TONICITY-VOLUME RELATIONS IN PARTIALLY HEMOLYZED HYPOTONIC SYSTEMS

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The volume V which a red cell of unit volume attains at equilibrium when immersed in a medium of tonicity T and when it behaves as an osmometer and swells by the transference of water alone is

$$V = RW\left(\frac{1}{T + 1/a} - \frac{T}{T + 1/a}\right) + 1 = RW \cdot f(T, a) \qquad (1)$$

where a is the ratio of the volume of the surrounding medium to the volume of the cell water. A straight line of slope RW results from plotting V against $f(T, a)$; here W is the water content of the cell expressed as a fraction of unity and R is a constant which determines the "perfection" of the osmometer, and which has had various meanings attributed to it (see Ponder, 1949 *a*).

Volume determinations in hypotonic media have usually shown the relation between V and $f(T, a)$ to be linear, although the values of R are usually smaller than 1.0, the value which would be expected on the basis of a simple theory of osmotic behavior; most of the observations, however, have been restricted to systems in which the tonicity T is not small enough to produce hemolysis. It is possible to extend them by using media of lower tonicity and by measuring the volume $V/(1 - p)$ of the $(1 - p)$ cells which remain intact after the lysis of a fraction p of the total. Observations of this kind (Guest and Wing, 1939, 1942; Guest, 1948) have shown that the linear relation does not hold once p has attained values in the neighborhood of 0.2. I, too, have found marked departures from the linear relation when there is appreciable hemolysis in the systems (Ponder, 1949 *a*, footnote 8), and this investigation has been carried out in an attempt to obtain more information about volume changes in such partially hemolyzed systems.

Methods

Hemolytic Systems.—The systems are similar to those already described (Ponder, 1949 *a*). A suspension of washed red cells from human heparinized blood is prepared in 1 per cent NaCl-buffer,[1] the volume concentration being adjusted to $\rho = 0.4$ (to

[1] The composition of the NaCl-buffer used is 80 ml. of 1 per cent NaCl plus 20 ml. of a buffer mixture of M/15 NaH_2PO_4 and M/15 Na_2HPO_4. Most of the experiments

be used in dense systems) or to $\rho = 0.067$ (to be used in dilute systems). The systems consist of 0.5 ml. of one of these suspensions added to 2 ml. of a series of NaCl solutions with tonicities varying from $T = 1.0$ to $T = 0.15$, the decrease in tonicity being by steps of 0.1 in the range in which there is no hemolysis and by steps of 0.02 in the greater part of the range in which hemolysis occurs.[2]

Measurement of Volume.—The volume V occupied by the intact cells of each system is measured by a hematocrit method which uses Hamburger hematocrit tubes (Guest and Wing, 1939, 1942; Guest, 1948; Ponder, 1948 *a*) and a specially designed centrifuge.[3] This consists of a compact aluminum head, carrying six metal containers for the hematocrit tubes, and mounted directly on the vertical shaft of a series-parallel wound 115 volt, 5 amp. Dumore motor. The speed of the motor is controlled by a variac, and can be adjusted at any value up to 12,000 R.P.M. At this speed, the cells in the middle of the column of packed cells are subject to a centrifugal force of $2 \times 10^4 G$. The hematocrit tubes are constructed so that the volume of the cup at the upper end (1.5 ml.) is either about 5 times, or about 30 times, the volume of the 8 cm. capillary in which the cup terminates; the former ratio is used when the systems are dense with respect to cells, and the latter when they are dilute.[4]

The volume V_0 of the cells in the system of $T = 1.0$ is put equal to unity, and the volumes of the intact cells in systems of lower tonicities are plotted, using an ordinate on the left of the graph (Fig. 1), against values of the function $f(T, a)$, which is a function of the tonicity T and of the ratio a of the volume of the external medium to the volume of the cell water (Ponder, 1949 *a*, Expression 3). When the cell behaves as an osmometer, the relation between the volume V and $f(T, a)$ is a straight line passing through the origin with a slope RW. As larger values of $f(T, a)$ are approached (these corresponding to smaller values of T), the tonicity-volume relation may depart from its initial linearity.

Measurement of Percentage Hemolysis.—When the spinning in the centrifuge is ended, the Hb concentrations in the fluids in the cups of the hematocrit tubes are determined colorimetrically as a fraction p of the concentration corresponding to complete hemolysis ($p = 1.0$). If hemolysis is all-or-none, the fraction of cells remaining intact is $(1 - p)$, and the fractional increase in volume of the average intact cell is $V/(1 - p)$.

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described in this paper were carried out at pH 7.0, but the proportions of the two phosphates can be varied to give a pH in the final mixture of from 6.0 to 8.0. These variations in pH produce the familiar effects on cell volume, but the tonicity-volume relation, in which the volume in a tonicity of $T = 1.0$ is put equal to unity, is substantially unaffected.

[2] A suitable series for dense systems is 2 ml. each of 1.0, 0.7, 0.6, 0.5, 0.4, 0.38, 0.36, 0.34, 0.32, 0.30, 0.25, and 0.20 per cent NaCl. To each, 0.5 ml. of the cell suspension in NaCl-buffer is added. The calculated tonicity of the mixture (the volume occupied by the cells being known from the volume concentration) is denoted by T. For dilute systems, a suitable series is 2 ml. each of 1.0, 0.7, 0.6, 0.45, 0.36, 0.34, 0.32, 0.30, 0.28, 0.25, 0.20, and 0.15 per cent NaCl.

[3] Constructed by Mr. Paul Cutajar of New York University Machine Shop.

[4] These hematocrit tubes are made by E. Machlett and Son, New York City.

The values of p are plotted against values of $f(T, a)$, using an ordinate marked off at the right of the graph (Fig. 1). Since the curve relating p to $f(T, a)$ is to be differentiated graphically, some attention has to be paid to the scales; that used in Fig. 1, in which 1 unit represents 0.1 in V, 0.2 in p, and 0.1 in $f(T, a)$, is satisfactory. The relation between p and $f(T, a)$ is nearly always sigmoid, sometimes with secondary changes in curvature which correspond to polymodalities. A smooth curve is drawn through the experimental points, and this curve is differentiated graphically, on the same piece of paper, by the method already described (Ponder, 1948 *b*) to give the frequency distribution of red cell resistances on a base of units of $f(T, a)$. The dis-

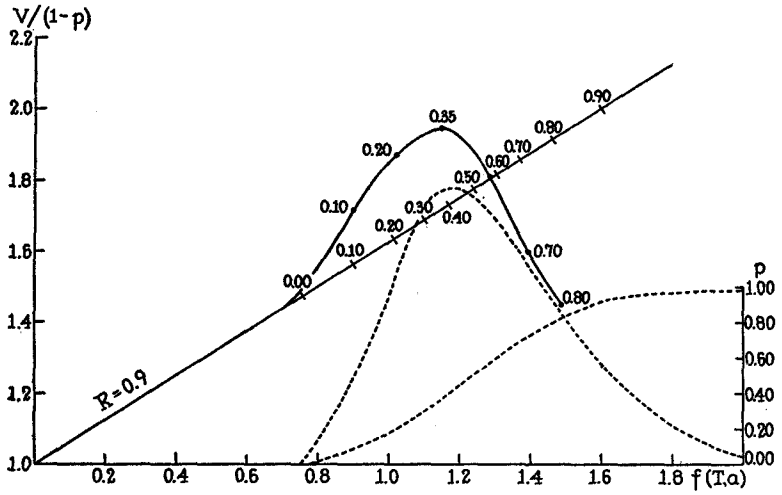

FIG. 1. A linear tonicity-volume relation and the relation observed experimentally in a dilute system (solid line). The values of p are marked off on both relations. The percentage hemolysis curve is the dotted sigmoid curve referred to the ordinate at the right of the figure; the other dotted curve is its differential. For further explanation, see text.

tribution of p as a function of $f(T, a)$ differs from the familiar distribution of p as a function of T in that it is more negatively skew.

I. Tonicity-Volume Relations in Dilute Systems

Fig. 1 shows a percentage hemolysis curve obtained by plotting p (right hand ordinate) against $f(T, a)$, together with its differential, the frequency distribution on a base in units of $f(T, a)$.[5] The straight line with a slope of RW represents the relation which would be expected between $f(T, a)$ and the volume of

[5] The data used for constructing Fig. 1 have been selected for illustrative purposes because the percentage hemolysis values are substantially the same as those given (Ponder, 1948 *b*) as average values for human red cells.

the intact cells of the system, $V/(1 - p)$, if the cells were to behave as osmometers of a degree of perfection measured by R; in this particular case, $R = 0.9$, the cell water occupying 0.7 unit of volume. Up to the point marked 0.00 on the line and corresponding to the tonicity 0.77, all the cells of the system are intact; if the tonicity is decreased below this, increasing hemolysis occurs and the values of p corresponding to the various tonicities, read off from the percentage hemolysis curve, are marked along the straight line.

In systems containing 0.5 ml. of a red cell suspension of volume concentration 0.067, and until the tonicity approaches that for commencing hemolysis, there is a good linear relation between the volume V of the cells, in terms of an initial volume V_0 put equal to unity, and the tonicity expressed as $f(T, a)$. The slope RW of the line is usually less than 0.7, and since the fraction of cell water W is usually about 0.7,[6] R is usually less than 1.0. Most of the values of R lie between 0.75 and 0.9.

When the tonicity is reduced to about $f(T, a) = 0.8$, at which point hemolysis usually begins, the tonicity-volume relation departs from linearity. Examined in detail, the departure is variable from experiment to experiment,[7] but its general course is quite apparent; in tonicities which give values of p from about 0.00 to about 0.35, the values of $V/(1 - p)$ are greater than those which correspond to the linear relation, whereas in tonicities which give values of p of 0.50 and more, the values of $V(1 - p)$ tend to be smaller than those which correspond to the linear relation. The volume $V/1 - p)$ of the intact cells of the system accordingly goes through a maximum in a tonicity for which p is in the neighborhood of 0.35. It should be pointed out that the position of the maximum and the course of the part of the curve beyond it are usually determined by 4 to 6 experimental points; the points corresponding to the lowest tonicities, in which p is 0.8 or more, may be somewhat unreliable, but the others are not.

The tendency for values of $V/(1 - p)$ to be unexpectedly small in low tonicities has been observed by Guest and Wing (1939, 1942), and is clearly shown in their figures. They have proposed that the maximum value of $V/(1 - p)$ is determined by the volume to which the cell can swell with its surface remaining unstretched, as has been suggested by Castle and Daland (1937) and by Ponder (1937). Probably because their method of plotting their results (V against T instead of V against $f(T, a)$, which

[6] W has been taken as 0.7 throughout this investigation.

[7] The relations obtained between $f(T, a)$ and V show considerable variation both as regards the values of R, the tonicity in which V is at a maximum, and the value of V at the maximum. The underlying reason is the minor variability in the relation of p to $f(T, a)$ in different experiments with different cell suspensions; this results in one never being sure that the experimental points will be desirably placed on the final curve, and in a good many experiments having to be discarded.

in their systems is very nearly the same as $1/T$) does not make it conspicuous, they do not comment on the preliminary upward departure of the experimental values from the linear relation, although it can be seen in their Fig. 2 (Guest and Wing, 1942; the experimental curve lies over the dotted theoretical curve before turning downwards). The complicated course of the experimental curves makes it difficult to decide what point corresponds to Guest and Wing's "maximum," but if one takes the point at which the experimental curve cuts the straight line, after having passed through the maximum and before descending below the linear values, a relatively constant value of $V = 1.70 \pm 8$ with a corresponding value of p of 0.50 ± 8 is found (10 consecutive experiments). Since the human red cell has an initial area of 163 μ^2 and an initial volume of 87 μ^3, and since the volume of a sphere of area 163 μ^2 is 196 μ^3, the volume to which a cell could swell without its surface being stretched is 193/87 or 2.22; this is much larger than 1.70, and so it is not at all clear that the stretching of the surface determines the critical volume. Guest and Wing's value, 1.75, is also smaller than 2.22, and so it seems likely that the average cell hemolyzes when its volume is substantially less than that of a sphere with a surface equal to the initial red cell area.

Occasionally, the tonicity-volume relation may seem to be linear up to values of p of 0.5. Two apparent cases are shown in Ponder (1949 *a*, Fig. 1), although the present investigation had its origin in the repeated failure to obtain strictly linear relations. If the tonicities are too widely spaced in the range in which p varies from 0.0 to 0.5, it is possible that the only points obtained will be those which lie on the line before the curve has risen towards its maximum and those which lie near the line and which belong to the curve after it has passed through its maximum and is descending to cut the line. In this way the maximum may be missed altogether.

It will be clear that the value of R will be overestimated if one or more of the points on the portion of the curve which departs from linearity in an upward direction are included when an average value of R is obtained. This may partly explain some of the high values of R which have been reported. It is difficult, indeed, to be sure when the departure from linearity begins, and one often gets the impression that the course of the $f(T, a)$, volume relation is initially a very flat curve concave to the abscissa instead of a straight line (see section 4). The accuracy with which the experimental points can be determined is not sufficiently great to decide this question.

II. Tonicity-Volume Relations in Dense Systems

In systems containing 0.5 ml. of a red cell suspension of volume concentration 0.4, there is a good linear relation between $f(T, a)$ and V until the tonicity is a little greater than that required to produce just commencing hemolysis. The slope RW of the line is usually a little greater than that for dilute systems, most values of R lying between 0.8 and 0.9; occasionally even higher R values (0.95 to 1.0) are found.

When the tonicity is low enough to produce commencing hemolysis, the tonicity-volume relation departs from linearity by turning upwards (Fig. 2); *i.e.*, the volumes observed are consistently larger than those to be expected on the basis of the cell's behavior as a simple osmometer. It is usually impos-

sible to define a maximum such as that found regularly in dilute systems; this failure, however, is probably due to the course of the extreme right-hand portion of the curve being determined by one, or at most two, not very reliable experimental points.

III. Possible Explanations for the Departures from Linearity

Two aspects of the situation require consideration. The first is the upward departure from linearity which is observed both in dilute and in dense systems,

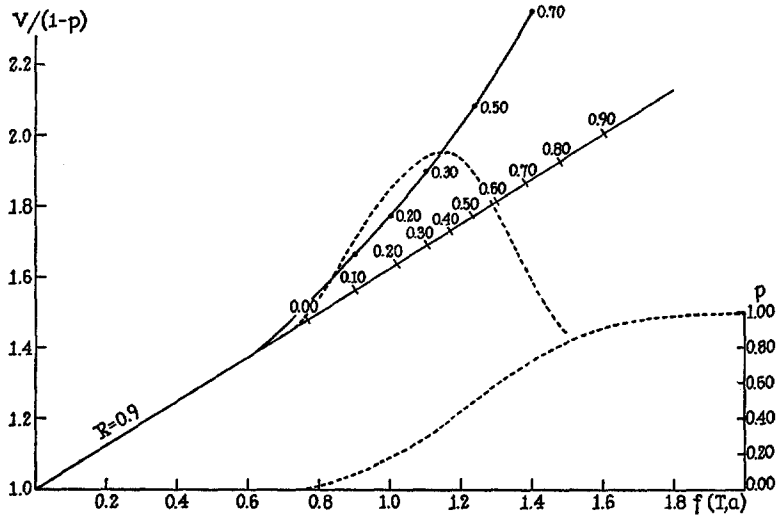

FIG. 2. Similar to Fig. 1, but for a dense system. Solid lines, the linear relation expected and the experimental relation deviating upwards from it; dotted sigmoid curve, the percentage hemolysis curve referred to the ordinate at the right. The other dotted curve is a tonicity-volume relation for a dilute system, shown for purposes of comparison.

and the second is the decline from a maximum to values much smaller than the linear values; it is usually only in dilute systems that the observations corresponding to this terminal portion of the curve are reliable.

The Upward Departure from Linearity.—(1) The first possibility which comes to mind as an explanation for the unexpectedly high values of $V/(1 - p)$ is that the cells are insufficiently packed. The course of the curves, however, is substantially the same when the measurements are made at a centrifugal force of $2 \times 10^3 G$ as it is when the force is $2 \times 10^4 G$; so far as the shape of the curve is concerned, there is no indication of a systematic effect of the rate of spinning, although the absolute volumes and the value of R become smaller as the rate is increased. Further, a centrifugal force of $2 \times 10^4 G$ is more than sufficient

to give Koeppe's criterion (a translucence of the column of packed cells, generally taken as an indication of adequate packing). Lastly, any explanation based on a supposed incompleteness of packing breaks down when one considers the course of the curve either before or after the upward departure from linearity.

(2) A possibility to be considered more seriously is that the transformation of the red cell into the ghost is a more complex process than simple osmotic theory represents it to be, and particularly that it is not all-or-none. There are two distinct situations to be considered. The first is that the intact cells of the system lose their Hb in a stepwise, as opposed to an all-or-none, manner; this would lead to p being overestimated and to V being overestimated also. Parpart's measurements (1931), however, show that the red cells of the ox and of man in large volumes of hypotonic saline do not lose Hb until they hemolyze to become ghosts which are so unsubstantial that they are not included in a red cell count. The evidence is less conclusive when the suspension medium is smaller in volume, the contradictory results of Baron (1928) and of Saslow (1929) being incapable, for technical reasons, of excluding the small partial loss of Hb which would be required to make the difference between a value $V = 1.75$ expected on the basis of a linear $f(T, a)$, V relation and the maximum value $V = 1.95$ found; a loss of Hb amounting to $p = 0.08$ from the 72 per cent of intact cells remaining would account for the difference provided the cells losing the pigment were able to maintain their volume unchanged.[8] This would be possible if the partially hemolyzed cells had a certain degree of rigidity (see below). The best evidence against such a partial hemolysis is that the mean corpuscular Hb of cells which have been exposed to tonicities as low as 0.4 to 0.32 is the same as that of cells suspended in isotonic media;

[8] The amount of Hb, p', which would have to be lost from the $(1 - p)$ intact cells of a system in order to account for a volume V being found in place of the volume V_1 expected on the basis of a linear relation between $f(T, a)$ and volume is

$$p' = 1 - p - \frac{V}{V_1}(1 - p) \qquad (2)$$

It is worthwhile considering a point which arises in connection with the volume and Hb determinations, and which has been mentioned both by Parpart and by Saslow in criticism of Baron's results. At the end of the spinning, a few swollen but intact cells may be left in the supernatant fluid along with the many ghosts which are not thrown down. These swollen cells contain Hb in excess of that in the surrounding medium, and so the value of p obtained by measurement of the Hb in the medium will be greater when such cells are present than when they are absent. This will lead to a systematic overestimation of p. But because the swollen cells are present in the supernatant fluid, they are absent from the column of packed cells, and V is underestimated in consequence. It is easy to show that the two errors cancel out almost completely.

this result agrees with those of Guest and Wing (1942) and of Hendry (1947), both of whom have made similar determinations.

The second possibility is that the red cell ghost retains some of its Hb, that most, if not all, of the ghosts are sufficiently dense to be carried down into the column of "intact cells," and that these ghosts have a rigidity sufficiently great to allow the loss of considerable amounts of Hb without a proportional decrease in volume (the lost Hb being replaced by water and salts). There is

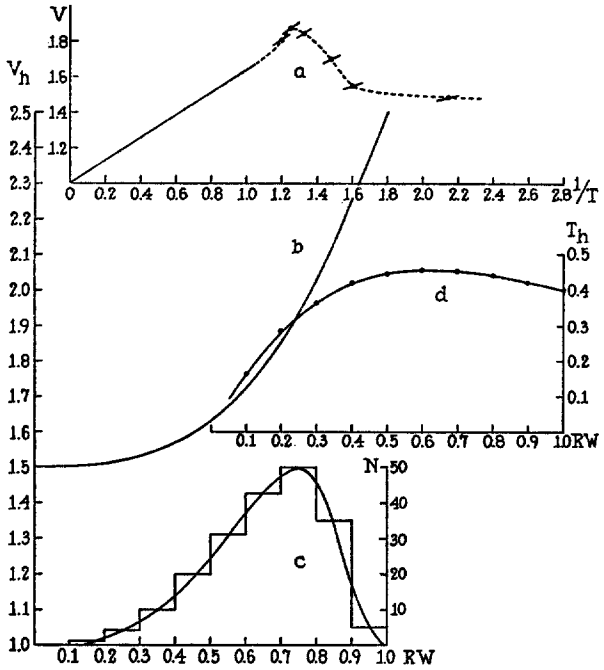

FIG. 3. Steps in the analysis of the effect of heterogeneity with respect to *RW* and an assumed relation between V_h and *RW*. For explanation, see text.

ample evidence that red cells can hemolyze and still contain a much higher concentration of Hb than is present in the medium surrounding them (Williams, Erickson, and Macy, 1941; Ponder, 1942). This is particularly true of red cells in hypotonic media of limited volume; there are even indications that the Hb is bound to other components of the ultrastructure of the ghost (Hunter, Stringer, and Weiss, 1940; chicken cells) and that the pH of the system plays a part in determining the quantity bound. What concerns us at the moment is that the retention of any considerable amount of Hb would result in the ghost being comparatively voluminous and dense, and in a difficulty in distinguishing experimentally, and even as a matter of definition, between an "intact cell" which has lost some of its Hb and a ghost which retains an appre-

ciable fraction of the cell's initial Hb. The consequences of this second possible situation will be discussed further when the downward departures from linearity are considered (see section 6, below).

(3) Remembering that the population of red cells under consideration is heterogeneous with respect to a number of properties, we can try to explain the upward, and also the downward, departures from linearity in an altogether different way. Let us suppose that the relation between V and $f(T, a)$ is always linear, but that the slope of the line; *i.e.*, the value of RW, varies; the cells of the system will then be distributed in some way with respect to RW. The highest possible value of RW is 1.0; the lowest experimental value is in the neighborhood of 0.5, and so the distribution may be supposed to have an appearance such as that shown in Fig. 3 *c*, in which the mean value of RW is 0.65. As long as all the cells of the system are intact, the relation between V and $f(T, a)$ will be a straight line with a slope of $RW = 0.65$.

Next introduce the condition that the critical volume V_h at which a red cell hemolyzes is a function of RW, V_h being, in general, small when RW is small.[9] The relation may take a variety of forms, but one of these is such that the group of cells which hemolyze at the highest value of T has a value of RW which is smaller than the mean value; the lysis of these cells, the swelling of which contributes relatively little to the average swelling of the population, would result in the average swelling of the remaining groups of cells proceeding along a line with a slope somewhat greater than before lysis began, and there would be an *upward* departure from the linear relation which describes the swelling of the population when intact. Again, the cells with the lowest value of RW may have a value of RW so small that they hemolyze in the lowest tonicity although their critical volume V_h is small also, under which circumstance the last group of cells remaining will be those which swell along a line of a much smaller slope than that which describes the swelling of the intact population; this will lead to a final *downward* departure from the initial linear relation.

The tonicity T_h in which the critical volume V_h is reached is

$$T_h = \frac{RW}{V_h - 1 + RW} \tag{3}$$

[9] Such a relation between V_h and RW could have its origin in properties of the red cell ultrastructure. The paracrystalline forms of the red cell tend to have small values of RW, and it is not unlikely that their compactly arranged molecules are capable of undergoing comparatively little separation before the continuity of the structure breaks down. Looked at in this way, a relation between V_h and RW would be one aspect of the enquiry: To what extent can the molecules of the cell interior, or surface plus interior, be separated from each other before their cohesion becomes insufficient to maintain an orderly structure? (see Ponder, 1948 *c*, p. 245).

and the required condition is that this function shall pass through a maximum at a value corresponding to a group of cells in the frequency distribution with respect to *RW*. Fig. 3, in which the distribution is shown in the curve and polygon marked *c*, a relation between V_h and *RW* in the curve marked *b*,[10] and the values of T_h corresponding to different values of *RW* in the curve *d* derived from curve *b* and expression (2), illustrates one of the possibilities.

Working from the polygon and curve *b*, a table is constructed showing *N*, the number of cells in successive groups, each with a mean value of *RW* and each limited by two values of T_h calculated from expression (2); the average of these is taken as the value of T_h at which the group, characterized by *RW* with respect to its swelling, hemolyzes. At the lowest tonicity, only the most resistant cells are left intact; their number is N_1, their volume change takes place along the line RW_1, and they hemolyze at T_{h_1}. The next most resistant group has number N_2 and its volume change takes place along the line RW_2; so for this together with the most resistant group, the mean volume change takes place along the line $(N_2RW_2 + N_1RW_1)/(N_2 + N_1)$. And so on, adding more and more resistant groups until we finally arrive at a value of T_h at which all the groups, with their various values of *N* and of *RW*, are intact; for higher values of *T*, the population as a whole swells along a line with a slope *RW* which is the average for the whole population. Fig. 3 *a* shows the resulting relation between V_h and $1/T$; this relation passes through a maximum because the first groups to hemolyze (*cf.* polygon *c* and curve *d*) are relatively large groups for which *RW* is smaller than the average for the population as a whole.

A somewhat analogous situation would be one in which V_h is a function of the initial volume of the cell, and in which the heterogeneous population contains a number of small cells with a smaller eccentricity than the average. Such cells might be expected to hemolyze at relatively high tonicities, and since they are less than average volume, their removal would result in relatively large cells remaining intact. In order to be able to account for the upward departure of the $f(T, a)$, *V* relation in this way, about 35 per cent of the population would have to consist of these small cells of low eccentricity, and their contribution to the initial volume would have to be such that their removal would increase V_0 by about 10 per cent. Using a frequency distribution of diameters with a mean of 8.5 μ and a standard deviation of ± 0.5 μ (see Ponder, 1948 *c*) and computing the volume distribution, it can be shown that the early lysis of a group of small cells numbering 32 per cent of the whole would result in the value for the mean volume increasing by about 10 per cent (98 μ^3 to 108 μ^3). Such a preferential lysis of small cells could therefore account for the upward departure of the $f(T, a)$, *V* relation towards its maximum. The passage through the maximum and the descent towards and below the linear relation would, however, remain unaccounted for, and such measurements as have been made of the diameter and thickness of red cells have not suggested a negative correlation between volume and eccentricity.

There is, of course, no independent evidence that the critical volume and *RW* are related in this particular way, and no relation which I have been able

[10] The particular function represented here is $V_h = RW^3 + \text{constant}$.

to find accounts for such large upward departures from linearity as are observed experimentally. In particular, points on the experimental curves which correspond to values of *RW* greater than 1.0 cannot possibly be accounted for on this basis, and such points are quite often found, particularly in dense systems. The purpose of the foregoing discussion is accordingly to show that upward and downward departures from linearity are possible under conditions of heterogeneity of the population, but it cannot be concluded that this accounts for the observations.

The Downward Departures from Linearity.—(4) These can be readily enough accounted for in terms of there being a relation between the critical volume and *RW* in a heterogeneous population (see above); this explanation is one of those suggested by Guest and Wing. The objection to it is that if the population contains a group of any size characterized by a very low value of *RW*, it is necessary to suppose, in order to account for the mean value of *RW* for the population while intact, that it also contains another group of similar size with a very high value of *RW*. The mean value of *RW* for the population, when all its cells are intact, is often nearly 0.7 (this is Guest's (1948) most usual value), and so, in order to balance the effect of a group numbering 15 to 20 per cent of the total and having a value of *RW* of 0.35 or less, we require to have an equal number of cells with a value of *RW* of 1.05 or more. In normally hemoglobinized cells, however, *RW* cannot increase much above 0.7, and values even approaching 1.0 are, from the standpoint of osmotic theory, impossible.

(5) There is no experimental evidence to support the suggestion (Ponder and Robinson, 1934, Guest and Wing, 1942) that exchanges of salt as well as of water account for the downward departures from linearity. Slow K losses and Na gains occur, but rapid and relatively large exchanges would be required to account for the volume changes, and these are not observed when either the K lost into the suspension medium or the K content of the intact cells is measured. These measurements have been carried out at various temperatures in systems, dense with respect to cells, in which the tonicity of the suspension medium is reduced to 0.6 (Ponder, 1949 *b*), as well as at 25°C. in systems in which the tonicity in the suspension medium is between 0.3 and 0.4.

(6) An important point about the relation between $f(T, a)$ and V is that its form is not the same in dense as in dilute systems. In the latter, the ascent to a maximum and the later decline are found regularly, whereas in the former the departures from linearity are predominantly upwards; a true maximum is observed only rarely, and even then only at the lowest tonicities. This suggests that the properties of the cell which determine V are affected in one way in dense systems but in a different way in dilute systems.

Returning to the idea of a retention of Hb by the ghosts of the system, the loss of a small amount of pigment either may or may not be accompanied by

a proportional decrease in red cell volume; two extreme situations are possible. In the first, the ghost can be imagined as a rigid body, the shape of which is determined by surface and internal ultrastructures; Hb might be lost from such a structure, being replaced by salts and water, without a proportional volume decrease occurring. In the second, in which the ghost would be imagined as non-rigid, the loss of Hb would necessarily be accompanied by a proportional volume decrease.

Let a fraction f of the volume $V_{obs.}$ of the mass of cells plus ghosts be occupied by ghosts, and let a fraction p of the cells of the system be hemolyzed. If $f = p$, the ghosts are entirely rigid, if $f = 0$, the ghosts are entirely non-rigid, and if $f > 0 < p$, the ghosts are partially but not completely rigid. If we ignore the fact that a fraction f is occupied by ghosts, and treat the whole volume as being composed of cells, the apparent volume per unit cell will be

$$\frac{V_{obs.}}{1 - p}$$

but if we allow for the volume of the ghosts, the volume per unit cell will be

$$\frac{V_{obs.} - f \cdot V_{obs.}}{1 - p}$$

The unrecognized presence of the fraction f of rigid ghosts accordingly increases the value for the true volume per unit cell from V_1, the volume corresponding to the linear relation between $f(T, a)$ and volume, to

$$V_1/(1 - Kf) \tag{4}$$

where K is a constant which can vary from zero for non-rigid ghosts to unity for completely rigid ones.

Since f is a function of p, and since $f = 0$ when $p = 0$, $V_1/(1 - Kf)$ will be equal to V_1 at least until some of the cells in the system hemolyze. Thereafter, $V_1/(1 - Kf)$ may exceed V_1, but as large values of p are reached, the rigidity of the ghosts may undergo a decrease and $V_1/(1 - Kf)$ may tend to become equal to V_1 once more. The maximum in the relation between $f(T, a)$ and V as well as the return to the value of V expected on the basis of the linear relation can thus be accounted for by supposing that the ghosts behave as relatively rigid bodies in media of a relatively high tonicity, and that in media of lower tonicity their rigidity is less, the rigidity being always greater in dense systems than in dilute ones.[11] Such a change in rigidity, if it really occurs, may

[11] One might think that the question of whether the column of packed cells is partly composed of Hb-containing ghosts could be settled by determining the mean corpuscular Hb concentration of the cells in the column after resuspending them in isotonic NaCl. Guest and Wing have made determinations of this kind, but a real difficulty arises as to the amount of isotonic fluid to be added. If this is small, the

be related to the leaching-out of the components of the ultrastructure in very hypotonic media.[12] The result obtained in expression (4), however, does not account for the relation between $f(T, a)$ and V cutting the linear relation and descending below it to values of V fully one-half of those on the straight line. There is the possibility, of course, of combining the result in expression (4) with the results of the computation of the effect of heterogeneity in the system with respect to RW (see 3, above).

These attempts at an explanation for the departures from linearity have been discussed more in order to see where they lead and what they imply than because any one of them is satisfactory. The one thing that seems certain is that the structures involved in the hemolytic process ("intact cells" and "ghosts") have different properties in different tonicity ranges and even when the same tonicity is established in dense as opposed to dilute systems. The most non-committal statement that can be made about the experimental results is that the swelling of human red cells in hypotonic media is not a good example of an osmotic process, because large correction terms of a dubious nature have to be introduced in order to reconcile the observed volumes with the volumes expected on the basis of the van't Hoff-Mariotte law.

The observation that V is not linear with $f(T, a)$ raises a point in connection with the distribution of red cell resistances to hemolysis by hypotonic media. The frequency distribution is conventionally given on an abscissa in units of T, but, on the basis of the van't Hoff-Mariotte law, either the process of swelling or the process of stretching of the surface is a simpler function of $1/T$ (or of $f(T, a)$ when the volume of the medium is limited) than of T itself. The most obvious scale for an abscissa would therefore be one in units of $1/T$ or of $f(T, a)$. If the relation between V and $1/T$ goes through a maximum, however, there is no simple unit proportional to V or to

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initial volume will be more completely restored if V is small than if V is large, and one has to calculate the shrinkage of swollen cells in a relatively hypertonic medium of finite volume. If the amount of fluid added is large, the accuracy with which the cell volumes can be determined falls off rapidly. If one washes with an isotonic fluid, one tends to lose the ghosts and to vitiate the experiment. Determinations of mean corpuscular Hb concentration in these systems are not accurate enough to decide questions regarding small Hb losses or small contributions to volume by ghosts (*cf.* Hendry's determinations, which show inconsistencies amounting to nearly 20 per cent).

[12] Large quantities of both proteins and lipids are leached out from the red cell standing in hypotonic media (Waugh and Schmitt, 1940), and it is possible that some kind of steady state is slowly reached as regards the distribution of materials in the ultrastructure of the cell or ghost and in the surrounding medium. The composition of the latter is different in dense and in dilute systems in which the same percentage hemolysis has occurred, and the former may be different also. Like most workers in the field, Hendry (1947) has noticed slow progressive increases in percentage hemolysis as cells stand in hypotonic media.

$V^{2/3}$ and therefore possessing a real physical meaning, which can be used on the abscissa of the frequency distribution; p can of course be plotted against $1/T$ or against $f(T, a)$, but this results in there sometimes being two groups of cells which hemolyze at the same volume, one in a high tonicity and the other in a low one. The distribution obtained by differentiation (as in Fig. 1) has accordingly no clear meaning in terms of what is supposed to be the mechanism of the hemolysis. The only escape from this difficulty lies in a clearer understanding as to why the $f(T, a)$, V relation is not linear in the range in which lysis occurs, and the difficulty is further increased by the $f(T, a)$, V relation being different in dense as opposed to dilute systems.

IV. Experimental Modifications of the Form of the Tonicity-Volume Relation

Many substances, *e.g.* the monovalent cations (Ponder, 1949 *a*) and non-electrolytes, produce small changes in the $f(T, a)$, V relation and associated changes in the values of p corresponding to various tonicities. When the curve is considered as a whole, however, the general form of the relation between $f(T, a)$ and V is very stable; it is not changed, except in minor respects, by allowing the cells to stand for 24 hours at 25°C. or at even higher temperatures, by adding lysins such as saponin in hypolytic concentration, by changing the pH of the systems between pH 6.0 and 8.0, or by substituting hypotonic plasma for hypotonic NaCl-buffer. The only ways in which I have been able to produce consistent and marked changes in the form of the tonicity-volume relation are treatment of the cells with resorcinol or related alcohols, with colloidal silicic acid, with iodoacetate, and with sodium oxalate.

1. Resorcinol.—When added to a dense suspension in sufficient quantity to provide a 0.064 M concentration in the suspension medium and allowed to remain in contact with the cells for 18 to 24 hours at 4°C. and then removed by washing with NaCl-buffer, resorcinol increases the extent of the upward departures from the theoretical linear relation between $f(T, a)$ and V. The volume of the cells (Fig. 4 *b*) is increased over that of untreated cells (Fig. 4 *a*) even in a tonicity of 1.0, the value of R is increased, and at low tonicities the intact cells are much more voluminous. A maximum is rarely observable, and the upward departure from linearity is often so pronounced, and begins at such high tonicities, as to give the impression that the real relation between $f(T, a)$ and V is a flat curve convex to the abscissa rather than a straight line. The effect of resorcinol is observed both in dense and in dilute systems. Small concentrations of resorcinol have smaller effects on the shape of the curve; larger concentrations produce increasing hemolysis and progressive increases in the volumes of the intact cells (*cf.* Ponder, 1948 *a*).

2. Colloidal Silicic Acid.—When added to dense suspensions in sufficient concentration to provide a 1 in 300 dilution in the suspension medium colloidal silicic acid sols (for method of preparation of the sol, see Ponder, 1932) increase the upward departures from linearity in much the same way as resorcinol does. In partially hemolyzed systems in which p lies between 0.1 and 0.5, the intact red cells are very voluminous. Smaller concentrations of colloidal silicic acid produce smaller effects (and less agglutination); larger concentrations tend to produce lysis as well as agglutination. The same general effects are observed in both dense and dilute systems.

3. Iodoacetate.—When sodium iodoacetate (pH 7.0) is added to dense suspensions in such quantities as to give from 1 per cent to 0.005 per cent concentration in the suspen-

sion medium, and when the cells are allowed to stand in contact with the iodoacetate for 18 to 24 hours at various temperatures, remarkable changes in the resistance of the cells to hypotonic hemolysis are observed. These can be demonstrated by adding 5 ml. of water to 0.2 ml. of the washed cells of the systems, the volume concentration

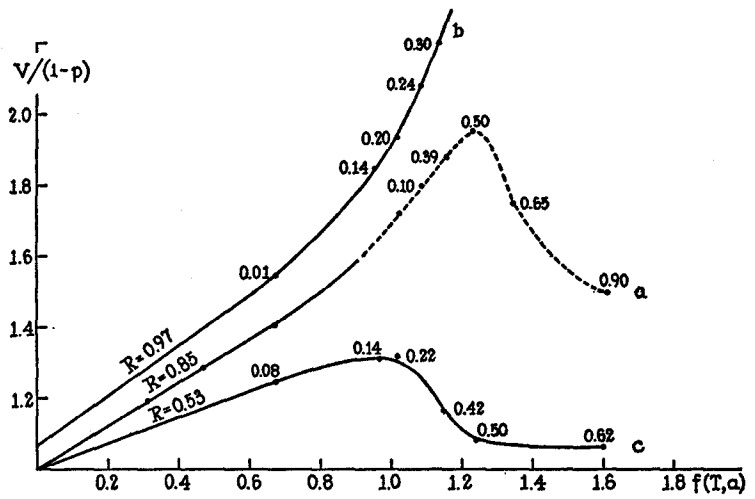

FIG. 4. The effect on the usual tonicity-volume relation (curve *a*), in dilute systems, of treating the cells with resorcinol (curve *b*), and with iodoacetate (curve *c*). Values of p are marked off along the relations.

TABLE I

Iodoacetate in system	Iodoacetate and cells kept 18 hrs. at		
	4°C.	25°C.	37°C.
per cent			
1.0	1.00	1.00	1.00
0.5	1.00	1.00	1.00
0.1	1.00	1.00	1.00
0.05	1.00	0.95	0.88
0.01	1.00	1.00	0.73
0.05	1.00	1.00	0.70

being adjusted to 0.4 after the washing by the addition of isotonic NaCl-buffer. Table I shows the values of p observed 15 minutes after the addition of the water.

No lysis occurs as a result of the cells remaining in contact with the iodoacetate, except for a small amount which takes place in the systems kept at 37°C. and containing 1.0 to 0.5 per cent iodoacetate. Addition of water brings about complete lysis, as it would in the case of untreated cells, except when the systems contain about 0.05 per cent iodoacetate at 25°C. or about 0.005 per cent iodoacetate at 37°C.;

in these systems lysis remains incomplete for hours, some of the cells apparently being very resistant to hypotonic hemolysis.

The relation between $f(T, a)$ and V for a system containing red cells allowed to stand for 18 hours at 37°C. in contact with 0.005 per cent iodoacetate at 37°C., and then washed with isotonic NaCl-buffer, is shown in Fig. 4 c. The value of R is small (0.53), lysis begins in quite a high tonicity, and the curve passes through a maximum at $f(T, a) = 0.95$. The volumes corresponding to lower tonicities are smaller and tend to become constant at about $V = 1.06$; lysis increases up to $p = 0.60$ to 0.70, and the remaining cells are unhemolyzed even in very low tonicities. The factors responsible for this increase in resistance to hemolysis remain to be investigated.

4. Sodium Oxalate.—The use of sodium oxalate (15 to 30 mg. per 5 ml. of blood) as an anticoagulant tends to decrease the value of R, particularly if the preparations are allowed to stand for some time. The height of the maximum in the $f(T, a)$, V relation is lowered in proportion, so that the general shape of the curve remains substantially unchanged. The connection between the decrease in R and the appearance of crenation has already been discussed (Ponder, 1944).

SUMMARY

The linear relation between the red cell volume V and the reciprocal of the tonicity T of a hypotonic medium is not the linear one expected on the basis of the van't Hoff-Mariotte law, particularly when a fraction p of the cells are hemolyzed and the volume $V/(1 - p)$ of the $(1 - p)$ cells which remain intact is considered. In systems of relatively high tonicity in which p is zero, the relation is linear but its slope is usually too small; at lower tonicities in which p has a value between zero and 0.35, the volumes are larger than those expected on the basis of the van't Hoff-Mariotte law, while at still smaller tonicities they are much smaller than expected. The volume measurements referred to are made with a high speed hematocrit; the results obtained in systems containing relatively high and relatively low volume concentrations of cells are contrasted with each other, and allowance is made in the calculations for the volume of the hypotonic medium surrounding the cells being limited.

Attempts are made at an explanation of the anomalous results in terms of incomplete packing, of a stepwise as opposed to an all-or-none loss of Hb from the cells, of a heterogeneity in the swelling properties of the cells of the population, of a loss of osmotically active substances from the intact cells, and of the red cell ghost having some degree of rigidity. These explanations are not satisfying, although some of them in combination may account for the phenomena observed. It seems likely that the structures involved in the hemolytic process (cells and ghosts) have different properties in different tonicity ranges and even when the same tonicity is established in systems which are dense, as opposed to dilute, with respect to cell concentration.

The form of the tonicity-volume relation can be changed substantially,

although not in the direction of greater linearity, by treating the cells with resorcinol, colloidal silicic acid, iodoacetate, or sodium oxalate. Small changes in pH, exposure to temperatures as high as 37°C., allowing the cells to stand for periods up to 24 hours, or the substitution of hypotonic plasma for hypotonic NaCl-buffer, produce, on the other hand, only minor changes in the tonicity-volume relation.

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