The Behavior, as Regards Shape and Volume, of Human Red Cell Ghosts in Fresh and in Stored Blood

By Eric Ponder and Delia Barreto

This paper is concerned with the way in which the volume and shape of human red cell ghosts, two properties related to the structure of the ghost and probably related to the structure of the red cells from which they are derived, behave as the red cells from which the ghosts are prepared are stored for increasing periods of time at 4°C. The problem will be approached first by considering (a) certain fundamental properties of ghost volume and shape as they are observed in freshly drawn heparinized blood, (b) how these properties are modified as the heparinized blood is stored at 4°C, (c) the modifications which appear in blood stored in ACD solution, and (d) the modifications which occur in blood stored in ACD plus inosine.

The simplest way of causing the volume of red cells and the volume of ghosts prepared from them to vary is to suspend the cells or ghosts in media of different tonicitics, T. Under such circumstances, the van’t Hoff-Mariotte law, which is based on the assumption that the volume changes are the result of the passage of water alone across a nonelastic cell surface, is supposed to apply. If it does, plotting the relative mean volume of the red cell or the ghost against the reciprocal of the tonicity (1/T) results in a straight line, the slope of which ought to be \( W \), the fractional water content of the cell or ghost.* So far as red cells are concerned, the slope is usually less and equal to \( RW \), where R is a constant introduced to reconcile observation with theory. The necessity for introducing it has been explained in various ways (existence of bound water, elasticity of the surface ultrastructure, etc.) and there are departures from linearity in addition, particularly in low tonicitics. So far as red cell ghosts are concerned, Teorell has found that ghosts produced by hemolysis in a medium of a tonicity of about 0.17, and subsequently added to media (NaCl, KCl, and sucrose) of tonicitics between 0.07 and 6.0 change their volume with change in tonicity according to a modified van’t Hoff-Mariotte law which contains two constants, both of which are to some extent arbitrary and the meaning of one of which is obscure. Teorell points out that all volumes of ghosts ought to be thought of as averages of a somewhat heterogeneous population, but concludes that the modified van’t Hoff-Mariotte law is a useful approximation.
Mariotte law is a real description of the physical process of shrinking and swelling in media of different tonicities.

It will now be shown that while a linear relation between the mean volume of red cells and the reciprocal of the tonicity may possibly justify the description of the volume changes of red cells by a modified van't Hoff-Mariotte law, a similar linear relation cannot be used as evidence that the volume changes of ghosts are described by a similar law. This is because a population of red cell ghosts consists of several distinct populations, each with its own shape and its own mean volume in the same tonicity. The most likely reason for this is that the volume and shape of a ghost depends partly on its structure, and not altogether on the tonicity of the surrounding medium. It has even been suggested (several private communications) that these differences in structure may be related to the age of the red cell from which the ghost is derived.

There are several ways in which the volumes and shapes of ghosts can be studied. One of the most informative is illustrated in figure 1. The procedures are as follows.

**Methods**

(a) **Measurements of Volume.** Human blood is drawn into heparin as an anticoagulant, the red cell volume concentration is adjusted to 0.4, and 0.5 ml. is added to 3.0 ml. of a series of NaCl solutions of tonicities varying from 1.0 to zero. The tonicity of the mixture of blood and NaCl solution can be calculated. The mean volume of the red cells is measured by using Hamburger hematocrit tubes and a centrifuge which gives $2 \times 10^4$ G, spinning for 90 minutes at less than 10 C. The low temperature prevents active cation transfer. The volume $V_0$ of the red cells in a tonicity $T$ of 1.0 is put equal to unity, and the relative volumes, $V/V_0$, of the swollen cells is plotted against $1/T$. A straight line, $a$ in figure 1, results until the "anomalous region" is reached, where a fraction $p$ of the red cells hemolyze; $V/V_0(1-p)$ is then plotted against $1/T$, and the small upward and large downward departures from linearity are met with. Finally, in a tonicity as low as 0.167, almost all the red cells are hemolyzed, and the population of red cells is replaced by populations of ghosts.

It is convenient to prepare a large volume of this almost completely hemolyzed system by adding 5.0 ml. of blood to 25 ml. of water. One ml. of solutions of NaCl of varying concentration is added to 5.0 ml. of this hemolyte (which has a tonicity of 0.167), so as to increase the tonicity to values such as 0.3, 0.5, 1.0, 1.7, etc. After waiting for 15 minutes, the apparent volume concentration of the ghosts, $\phi_p$, is found with Hamburger hematocrit tubes and the same high speed centrifuge spinning for 90 minutes. The "corrected" volume concentration of the ghosts, $\phi_{pc}$, is found by measuring the conductivity of the column of ghosts and of the medium in the cup of the hematocrit tube, the assumption being that the ghosts are non-conducting. Plotting $\phi_p$ against $1/T$ gives the line $b$ in figure 1; plotting $\phi_{pc}$ against $1/T$ gives the line $c$ in figure 1.

(b) **Measurement of Shape.** Inspection with phase optics of the ghosts derived from red cells by placing the latter in a tonicity of 0.167 and then adding NaCl to restore the tonicity to values between 0.3 and 1.7 shows that there are at least three kinds of ghosts present in any tonicity between 0.167 and 1.7. These are (1) spherical ghosts which appear as an extremely thin circular line, (2) biconcave ghosts, and (3) ghosts which are so crenated that their shape cannot be defined.

By illuminating the phase contrast microscope with an electronic flash which gives an exposure of 0.001 second (supplied by a Heiland Strobomiar IV), and by photographing...
Fig. 1.—Ordinates, height of hematocrit column, $h$, and the corresponding values of $V$ in $\mu^3$. Abscissa, reciprocal of the tonicity $T$. Line $a$, for red cells, corrected for the amount of hemolysis in the lower tonicities. Line $b$, for ghosts derived from red cells by hemolyzing them at a tonicity of 0.167, and then restoring the tonicity to the values on the abscissa by adding NaCl in appropriate amounts. Line $c$, similar to line $b$, but with a dubious correction for the incompleteness of packing in the systems to which line $b$ refers. The "anomalous region" is indicated schematically. Human red cells drawn into heparin; observations made on the same day as the withdrawal.

individual ghosts, the radius of the spherical ghosts* can be measured and the mean volume calculated from the radius. Similarly, pictures can be obtained of the biconcave ghosts seen on edge, and by using Pappus' Theorem sym their individual volumes and areas can be found by treating them as solids of revolution. Finally, by examining a sample of the system with phase optics, the fractional number of spherical forms, $f_s$, of biconcave discoidal forms, $f_d$, and of crenated forms, $f_c$, can be determined by observing about 200 ghosts and separating them into the three categories.

These observations were repeated, at intervals of from 4 to 7 days, on the blood in each stored container until it became evident (see below) that the structure of the ghosts was no longer being maintained.

Mean Volume of Ghosts in Different Tonicities, and the Volumes Associated with the Different Shapes (Fresh Heparinized Blood)

Figure 1 shows that the mean volume, $v_m$, of the ghosts found in the hematocrit column is smaller than that of the red cell from which it is derived, this being true in all tonicities. At $T = 1.0$, for example, the mean volume of the ghost is 50 per cent of that of the red cell if we compare the value in $\mu^3$ on line $a$ with the value on line $b$, or 40 per cent if we compare the values on line $a$ and line $c$. Figure 1 also shows that the mean volume of the ghost tends to be linear with $1/T$, irrespective of whether the values on line $b$ or on line $c$ are considered, just as line $a$ shows that the mean volume of the red cells tends to be linear with $1/T$.

* If a species of ghost never presents itself on edge, it is necessarily spherical.9
until the "anomalous region" is reached. While the van't Hoff-Mariotte law may be a description of the physical processes operating when red cells are suspended in media of varying tonicity, the linear relation between \( r_m \) and \( 1/T \) cannot have any real meaning in terms of this law because \( r_m \) is the mean volume of three different populations, each of which has its own mean volume in any selected tonicity.

Table 1 shows the mean values for the diameter, the greatest thickness, the least thickness, the surface area and the volume of the biconcave discoidal ghosts in the tonicities 0.3, 1.0 and 1.7. The means are based on the measurement of 17 individual ghosts on edge in each tonicity. The scatter, \( s \), follows each mean value. The unexpected observation is that the dimensions of the biconcave ghosts do not vary much in the three very different tonicities, and so it seems that the kind of ghost which can resume its discoidal biconcave form has nearly the same diameter, the same area, and the same volume as the red cell has in a tonicity of 1.0. The dimensions, moreover, and particularly the volume, of these discoidal ghosts do not seem to change with the duration of storage of the red cells from which the ghosts are prepared. The only marked difference is found in the value for the least thickness, which is appreciably less than that of the human red cell in an isotonic medium (1.0 \( \mu \)). The similarity between the mean volume of the biconcave ghost and that of the biconcave red cell in an isotonic medium suggests that the loss of Hb which accompanies hemolysis is compensated for by an intake of fluid from the surrounding medium, but that this exchange results in a pinching of the ghost in the region of its least thickness and to a lesser extent in the region of its greatest thickness (mean value for the cell, 2.4\( \mu \), mean value for the ghost, 2.1 \( \mu \)). The general similarity of shape of these biconcave ghosts and of biconcave red cells leads one to attribute to the former something of the structure possessed by the latter.

Turning next to the spherical ghosts, these are again found to have substantially the same mean volume, 150 \( \mu^3 \), in all tonicities between 0.3 and 1.7. Again, this mean volume does not seem to change with the duration of storage of the red cells from which the ghosts are formed. Finally, the mean volume of the crenated ghosts cannot be measured directly because their shape is too irregular, but their volume \( v_c \) can be computed from the fractional number of discoidal forms \( f_d \) and their mean volume \( v_d \), the fractional number of spherical forms, \( f_s \) and their mean volume \( v_s \), together with the fractional number of crenated ghosts, \( f_c \), and the mean volume \( v_m \) of all the kinds of ghosts taken together. In all tonicities,

\[
v_c = v_m - \frac{f_d \cdot v_d + f_s \cdot v_s}{f_c}\]

<table>
<thead>
<tr>
<th>( \mu )</th>
<th>Diameter ( \mu )</th>
<th>Greatest Thickness ( \mu )</th>
<th>Least Thickness ( \mu )</th>
<th>Surface 2 ( \mu )</th>
<th>Volume 3 ( \mu^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>9.26 ± 0.5</td>
<td>2.10 ± 0.22</td>
<td>0.36 ± 0.08</td>
<td>174 ± 17</td>
<td>99.8 ± 16</td>
</tr>
<tr>
<td>1.0</td>
<td>8.9 ± 0.5</td>
<td>2.08 ± 0.26</td>
<td>0.58 ± 0.18</td>
<td>156 ± 26</td>
<td>88.0 ± 19</td>
</tr>
<tr>
<td>1.7</td>
<td>8.9 ± 0.4</td>
<td>2.14 ± 0.19</td>
<td>0.39 ± 0.02</td>
<td>161 ± 13</td>
<td>93.0 ± 11</td>
</tr>
</tbody>
</table>
which can be re-arranged as
\[ v_m = (f_d \cdot v_d + f_s \cdot v_s + f_c \cdot v_c) \quad (3) \]

Table 2 shows \( f_d, f_s \) and \( f_c \) found in systems of tonicity 0.3, 1.0, and 1.7 (fresh heparinized blood) while table 3 shows \( v_m \) and the calculated values of \( v_c \) in \( \mu^3 \) in the same three tonicities, first using the values of \( v_m \) given by line \( b \) of figure 1, and then the values of \( v_m \) given by the line \( c \) of figure 1. These tables are inserted as illustrations as to how the work proceeds, and not because the individual values have any particular significance.

A Quantitative Expression of the Failure of the Structure of the Ghost to Be Maintained

A failure in the maintenance of the structure of the ghost, as it develops when the ghosts are prepared from red cells stored at 4 C for increasing periods of time, can now be expressed simply. Discoidal and crenated ghosts certainly retain some of the elements of red cell structure, and even the large almost Hb-free ghost still has a structure of its own. When the spherical ghost breaks into fragments and myelin forms, anything resembling the original structure must be lost.

Returning to expressions (2) and (3), it will be clear that \( v_c \), the volume of the crenated ghost, cannot conceivably be negative, but must have a positive value, however small. The value for the “fixed framework” of the red cell\(^8\) is about 2.5 per cent, dry and excluding the contribution of residual Hb. When the contribution of water\(^7\) and the amount of residual Hb are allowed for, the volume of the smallest crenated ghost can scarcely be less than about 15 \( \mu^3 \), a figure which agrees with an estimate based on the spontaneous contraction of ghosts.\(^8\) A limit can now be placed on the value of \( v_c \) which is compatible with physical reality, and, using 15 \( \mu^3 \) as the smallest permissible value for the volume of a crenated ghost, we have an expression (4), analogous to expression (3), in which

**Table 2.—Fractional Number of Different Kinds of Ghost in Three Different Tonicities**

<table>
<thead>
<tr>
<th>( T )</th>
<th>( f_d )</th>
<th>( f_s )</th>
<th>( f_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.44</td>
<td>0.22</td>
<td>0.34</td>
</tr>
<tr>
<td>1.0</td>
<td>0.29</td>
<td>0.03</td>
<td>0.68</td>
</tr>
<tr>
<td>1.7</td>
<td>0.25</td>
<td>---</td>
<td>0.75</td>
</tr>
</tbody>
</table>

In determining \( v_c \) from expression (2) the assumption is made that \( v_d = 85 \ \mu^3 \) and \( v_s = 150 \ \mu^3 \).

**Table 3.—Mean Volume of Ghosts and Volume of Crenated Ghosts in Different Tonicities; taken from lines \( b \) and \( c \)**

<table>
<thead>
<tr>
<th>Line ( b ), ( T )</th>
<th>( v_m )</th>
<th>( v_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>92 ( \mu^3 )</td>
<td>63 ( \mu^3 )</td>
</tr>
<tr>
<td>1.0</td>
<td>46 ( \mu^3 )</td>
<td>18 ( \mu^3 )</td>
</tr>
<tr>
<td>1.7</td>
<td>34 ( \mu^3 )</td>
<td>17 ( \mu^3 )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Line ( c ), ( T )</th>
<th>( v_m )</th>
<th>( v_c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>73 ( \mu^3 )</td>
<td>8.8 ( \mu^3 )</td>
</tr>
<tr>
<td>1.0</td>
<td>34 ( \mu^3 )</td>
<td>13 ( \mu^3 )</td>
</tr>
<tr>
<td>1.7</td>
<td>27 ( \mu^3 )</td>
<td>8.0 ( \mu^3 )</td>
</tr>
</tbody>
</table>
V, the smallest permissible mean ghost volume as determined from the distribution of shapes and volumes of the ghosts, replaces \( v_m \), the mean ghost volume found by the hematocrit:

\[
V = (f_d \cdot v_d + f_s \cdot v_s + f_{cr} \cdot v_{cr})
\]

in which there is the restriction that \( v_{cr} \) cannot be less than 15 \( \mu^3 \). Inserting the values \( v_d = 85 \, \mu^3 \), \( v_s = 150 \, \mu^3 \), and \( v_{cr} = 15 \, \mu^3 \), shows that this expression is a simple piece of arithmetic,

\[
V = 85 \left( f_d + 1.76 \cdot f_s + 0.176 \cdot f_{cr} \right)
\]

\( f_d, f_s, \) and \( f_{cr} \) being experimental observations of the fractional number of discoidal ghosts, spherical ghosts, and crenated ghosts present in the system under consideration.

Even allowing for the standard errors which effect the distribution of ghosts into the three categories, values such as 8.8 \( \mu^3 \), 13 \( \mu^3 \), and 8.0 \( \mu^3 \) (table 3, data from line c) are improbably small. Further, experience with the ghosts of red cells stored for longer and longer periods of time has shown that the apparent value of \( v_m \) tends to become smaller and smaller, and ultimately negative. These very small and negative values occur particularly frequently when the values of \( v_m \) taken from line c are used in expression (2). It can be concluded from this that, as the duration of storage increases, some of the ghosts prepared from the cells become conductive. This is a good reason for eliminating line c from long time experiments. At the same time, the development of some degree of conductivity in initially non-conducting bodies implies a modification of their structure.

If \( v_m \), the mean ghost volume as measured by the hematocrit, is less than \( V \), some of the ghosts (or their breakdown products) have not been thrown down into the ghost column, the simplest reason for this being that some of the ghosts have been replaced by small, light fragments and myelin forms, i.e., that the structure of some of the ghosts has been irreversibly lost. The general validity of this conclusion can be demonstrated by observing the contents of the cup of the Hamburger hematocrit tube with phase optics.

Finally, the difference between \( V \) and \( v_m \) measures the loss of ghost structure neatly if expressed as

\[
\frac{V - v_m}{V} = \lambda
\]

where \( \lambda \) is the fractional number of ghosts which have lost their structure to the extent that they (or rather their breakdown products) are not thrown down into the ghost column in the hematocrit tube under the centrifugal force used. Note that \( \lambda \) becomes increasingly large (and positive) as the ghost structure is increasingly lost, and that it is a minimum value for the loss. Large negative values of \( \lambda \) probably mean that the mean volume of the crenated ghosts is much greater than 15 \( \mu^3 \), but this is a subsidiary point.

Table 4 shows the values of \( \lambda \) computed for ghosts prepared from heparinized human blood stored at 4 \( ^\circ \)C for various periods of time. On the 26th day of the values of \( \lambda \) (previously negative) are all positive, a typical value being 0.12. This means that by the 26th day at least 12 per cent of the ghosts have lost their structure in the sense defined above, and that some time a little shorter
than 26 days is the longest time during which ghost structure can be considered as being maintained under the conditions of this experiment. *

Table 5 summarizes the experimental results. In human blood rendered incoagulable with heparin, structural breakdown in the preceding sense occurs after about 26 days. In human blood preserved in ACD, structural breakdown measured in an identical manner occurs after about 55 to 60 days. In human blood preserved in ACD-inosine, structural breakdown does not occur until about 75 to 80 days. These observations tend to support the results obtained by Finch and his collaborators which show a longer in vivo survival of red cells after storage in ACD-inosine, but it is impossible to reduce the results of in vitro survival and the results obtained in this paper to common terms because the criteria for the maintenance of structure are entirely different.

**SUMMARY**

(1) When human red cells are hemolyzed in very hypotonic media (NaCl of a tonicity of 0.167) and when the tonicity is restored, by adding appropriate

* Like tables 2 and 3, table 4 is intended to be nothing more than illustrative of the results obtained by the procedures described in the text, and in this case, of the way in which λ changes sign, relatively suddenly, when the period during which ghost structure is maintained is exceeded. While of no particular significance as mere numbers, the values, of course, are values actually obtained experimentally, as are those in tables 1, 2, and 3.
amounts of NaCl, to tonicities such as 0.3, 0.5, 1.0, and 1.7, the mean volume of the ghosts appears to be linear with the reciprocal of the tonicity. This might lead one to conclude that the ghosts are osmometers and that their volume is governed by simple osmotic considerations such as those expressed by a modified van’t Hoff-Mariotte law. Examination of the shapes and volumes of individual ghosts by a technic which combines phase optics, electronic flash (exposure time 0.001 second) and photography shows that three distinct populations of ghost coexist in any tonicity. These are spherical ghosts with a mean volume of 150 μ³, discoidal biconcave ghosts with a mean volume of 85 μ³, and crenated ghosts with a smaller volume which can be calculated. The most likely reason for this complexity is that the shape and volume of the ghost depends partly on its structure, and not altogether on the tonicity of the surrounding medium. Simple osmotic laws have no real application to systems of this kind.

(2) The changes in ghost volume and shape, as they depend on the duration of storage of the blood, at 4 °C, from which the ghosts are prepared, and as they reflect changes in ghost structure, can be expressed simply. Crenated and discoidal ghosts certainly have some of the elements of red cell structure; the spherical ghost, which soon fragments and gives rise to myelin forms, may also retain some of the original elements of structure, but the fragment and the myelin form have certainly lost them. The latter objects are so small and light that they are not thrown down into the ghost column in the hematocrit tube, and so, as ghost structure disappears with increasing time of storage of the red cells from which they are prepared, a discrepancy appears between the volume of the ghost column as measured by the hematocrit and the volume which one would expect. This discrepancy can be used as a measure of the extent to which ghost structure is lost, and there comes a time, as the duration of red cell storage is increased, when the ghosts prepared from these red cells begin to be replaced by breakdown products such as myelin forms, etc.

(3) The less the efficiency of the conditions of red cell preservation, the shorter is this time. In human blood rendered incoagulable with heparin, structural breakdown in the preceding sense and measured by a simple expression which changes sign when the loss of structure has reached a certain point, occurs after about 26 days. In human blood preserved in ACD, structural breakdown measured in an identical manner occurs after about 55 to 60 days. In human blood preserved in ACD-inosine, structural breakdown does not occur until about 75 to 80 days.

These results are based on a large amount of preliminary work of an exploratory nature and then on three runs with heparin, five runs with ACD and five runs with ACD-Inosine.

**Summario in Interlingua**

1. Quando erythrocytos human es hemolysate in medios multo hypotonic (NaCl de un tonicitate de 0.167) e quando postea le tonicitate es restablite per le addition del appropriate quantitates de NaCl a nivellos de 0.3, 0.5, e 1.7, il pare que le volumine medie del stromas es un function linear del valores reciproc del tonicitate. Iste observation poterea inducer nos a concluder que le stromas es osmometros e que lor volumine es governate per simple considerationes osmotic del typo de illos exprimite per un modifies lege de van’t Hoff-Mariotte. Le exa-
mine del conformatione del volumine de stromas individual per un technica in que optoe phase, exposition electronic a breve durantia (0,001 secundas), e photographia es combineate mostra que tres distincte populationes de stromas coexiste a omne nivello individual de tonicitate. Le tres es (1) stromas spheric con un volumine medie de 150 $\mu^3$, (2) stromas biconcave discoide con un volumine medie de 85 $\mu^3$, e (3) stromas crenate de un plus parse volumine que pote esser calculate. Le plus probable ration pro iste complexitate es que le conformation e le volumine del stromas depende in parte de lor structura e non exclusivamente del tonicitate del medio ambiente. Simple leges osmotie non es vermente applicabile a systems de iste genere.

2. Le alterationes del volumine e del conformatione del stromas—in tanto que illos depende del tempore durante le qual le sanguine ab que le stromas es preparate esseva immagasinate a 4 C e in tanto que illos reflecte alterationes del structura stromal—pote esser exprimite simplemente. Stromas crenate e discoide retene certemente un numero del elementos del structura erythrocytic. Etiam le stroma spheric, que tosto se disintegra in fragmentos e resulta in formas myelinic, pote retener certes del elementos structura original, sed iste elementos structura es certo absente ab le fragmentos e formas myelinic. Iste ultime objectos es si parve e si leve que in le tubo hematocritic illos non es precipitate a in le columna stromal. Per consequente, in tanto que le structura stromal dispare con le prolongation del tempore de immagasinage del erythrocytos original, un discrepantia se manifesta inter le volumine del columna stromal mesurate per le hematocritic e le volumine que on expectarea. Iste discrepantia pote esser usate como mesura del grado a que le structura stromal es perdite. De facto, le prolongation del immagasinage del erythrocytos ab que le stromas es preparate resulta finalmente in un stadio ubi le stromas es reimplaciate per products disintegratori, como per exemplo formas myelinic, etc.

3. Quanto minus efficace le conditiones del preservation del erythrocytos, tanto plus breve le tempore post que le stromas es reimplaciate per products de disintegration. In le caso de sanguine human rendite incoagulabile per medio de heparina, le supra-mentionate disintegration structural—mesurate per un simple expression que cambia su signo quando le perdita de structura attinge un certe puncto—occurre post circa 26 dies. In le caso de sanguine human preservate in acido-citrate-dextrosa (A.C.D.), le disintegration structural—mesurate in le mesme maniera—occurre post circa 55 a 60 dies. In sanguine human preservate in A.C.D. e inosina, le disintegration structural non occurre usque post circa 75 a 80 dies.

Iste resultatos es basate super un grande quantitate de labores preliminar de natura exploratori, sequite per tres series con heparina, cinque series con A.C.D., e cinque series con A.C.D. e inosina.

REFERENCES

Appendix

Densitometry Measurements of the Column of Ghosts in Hematocrit Tubes

It has been shown above that the ghosts of human red cells, resulting from hemolysis in a medium of tonicity 0.167 and afterwards brought to various tonicities from 0.3 to 1.7 by the addition of appropriate quantities of NaCl, make up at least 3 populations, distinguishable by their shape as seen with phase optics. These three kinds of ghost have different densities (density being always considered as that in excess of the density of the surrounding medium), that of the spherical ghost being the smallest and that of the crenated ghost the greatest. Comparison of the density of the former with that of the latter shows that the density of crenated forms may be 50 times as great as that of spherical ghosts, while the density of the latter may be as little as 2 per cent in excess of that of the medium surrounding them.

Consider a situation in which the ghosts of all three populations are thrown down by a centrifugal force of $2 \times 10^4$ G from the cup of a Hamburger hematocrit tube into the stem of the tube. The starting position in the 1.0 cm. cup may be considered as substantially the same for all the ghosts in the cup, since the stem, into which the ghosts have to fall, is about 8 cm. long. The densest ghosts reach the closed bottom of the stem first, those less dense form a column above them, while the least dense ghosts may not be thrown down at all and are found, by phase optics, in the apparently translucent fluid in the cup and in the upper part of the stem. As the densities and fractional number of the three populations vary, the state in the stem of the capillary varies, and this variation can be investigated by making densitometry measurements along the stem of the hematocrit tube.

The systems are prepared from human blood preserved at 4 C in ACD exactly as described in the paper to which this is an appendix. The “reversed” systems contain the several kinds of ghosts described therein. About 1.0 cc. of each system is spun for 90 minutes in Hamburger hematocrit tubes at $2 \times 10^4$ G and at less than 10 C. The stem of each tube is inserted in the light-path of a densitometer, the characteristics of which are that measurements of optical density are made at 6500 A through successive mm. of the stem, the optical system of the densitometer receiving the light transmitted through a slit of 0.3 mm. The optical density $D$ is plotted against the distance $d$ in mm. from the closed end of the stem, giving the relations between $d$ and $D$ shown in figure 2, a, b, c, and d.

The fundamental relations are

$$I/I_0 = e^{-\kappa t}, \quad D = kc$$

The thickness $t$ of the stem being constant, this reduces to $D = k$, where $c$, if $k$ were constant, would measure the concentration of the ghosts or the number of ghost per unit thickness of the light-path, and where $k$, if $c$ were constant, would be what might be called a “characteristic” of the ghost with respect to its interference with the passage of light through the light-path. An obvious property of the ghost which would influence the value of $k$ is the residual Hb which the ghost contains; the measurements are made at 6500 A to reduce this effect to a minimum. The other obvious property is that of shape, the
BEHAVIOR OF HUMAN RED CELL GHOSTS IN BLOOD

Fig. 2.—Ordinates, optical density measured at various distances from the bottom of the Hamburger hematocrit tube; these distances are shown in mm. on the abscissa. a, systems prepared from stored blood hemolysed and brought to a tonicity of 1.7; b, systems prepared from stored blood hemolysed and brought to a tonicity of 1.0; c, systems prepared from stored blood hemolysed and brought to a tonicity of 0.5; d, systems prepared from stored blood, hemolysed and brought to a tonicity of 0.3. Human blood in AC10 stored at 4 C for 8 days in all cases.

surface reflections which occur in crenated ghosts contributing to the light-stopping power in the direction of the light-path in a way undefinable at present. The difference between figure 2, a, b, c, and d is that the four parts of the figure show the result of restoring the hemolysed ghosts, derived from red cells stored for eight days at 4 C, to varying tonicities.

Figure 2 a shows the d-D relation at a tonicity of $T = 1.7$, in which the analysis is most easily made. There are three distinguishable regions. The first is a plateau corresponding to $D = 2.0$ and extending for a distance $d = 5.0$ mm. The ghosts in this region are maximally packed, and consist principally of crenated ghosts with some admixed discoidal forms. The second region extends from $d = 5.0$ to $d = 9.5$ mm., the values of $D$ falling (with irregularities which may be experimental) from 2.0 to 0.4. The light-stopping power in this region decreases as $d$ increases. The third region extends from $d = 9.5$ to all greater values of $d$, $D$ being simply the optical density of the Hb-containing and relatively ghost-free fluid in the upper part of the stem, from which the great majority of the light-stopping ghosts have been thrown down. Note that $D$ is not zero in this region; this is principally because the optical density of the contained Hb is not zero at 6500 A. A very subsidiary factor is that the fluid in the upper part of the stem is not entirely free of ghosts, fragments, and myelin forms not dense enough to be thrown down; these objects can be seen with phase optics.

Between $d = 0$ and $d = 5.0$, $D$ is constant, i.e., $kc$, and probably both $k$ and $c$, is constant. If so, the total number $N_1$ of ghosts in the region between $d = 0$ and $d = 5.0$ is given by the area $A_1$ underlying the plateau, less the area underlying the extension of the line at $D = 0.4$ ($D = 0.4$ is not an attribute of the ghost column, but of the column of Hb solution in the upper part of the stem).
Since $A_1 + A_2 = 1.0$, the region between $d = 5.0$ and $d = 9.5$ must be occupied by ghosts of a lesser density, the fractional number of which is $N$. Their attributes, however, cannot be constant, i.e., either $k$ or $c$, and most likely both $k$ and $c$, are variable, some ghosts varying in their density, some in their “characteristic”, and some in both. In terms of $D$, there is a variation between 2.0 and 0.4, i.e., the light-stopping power of this part of the ghost column varies with $d$. Since $A_2 = 1 - A_1$, the fractional number of ghosts with variable properties is about 0.29.

The third region, from $d = 9.5$ onward, requires no further explanation.

Figure 2b ($T = 1.0$) can be analysed in a manner similar to that applied to figure 2a, to which it is similar except that there is a less definite plateau. $D$ is again 2.0 at $d = 0$, but the slope of the line forming the upper limit of the area $A_2$ is less than in figure 2a. The fractional number of ghosts with variable properties is about 0.28.

Now consider Fig. 2c and d ($T = 0.5$ and 0.3 respectively). There are so many ghosts which have dissimilar properties that there is no plateau, and densitometry cannot distinguish $A_1$ from $A_2$. All kinds of ghost are mixed together, probably because, in these low tonicities, the density of all kinds of ghost is small. There is still a tendency, however, for the denser crenated ghosts to reach the lowest part of the hematocrit stem. $A_1$ can be imperfectly distinguished from $A_2$ by sampling the ghost column at different levels and observing the form of the ghosts, their degree of hemoglobinisation, etc., with phase optics. An important finding, however, is that $A_1 + A_2$ decreases with lower tonicities so that if it is 1.0 at $T = 1.0$, it is 0.63 at $T = 0.5$ and 0.5 at $T = 0.3$. This means that ghosts have been lost in the lower tonicities, and examination with phase optics of the apparently clear fluid in the cup and in the upper part of the stem of the hematocrit tube shows that there are many spherical ghosts, fragmented ghosts, and myelin forms.

These observations of the optical properties of the ghost column in hematocrit tubes confirm the observations made independently on the form of the red cell ghost produced by lysis in low tonicities and then returned to tonicities between 0.3 and 1.7. Specifically, they confirm the observation that red cell ghosts prepared in this way are very heterogeneous as regards their properties (particularly, in these investigations, as regards their density, the independent observations being concerned principally with heterogeneity of shape), that hematocrit determinations cannot be relied upon for the determination of the mean volume of the ghost in hypotonic systems, and that there are losses, particularly in hypotonic systems, of the ghosts derived from red cells because the ghosts are replaced by myelin forms, fragments, and ghosts which have such a small density that they cannot be thrown down even at centrifugal forces as great as $2 \times 10^4$ G. As the length of time of storage at 4 C is increased, there is a tendency for the type of $d$-$D$ relation at tonicities such as 1.7 and 1.0 (fig. 2a and b) to be replaced by the flat type of $d$-$D$ relation shown in figure 2c and d. This again means that ghosts have been lost to an increasing extent in the lower tonicities as the time of storage is prolonged, and again, examination with phase optics of the apparently clear fluid in the cup and in the upper part of the stem of the hematocrit tube shows spherical ghosts, fragmented ghosts, and myelin forms which increase in number as the duration of the storage increases.
The Behavior, as Regards Shape and Volume, of Human Red Cell Ghosts in Fresh and in Stored Blood

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