932), indeed, regards crenation as one stage in the formation of intracellular crystalline hemoglobin. He observes that crenated red cells show more birefringence than normal cells do, and attributes this to an increasing degree of orientation of the hemoglobin which ultimately leads to intracellular crystal formation. This takes place in several stages, each more stable than the one before it. In the first stage, which corresponds to the reversibly crenated erythrocyte, local areas of gelation appear, and in these areas, which correspond to the individual crenations, the hemoglobin molecules are lined up along their axes of crystallization. The orientation is reversible at first, and the crenations can be made to disappear by mechanical, chemical, or osmotic means. In the more advanced stages the gel formation and the orientation of the hemoglobin are more permanent, and in the final stage crystals of hemoglobin are seen. Teitel-Bernard thinks that the gel which is formed in the earlier stages is actually a hemoglobin gel formed as a result of the destruction of an equilibrium of some kind, as by the withdrawal of water or the changes of pH towards 6.5, the isoelectric point of hemoglobin. It confers on the crenated cells a mechanical rigidity which can be demonstrated by microdissection methods, and they are sometimes so completely gelated that they do notl ose their contents even when torn by the microdissection

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needle.[4] The conclusion that the crenated red cell has an internal skeleton which gives it mechanical rigidity is in keeping with a number of other observations in the older literature, such as the observation of Schafer that red cells can sometimes be cut in two without losing their contents and the description by Rockwood of red cells which are penetrated by glass particles without their hemoglobin escaping.

Generally speaking, the literature treats the phenomena of crenation in a perfunctory fashion, and does not make sufficient distinction between one form of crenation and another. There seem to be at least three forms. (1) One kind of crenation is often associated with the abstraction of water from the cell and with a diminution in cell volume, although diminution in volume is not necessarily accompanied by crenation. This type occurs when the cells are exposed to hypertonic solutions, or when oxalates, fluorides, etc., are added to plasma as anticoagulants. There is no difficulty in seeing how it could be the result of gelation of hemoglobin and perhaps also of whatever other proteins exist in the cell interior. (2) Human cells in saline media containing acetate buffers begin to crenate at pH = 7.0 when the pH is reduced towards 6.5, the isoelectric point of hemoglobin, and the crenations persist until about pH 6.0, below which the cells are discoidal until they begin to hemolyze at about pH = 5.4 (Claudio, 1931; Teitel-Bernard, 1932). This type of crenation appears to be associated with the intracellular crystallization of hemoglobin, no doubt preceded by gel formation. (3) Washed human red cells in saline media become crenated if the pH is increased above 8.0, and assume the perfectly smooth spherical form at about pH = 9.2. The appearance of crenation and the assumption of the spherical form, at first crenated and later smooth, can be prevented between pH 8.0 and pH 10.0 by the presence of the antisphering substance of plasma, shown to be an albumin by Furchgott (1940) and Furchgott and Ponder (1940). This type of crenation is probably due to an inability of the red cell membrane to maintain its special shape rather than to a process of gelation.

[4] The changes observed when a crenated cell is pricked with the microdissection needle are different from those which follow the pricking of a normal cell. The normal cell becomes spherical, fades, and becomes a ghost, which momentarily reassumes the flattened shape of the original cell before shrivelling up into a viscous mass on the end of the needle. Some crenated cells behave similarly, but fade more slowly, as if their contents were more viscous. Others become spheres, but do not lose their contents, and some can be transfixed without hemolyzing. They seem to be irreversibly gelated (Teitel-Bernard, 1932).

No direct measurements of tension and elasticity similar to those of Cole (1932) for the *Arbacia* egg or of Norris (1939) for nucleated erythrocytes exist for the crenated mammalian red cell. It is not unlikely that the values for the normal mammalian red cell would be small, like Norris' values, but that those for crenated cells would be very much greater.

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The pH range in which crenation is absent in saline media is therefore small (pH 7.0 − 8.0), and it is only when blood is heparinized, hirudinized, or defibrinated that we can expect crenation to be absent in plasma. Even under these circumstances crenation may occur for reasons not yet understood and apparently peculiar to the individual, and when it occurs it may affect only a few of the red cells, or many.[5]

III. Crenation and R Values

Proceeding on the assumption that the appearance of crenation is a sign of gelation in the red cell interior, we may enquire whether the values of R are smaller when red cells are crenated than when they are not. To do this we require to measure the swelling of cells in plasma of known degrees of hypotonicity, and, while this may be done by a variety of methods, the procedure described by Shohl and Hunter (1941) is the most satisfactory. Their method is a colorimetric one which differs from the original method of Stewart (1899), and from Ponder and Saslow's modification of it (1930*a*), in that the dye Evans blue (T-1824) is used in place of a solution of hemoglobin. The method is used in the following way for obtaining R values in hypotonic plasma.

Evans blue (Eastman Kodak) is dissolved in distilled water in a concentration of 600 mg. of dye per liter. Exactly 0.5 ml. of the solution is placed in a series of tubes (100 mm. by 13 mm.) and is evaporated to dryness at 70°C. It is convenient to prepare a few dozen tubes at a time, and the success of the method depends on the accuracy with which the same volume of the dye solution is delivered into each. Blood is obtained in the usual way with either heparin or oxalate as the anticoagulant, and about 1.5 ml. is centrifuged to give plasma, of which 0.5 ml. is added to one of the tubes containing dye. The tube is shaken to dissolve the dye, and 0.1 ml. of the dye-stained plasma is transferred to a tube containing 10 ml. of saline (tube P_0). To another tube (tube O_0) containing 10 ml. of saline is added 0.1 ml. of the plasma itself. One ml. of whole blood is now placed in a tube containing dye, which is dissolved by stoppering the tube and shaking gently. The tube with its contents is then gently centrifuged, and 0.1 ml. of the supernatant dye-stained plasma is transferred to another tube containing 10 ml. of saline (tube B_0). Using the contents of tube O_0 for setting the zero, opacity readings for the contents of tube P_0 and B_0 are made with a lumetron photometer; the amount of plasma, in milliliters, contained in the 1 ml. of blood is then obtained by dividing the reading P_0 by the reading B_0

[5] Guest and Wing (1942) have recently described an ingenious variety of hematocrit method by means of which the swelling of red cells can be followed even in solutions so hypotonic as to produce lysis. They find that normal human red cells (heparinized blood) tend to swell as perfect osmometers. Dr. Guest writes me, however, to say that the red cells in sicklemia and Mediterranean anemia (Cooley) swell less than would be expected of a perfect osmometer in hypotonic saline. The interest attached to this observation, so far as this paper is concerned, lies in the close relation between sickling and extreme forms of crenation.

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and multiplying by 0.5. The volume V_0 occupied by the cells is obtained by subtraction.

Two ml. of whole blood is next placed in a test tube and centrifuged gently so as to produce a layer of clear supernatant plasma. A volume of water equal to the volume of plasma known to be contained in the 2 ml. of blood, or $2(1 - V_0)$ ml., is added in such a way as to form a layer over the supernatant plasma. The tube is then stoppered and the contents are rapidly mixed by shaking. One ml. of the mixture is added to a tube containing dye, and after the dye is dissolved 0.1 ml. is transferred to a tube containing 10 ml. of saline (tube B_1). The rest of the mixture is centrifuged to give diluted plasma, of which 0.1 ml. is added to 10 ml. of saline (Tube O_1) and 0.5 ml. to a tube containing dye. When this is dissolved, 0.1 ml. of the dye-stained diluted plasma is transferred to 10 ml. of saline (tube P_1). The photometer zero is set for the contents of tube O_1, and 0.5 P_1/B_1 gives V_1, the new volume occupied by the swollen cells, the number of which, however, has been reduced by the addition of water to the whole blood. The percentage swelling, accordingly, is

$$V_{\text{exp.}} = \frac{100V_1}{V_0 \cdot \dfrac{2}{2 + 2(1 - V_0)}} \tag{3}$$

$$= \frac{100V_1(2 - V_0)}{V_0} \tag{4}$$

which is to be compared with the swelling of a perfect osmometer in a medium of tonicity T, in these experiments 0.5

$$V_{\text{calc.}} = W \cdot \frac{p - pT}{pT + 1} + 100 \tag{5}$$

in which W is the quantity of water contained in the cell and expressed as a percentage by volume, and p the ratio of the volume of the hypotonic medium to the volume of the cell water. R, the constant under discussion, is equal to $(V_{\text{exp.}} - 100)/(V_{\text{calc.}} - 100)$.

This method is far more satisfactory than any other method for measuring red cell volume that I have used, and its accuracy seems to be limited only by the precision with which photometric readings and delivery from pipettes can be made.[6] It has all the advantages claimed for Ponder and Saslow's modification of Stewart's colorimetric method, with the additional advantage that dye is used instead of a hemoglobin solution, and so the risk of error due to lysis of a few of the cells of the system is minimized. I have compared the results given by Shohl and Hunter's method with those given by the hematocrit, and

[6] An important technical point is that the transfer of dye-stained plasma is carried out with the special pipettes described by Levy (1936), from which very accurate delivery can be made. For measuring out the dye, I use 0.5 ml. micro pipettes ("to contain") with a ground tip and a constriction in the region of the 0.5 ml. mark.

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have obtained good agreement within the limits of error to which the hematocrit method is subject. In previous investigations on the swelling of red cells in hypotonic media there has always been some question as to the reliability of the method used (speed in the case of the hematocrit, the value of the form factor in the case of conductivity measurements, the wave length, etc., in the diffractometric methods, the possibility of hemoglobin escaping from red cells in the original colorimetric method, and so on), but the results obtained by this colorimetric method are not open to question on any such grounds.

TABLE I

Experiment	Anticoagulant	R	Condition of cells
1	Heparin	0.97	No crenation
2	Heparin	0.95	No crenation
3	Heparin	0.93	No crenation
4	Heparin	0.93	No crenation
5	Oxalate	0.97	No crenation either in $T = 1.0$ or $T = 0.5$
6	Oxalate	0.96	Slight crenation in $T = 1.0$; no crenation in $T = 0.5$
7	Oxalate	0.89	Slight crenation in both $T = 1.0$ and $T = 0.5$
8	Oxalate	0.85	Slight crenation in both $T = 1.0$ and $T = 0.5$
9	Oxalate	0.75	Some crenation in $T = 1.0$; considerably less in $T = 0.5$
10	Oxalate	0.75	Some crenation in $T = 1.0$; a lot in $T = 0.5$
11	Oxalate	0.72	Every second cell crenated in $T = 0.5$
12	Oxalate	0.70	Like No. 11
13	Oxalate	0.69	Like No. 11
14	Oxalate	0.67	Like No. 11
15	Oxalate	0.59	Marked crenation in both $T = 1.0$ and $T = 0.5$
16	Oxalate	0.52	Marked crenation in both $T = 1.0$ and $T = 0.5$

This method was used to measure the swelling of human red cells from (*a*) heparinized blood (5 mg. heparin to 5 ml. of blood) and (*b*) oxalated blood (20 mg. sodium oxalate to 5 ml. of blood).[7] The degree of hypotonicity established was $T = 0.5$ throughout. The cells were examined microscopically both in the undiluted plasma and in the hypotonic plasma, the purpose of the examination being to see if crenation was present, and if so, to what extent. The results of sixteen experiments are shown in Table I.

[7] The quantity of sodium oxalate usually used as an anticoagulant is 10 mg. per 5 ml. of blood. In these experiments the quantity has been doubled because it is desired to exaggerate the effect. The amount of crenation produced, however, bears no simple relation to the amount of oxalate used. Factors of both time and temperature are involved, and very marked differences can be observed among the cells of different individuals. Crenation sometimes occurs in both heparinized and defibrinated blood.

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It will be clear that the values of R in Table I depend in a general sort of way on the amount of crenation observed. In heparinized human blood crenation is usually absent, and R values found are usually high (0.9 or more). In oxalated blood, on the other hand, crenation is very often present, although its extent seems to vary from individual to individual for reasons still unknown. In any individual case, it should be observed that all the cells are not necessarily crenated to the same extent, and that some of them may appear normally discoidal while others are crenated; in some cases, moreover, crenation persists even in hypotonic plasma, while in others it is still in its reversible state and disappears as the cells swell. The lowest values of R are found when crenation persists in the hypotonic medium.

In estimating the amount of crenation in a preparation, one is helped by the fact that normal discoidal cells tend to form rouleaux, even in hypotonic plasma, whereas crenated ones do not. The latter therefore tend to lie apart from the rouleaux formed by the former. This is reflected in the observation that sedimentation is usually slower in oxalated blood than in blood which has been heparinized.

IV. Calculation of the Bulk Modulus

Suppose that the red cell swells to a volume $V_{\text{exp.}}$ when placed in a hypotonic solution of tonicity T, instead of to the larger volume $V_{\text{calc.}}$ given by expression (5). There must be some other tonicity T_1, higher than T, in which a cell with an R value of 1.0 would be in equilibrium when it had attained the volume $V_{\text{exp.}}$, and this tonicity can be shown to be

$$T_1 = \frac{pW - (V_{\text{exp.}} - 100)}{p(V_{\text{exp.}} - 100 + W)} \tag{6}$$

which is the same as expression (1) when p is indefinitely great. Now T and T_1 could be converted into their corresponding osmotic pressures by multiplying by a suitable constant k, the value of which is about 7.5. The bulk modulus of the cell material, considered as a uniform gel, is therefore given by

$$K = \frac{k(T_1 - T)}{0.01(V_{\text{exp.}} - 100)} \tag{7}$$

which, to give K in the usual units of dynes/cm.^{2}, must be multiplied by 10^6.

Taking the result of Experiment 16 in Table I as an example, we have $T = 0.5$, $p = 6.4$ (the initial volume concentration being 0.40), $V_{\text{calc.}} = 146$, and $V_{\text{exp.}} = 124$. This gives $R = 0.52$. Substituting in expression (6), we get $T = 0.664$, and substituting this in (7) gives $K = 5.1$ atmospheres or 5.1 (10^6) dynes/cm^2. This is the highest value found in this series of experiments, whereas the lowest value is zero if we suppose 5 per cent of the cell water to be bound to hemoglobin. The values of K are accordingly of the same magnitude as those found for gelatin gels (Northrop, 1927).

The variation in R between zero and about 0.5 in a series of experiments

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which show crenation to an increasing extent may be due either to there being different degrees of crenation each associated with a different degree of rigidity and value of K, or to some of the cells being crenated while others are not. The value of K as measured is a mean value equal to $\Sigma nK/n$, and these experiments do not distinguish between the effect of different degrees of rigidity and that of the involvement of different numbers of cells. The second factor, however, clearly enters into such experiments as Nos. 11 to 14.

DISCUSSION

In surveying the many experiments which have been done on the changes in volume of the mammalian red cell in solutions of different tonicity, the result of one type of experiment has always been very difficult to explain. This is the observation that when swelling is measured in hypotonic plasma the value of R is usually much more nearly unity when the blood is defibrinated or heparinized than when it is oxalated (Ponder and Robinson, 1934). The difficulty has been the greater because in this type of experiment the possibilities of error are reduced to a minimum, and there is no doubt about the tonicity of either the plasma bathing the cells initially or the plasma which is rendered hypotonic. The anomalously small degree of swelling in oxalated blood is now satisfactorily accounted for by the observation that the cells are usually crenated, and by the demonstration that such cells swell as if they were elastic bodies with a bulk modulus of the same order as that for gelatin gels.

Whether the effects of crenation and gelation explain every low value of R which has been described is another matter. In many experiments in saline media and in media containing non-electrolytes, crenation must have been present without having been commented on. This is certainly true of many of my own experiments done between 1931 and 1940. Since crenation is part of the process by means of which the spherical form is produced, a certain amount of suspicion may now attach itself to those experiments in which diffractometric measurements of volume were made after the cells were converted into spheres between slide and coverglass (Ponder and Saslow, 1931), or by the addition of lecithin (Ponder and Robinson, 1934; Ponder, 1935). This suspicion, however, is probably not justified, for the type of crenation which occurs during the disk-sphere transformations seems to be distinct from that which occurs when water is abstracted from the cell or when the isoelectric point of hemoglobin is approached, and does not seem to be associated with gel formation.

In certain systems, the value of R may be less than that expected of a perfect osmometer for reasons other than the appearance of crenation with its associated elastic forces. In hypotonic glucose, for example, there is some loss of osmotically active substances in the case of the red cells of some species (Davson, 1934), and both Davson (1937) and I and my collaborators have found

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some loss of potassium into hypotonic solutions and into hypotonic plasma. Although the losses have been too small to account for the values of R, it must be repeated with some insistence that they constitute presumptive evidence for the existence of a mechanism by which small amounts of osmotically active substances can pass across the red cell membrane.[8] The swelling of ghosts in hypotonic media (Ponder, 1936) may be another example of a case in which more than a simple exchange of water is involved. While the problem of the anomalously small swelling of the red cell is greatly simplified by recognizing that crenation and gelation can result in the development of forces which oppose the osmotic forces, it cannot fairly be said that it is completely solved in the quantitative sense.

SUMMARY

The anomalously small swelling which the red cells of human oxalated blood undergo in hypotonic plasma is related to the extent to which the cells are crenated. Reasons are given for regarding crenation as corresponding to gelation, and the bulk modulus for crenated cells, calculated from the measurements of swelling in hypotonic plasma, is shown to be of the same order as that for gelatin gels.

ADDENDUM

A particularly stable form of gelation is observed when rat red cells are suspended in 3 per cent sodium citrate. Initially, the cells are discoidal or cup-shaped in this medium, and undergo the typical disk-sphere transformations between glass surfaces and on the addition of lecithin. When kept in the refrigerator (4°C.) for about 72 hours, the cells become irregularly crenated, and are apparently so gelated that they do not hemolyze even in distilled water, provided it is cold (10°C. or less). At higher temperatures, *e.g.*, 20°C., lysis occurs slowly, and at still higher temperatures, *e.g.*, 37°C., it is quite rapid; as the cells are left longer and longer in the refrigerator, hemolysis becomes progressively slower at all temperatures.

The gelated cells, while irregularly crenated, are essentially discoidal, but are unable to undergo disk-sphere transformations either between glass surfaces or when lecithin is added. The addition of 1 in 1000 saponin is followed by a slow hemolysis without the usual preliminary shape changes; the crenations do not disappear, nor does the cell become a prolytic sphere. In some cells one gets the impression that one part of the cell loses hemoglobin before other parts do, and the ghost, after lysis is complete, presents the same crenations as the original cell did. When the crenated cells are slowly warmed in the distilled water in which they have stood, most of them hemolyze without any change of shape, the pigment escaping and leaving a crenated ghost behind. A few cells, however, and usually those which are the most optically prominent

[8] One can easily imagine a crenated, gelated cell losing electrolyte through an injured membrane without losing hemoglobin. In this way crenation might complicate experiments in which K loss is measured under different conditions.

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in the field, lose their crenations, become prolytic spheres, and fade. Presumably these are the cells in which gelation is incomplete or still partly reversible.

This extreme resistance to hemolysis by water is observed when rat red cells are kept in the cold in 3 per cent and 4 per cent sodium citrate, but not when they are kept in 2 per cent citrate. It also occurs to a slight extent when the cells are kept at low temperatures in 1.5 per cent NaCl, and after several days some of the cells hemolyze only slowly when added to distilled water. When rat cells are kept in isotonic NaCl, NaCl to which oxalate has been added, or phosphate buffer at pH 6.5, hemolysis in water occurs as usual.

Human red cells kept at 4°C. for 5 days in the acetate buffer at pH 6.5 in which Teitel-Bernard finds crenation to be at a maximum, do not exhibit the extreme resistance to hypotonic hemolysis shown by rat red cells. Rabbit red cells resemble the cells of man rather than those of the rat.

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