A STUDY OF THE OSMOTIC BEHAVIOR OF THE HUMAN ERYTHROCYTE * †

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Despite many previous studies of the osmotic properties of erythrocytes, unequivocal answers have not been given to two important questions: *1*) Is the erythrocyte in osmotic equilibrium with its normal surrounding fluid? *2*) When the osmotic properties of the surrounding fluid are varied, does the erythrocyte gain or lose water to the extent necessary to remain in osmotic equilibrium with the new surroundings—that is, does the erythrocyte behave as a perfect osmometer over a wide range?

Previous work related to these questions has been of two general types: measurements of changes of volume of the erythrocytes as the osmotic concentration of the extracellular phase was altered; and measurements of one or more of the colligative properties of the solutions involved. The present study is of the latter type. The osmotic behavior of the normal human erythrocyte has been investigated over a wide range of concentrations by a cryoscopic method and a method for measuring the melting point of microscopic samples. The results give affirmative answers to both of the above questions and illustrate some of the difficulties which may be expected in studies of osmotic properties of any tissues.

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MATERIAL AND METHODS

All samples of blood were obtained from healthy, white male and female medical students or young physicians. Venous blood was collected anaerobically and transferred to a mercury-containing bulb, in which the blood was defibrinated by shaking. In experiments in which disruption of red cell membranes was desired, this was achieved by freezing the samples in a mixture of solid carbon dioxide and 95 per cent ethyl alcohol for five to seven minutes, followed by gradual warming to room temperature. This procedure resulted in complete hemolysis; no red cells could be identified microscopically in the samples and only an insignificant amount of red cell mass could be separated from the hemolyzed mixture by centrifugation.

In studies in which cells were separated from their surrounding fluid, this was achieved by centrifugation, under oil, at a relative centrifugal force of $600 \times G$ for 50 to 60 minutes in Tygon® tubing bent in the shape of a "U" and inserted in the cups of the centrifuge. After centrifugation, a small portion of the supernatant was reserved under oil. The tubing was then clamped a short distance below the surface of the packed cells, cut on the underside of the clamp and the cell mass expelled into an oil-containing tube. The volume of packed cells, where used, was measured in Daland tubes by centrifugation at a relative centrifugal force of $600 \times G$ for 50 to 60 minutes; this was shown to produce a constant volume. As noted in the discussion below, this measurement of volume of packed cells was considered to represent fairly accurately the cell volume in normal whole blood, but not in samples in which the osmotic activity had been significantly altered.

The first series of experiments involved measurement of freezing point depressions using a Fiske osmometer, calibrated as previously described (1). Reproducibility of the instrument on replicate samples, with care to achieve the same degree of supercooling before crystallization, was within 1 to 2 milliosmoles per Kg. water (mOsm.) throughout the range from 100 to 500 mOsm. Readings on each sample were repeated until at least two readings were within 2 mOsm. of each other; usually the first two readings were within these limits. The average of all readings is reported in the tables of Results.

The units of "milliosmoles" (per Kg. H_2O) are used in the first part of this study because the readings for freezing point depression were actually recorded in this form, because they can be readily converted to degrees

of depression of freezing point (1,000 mOsm. = freezing point depression of 1.86° C.) and because these units have become familiar as a way of expressing "osmotic activity" of biological solutions. However, the thermodynamic property of the solution which is actually being quantified by such measurements is the activity of water in the solution.

The second series of experiments involved measurements of the melting points of tiny samples of hemolyzed red cells and their former surrounding fluids. The apparatus used was made from the design of Ramsey and Brown (2) with slight modifications. A sample drop, about 1×10^{-6}-10^{-7} ml., is taken up in a microcapillary tube, preceded and followed by paraffin oil. It is frozen by immersion in dry-ice-alcohol for 15 to 20 seconds and placed in a bath of the apparatus which is already cooled below the melting point of the sample. With the aid of a microscopic attachment, crystals of ice can readily be seen in the sample, even in samples of pure hemolyzed red cells. The temperature of the bath is then raised very slowly, at a rate of 0.001 to 0.002° C. per minute, in the vicinity of the expected melting point. The disappearance of the last ice crystal is taken as the melting point of ice in that solution. With care and practice, replicate readings on a sample of serum agreed within less than 0.005° C. Variability in samples of hemolyzed red cells was somewhat greater but was rarely greater than 0.01° C. All readings are reported in the tables. A sample reading required 10 to 20 minutes.

TABLE I

A. Freezing points of whole blood and serum from the same samples

Sample	Whole blood	Serum	Difference
	*mOsm.**		
CL	288.8	288.0	+0.8
BL	283.5	283.5	0
PD	284.5	284.0	+0.5
ED	285.0	285.0	0
AM	289.5	289.5	0
JB	283.0	283.5	−0.5
H	282.0	282.0	0
WW	283.0	283.0	0
GN	278.0	278.5	−0.5
JM	279.5	280.5	−1.0

B. Freezing points of whole blood plus solutions of NaCl and the cell-free supernatants from the same samples

	Whole blood plus saline	Supernatant	
CL	521.0	521.5	−0.5
	228.0	228.0	0
BR	517.5	518.5	−1.0
ED	521.5	520.0	+1.5
	224.5	224.5	0
	520.5	520.0	+0.5
H	520.0	520.5	−0.5
AM	215.5	215.5	0
JB	212.5	213.0	−0.5
NH	213.0	212.5	+0.5

* In this and subsequent tables, mOsm. = milliosmoles per Kg. H_2O.

Although others (3, 4) have found that the freezing point of other tissues changes considerably within the first few minutes of exposure to temperatures near the freezing point, the melting point of samples of hemolyzed red cells in the present study was not detectably changed for at least one to two hours, at temperatures between 0 and 25° C., under oil. A very small change with time in the freezing point of nonhemolyzed blood was found (*vide infra*).

RESULTS

1. *Freezing point depression of whole blood and serum*

The finding of Hamburger (5) and Collins and Scott (6), that the depressions of freezing point of whole blood and the serum separated from the same blood are identical, was confirmed in 10 samples (Table I). This observation was extended to freezing point depressions of one-to-one mixtures of whole blood and solutions of sodium chloride, compared with the supernatant fluid removed from the same mixtures after centrifugation. In 10 such mixtures, the osmolality of which extended from 212.5 to 521.5 mOsm., the freezing point depression was the same for the whole mixture and the supernatant (Table I). In the subsequent studies to be described the freezing point depressions of samples containing *nonhemolyzed* red cells are therefore considered to be equal to the freezing point depressions of the extracellular phases of those samples.

2. *Freezing point depression of whole blood before and after hemolysis*

When the freezing point depressions of samples of whole blood were compared with those of the same blood after hemolysis, it was found that the osmotic activity after hemolysis was significantly lower. The mean difference in 13 samples not handled under oil was 4.46 mOsm. (Table II). The same comparison was repeated with five samples handled under paraffin oil throughout (including the determination of freezing point). A mean difference of 3.66 mOsm. was found (Table II). This mean difference was significantly different from zero but not significantly different from that of the samples not handled under oil (7).

In this study and in subsequent studies in which comparisons of nonhemolyzed and hemolyzed samples are made, 15 to 45 minutes elapsed

TABLE II

A. Freezing points of whole blood, unhemolyzed, and the same samples after hemolysis, not handled under paraffin oil

Sample	Unhemolyzed	Hemolyzed	Difference
	mOsm.		
WW	283.5	282.0	1.5
PE	282.5	279.5	3.0
JA	288.0	281.0	7.0
RW	288.0	281.5	6.5
AG	288.0	282.5	5.5
FW	285.5	281.5	4.0
EM	279.0	274.5	4.5
HE	285.5	282.0	3.5
S	287.0	283.0	4.0
TM	283.5	278.0	5.5
BR	283.0	279.0	4.0
CL	288.5	284.5	4.0
CP	286.0	281.0	5.0
	Mean = 285.2		Mean diff. = 4.46
			p = <.001

B. Same as A, but handled under oil throughout

FW	288.4	282.6	5.8
AT	279.5	278.0	1.5
C	285.8	281.8	4.0
TP	279.0	275.8	3.2
RC	280.8	277.0	3.8
	Mean = 282.7		Mean diff. = 3.66
			p = <.01

"t" Test of differences between the means of A and B: $t_{16\ d.f.} = 0.7686$, not significant.
"p" is the probability that the mean difference is zero (7).

between defibrination of the blood and the determination of freezing point on the hemolyzed sample. In 64 samples of various osmotic activities the freezing point of the nonhemolyzed aliquot was determined initially and again just after the determination of the hemolyzed sample. The second determination gave a slightly but significantly lower osmotic activity than the first—between 0.5 and 1.0 mOsm. lower. In the data reported in Table II and in subsequent similar tables the value for the nonhemolyzed sample is the value obtained for its freezing point immediately after the determination on the hemolyzed sample. Thus the slight change in value with time in both samples should be minimized or eliminated.

3. Freezing point depressions of mixtures of whole blood and solutions of sodium chloride or sucrose, before and after hemolysis

When the freezing point depressions of one-to-one mixtures of whole blood and various solutions of NaCl (5.0 Gm. per L. to slightly over 30 Gm. per L.) were compared with those of the same samples after hemolysis it was found that the osmotic activity after hemolysis was significantly lower. This difference was greater the higher the osmotic activity of the sample (Figure 1). Over the wide range of concentrations studied (208 to 747 mOsm.) the data for 83 samples not handled under oil fit the following linear equation with a high degree of significance:

$$C_H = 0.9728\ C_S + 4.09, \qquad 1)$$

where C_H = osmotic activity of hemolyzed samples (in mOsm.) and C_S = osmotic activity of unhemolyzed samples (in mOsm.). The proportion of variation explained by this equation is 0.99977 (8).

The study was repeated with 15 samples handled under oil throughout over the range 210 to 516 mOsm., with essentially the same result. Table III presents these data and is representative of the data obtained on the 83 samples not handled under oil. The data of Table III fit the following equation:

$$C_H = 0.9777\ C_S + 3.40. \qquad 2)$$

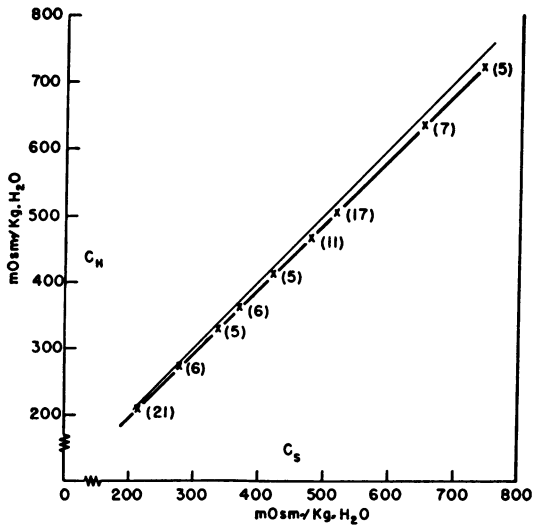

FIG. 1. THE RELATIONSHIP BETWEEN FREEZING POINT DEPRESSIONS BEFORE HEMOLYSIS (C_S) AND AFTER HEMOLYSIS (C_H) OF SAMPLES OF WHOLE BLOOD MIXED 1:1 WITH NACL SOLUTIONS OF VARIOUS CONCENTRATIONS

The figures in parentheses are the numbers of samples at each level. The points plotted are the mean values at each level; the range is too small to depict. The thin line represents $C_H = C_S$.

TABLE III

Freezing points of blood-plus-saline, unhemolyzed, and the same samples after hemolysis, handled under oil throughout

Sample	Blood + NaCl (5.0 Gm./L.)			Blood + NaCl (9.0 Gm./L.)			Blood + NaCl (22.22 Gm./L.)		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*			*mOsm.*		
AT	210.1	209.5	0.6	278.5	276.0	2.5	510.7	503.9	6.8
C	214.4	213.1	1.1	282.3	279.0	3.3	515.9	506.5	9.4
TP	210.6	208.9	1.7	279.0	276.0	3.0	509.5	500.7	8.8
B	217.4	216.6	0.8	284.5	282.3	2.2	516.2	508.2	8.0
RC	210.4	208.6	1.8	279.8	275.8	4.0	509.2	502.2	7.0

There is no significant difference in the slope or intercept of Equations 1 and 2. In the second experiment (under oil) the proportion of variation explained by Equation 2 is 0.99995 (8).

In a similar study in which solutions of sucrose instead of sodium chloride were used, at two levels of osmotic activity of the mixture (217 and 497 mOsm.), a comparable decrease in activity with hemolysis was found (Table IV).

4. The effects of addition of dry urea

Samples of whole blood were mixed one-to-one with a solution of NaCl, 5.0 Gm. per L., used previously to produce a hypotonic mixture (about 212 mOsm.). Various amounts of dry urea were then added to the mixture, the amounts so chosen that the whole range of osmolalities of the saline studies given above was reproduced with the urea-containing mixture. Freezing point depression was then measured with and without hemolysis. The results are given in Table V. These data indicate that, in contrast to the results with saline and sucrose, the differences obtained between unhemolyzed and hemolyzed samples are not significantly altered by the addition of varying quantities of urea. The average difference of similar samples without urea added was 1.73 mOsm.; the average difference of these containing urea is 2.09 mOsm.

Comments on Studies 2, 3 and 4. The osmotic activity—freezing point depression or any other measure of colligative properties—of a sample of blood would be expected to be the same after rupture of the red cell membranes as it was before rupture if: *1*) the red cell contents before rupture were in osmotic equilibrium with their surroundings; *2*) with rupture the mixing of the two previously separate solutions did not change appreciably the activity coefficients of the various solutes present; and *3*) no solutes or water were added to or lost from the solution with rupture. Since the present studies show a definite change in osmotic activity of the mixture after hemolysis, at least one of the above conditions does not hold. The change in osmotic activity with hemolysis is small but, within the limits of the design of the

TABLE IV

Freezing point of blood-plus-sucrose, unhemolyzed, and the same samples after hemolysis

Sample	Blood + sucrose (53.1 Gm./L.)			Blood + sucrose (243.9 Gm./L.)		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*		
D-1	218.5	216.5	2.0	498.0	488.0	10.0
D-2	218.5	217.5	1.0	501.0	489.5	11.5
D-3	221.5	221.0	0.5	501.5	489.5	12.0
D-4	214.5	212.5	2.0	491.5	485.5	6.0
D-5	215.0	215.0	0	496.0	488.0	8.0
D-6	216.0	213.5	2.5	500.0	484.0	16.0
D-7	217.5	217.0	0.5	495.0	486.5	8.5
D-8	216.0	213.0	3.0	497.0	486.0	11.0
D-9	217.0	214.0	3.0	489.0	479.5	9.5
D-10	213.5	212.5	1.0	496.0	482.0	14.0
		Mean diff. = 1.55			Mean diff. = 10.65	

TABLE V

*Freezing points before and after hemolysis of samples to which dry urea was added**

Sample	14 mg. urea added			31 mg. urea added		
	Unhemolyzed	Hemolyzed	Diff.	Unhemolyzed	Hemolyzed	Diff.
	mOsm.			*mOsm.*		
4G	278.5	276.5	2.0	346.5	344.0	2.5
5G	277.0	276.0	1.0	345.0	343.0	2.0
6G	271.5	270.5	1.0			
7G				355.0	354.0	1.0
8G						
9G				353.0	350.0	3.0
10G						
11G	268.5	266.0	2.5	349.5	347.5	2.0
12G	269.0	267.0	2.0	349.5	347.0	2.5
13G	270.5	267.5	3.0	345.5	343.0	2.5
14G	279.0	276.0	3.0	348.5	347.0	1.5
15G	272.5	271.5	1.0			
		Mean diff. = 1.94			Mean diff. = 2.13	
	48 mg. urea added			64.5 mg. urea added		
4G	426.5	425.0	1.5	497.5	492.5	5.0
5G	426.5	426.5	0			
6G	418.0	415.5	2.5	510.5	504.5	6.0
7G				505.0	499.5	5.5
8G				492.5	491.5	1.0
9G				504.5	502.0	2.5
10G				506.5	505.0	1.5
11G	421.0	421.0	0	494.0	494.0	0
12G	420.5	419.0	1.5			
13G	419.0	415.5	3.5			
14G	421.0	420.5	0.5	495.0	494.0	1.0
15G	418.0	417.0	1.0	484.5	481.0	3.5
		Mean diff. = 1.31			Mean diff. = 2.89	

* Each sample contained 2 ml. blood plus 2 ml. NaCl solution, 5.0 Gm. per L.

present study, highly predictable from sample to sample of normal blood. Furthermore, the difference between hemolyzed and unhemolyzed aliquots of the same sample varies in a predictable fashion as the osmotic concentration of the fluid outside the red cells is varied by changing the concentrations of solutes which will not readily cross the red cell membranes, *e.g.*, sodium chloride or sucrose. However, this difference is not affected by the addition of urea, a solute which diffuses easily into the red cell. Thus, the *total* osmotic activity of the solutions inside and outside the cell is not the determinant of the variation in differences of osmotic activity before and after hemolysis.

These results are consistent with, but more extensive than, previously reported studies. Thus Hamburger (9) found that the freezing point depression of horse and dog serum was 1 to 2 per cent lower than that of the hemolyzed blood. Mukai (10) obtained similar results in samples handled under paraffin oil, with a mean difference between serum and hemolyzed whole blood (expressed in the units of the present paper) of 5.9 mOsm. Utilizing Barger's vapor pressure method, which involves the measurement of change of length of drops of fluid, separated by air spaces in capillary tubes, Mukai found no measurable difference in the vapor pressures of a mixture of laked blood and serum (1 : 1), and serum alone. However, in the diluted blood samples with which he was working, a difference in vapor pressure between serum and hemolysate-serum mixture of a magnitude similar to that observed in the present study could have been missed.

Of the three conditions mentioned above, there is independent evidence that the second, absence of appreciable change in activity coefficients of the solutes present, does not hold. In his studies of the osmotic properties of hemoglobin, Adair (11) found that the activity coefficient of hemoglobin increased with increasing concentration of

hemoglobin; *i.e.,* the total osmotic activity of hemoglobin solutions increased at a progressively greater rate than the concentration of hemoglobin in the solution. Similar properties for plasma proteins have been described by Scatchard, Batchelder and Brown (12). From Adair's findings one would predict the following events: *1*) When whole blood is hemolyzed, and the *concentration* of hemoglobin in the solution falls from that of the interior of the red cell to a much lower level in the hemolyzed blood, the *osmotic activity* of the hemoglobin would decline even more. Thus the total osmotic activity of the hemolyzed blood would be expected to have a lower value than the activity produced by the same total amount of solutes in the same total amount of water when the hemoglobin was contained in the red cell. *2*) Similarly, when the blood is mixed with hypertonic NaCl, if water moves freely across the cell membrane in response to osmotic forces, water would leave the cell, and the concentration of hemoglobin would rise. However, the osmotic activity of hemoglobin would rise even more and thus osmotic equilibrium would be reached with less movement of water out of the cell than would have occurred if there were no change in the activity coefficient of the hemoglobin. When such cells are then hemolyzed, the concentration of hemoglobin falls more than it does when cells in isotonic media are hemolyzed, and the osmotic activity of the hemoglobin would be expected to decline even more. Thus a greater difference in osmotic activities, before and after hemolysis, would be expected under hypertonic conditions than under isotonic conditions. The reverse would be expected with cells exposed to hypotonic media.

Qualitatively, the present data are in agreement with such predictions based on Adair's findings. An approximate quantitative comparison can be made for the hemolysis of normal whole blood. For an average concentration of hemoglobin within the normal red cell of 34 Gm. per 100 ml. solution, Adair's data indicate that the osmotic activity of the hemoglobin would be about 18.7 mOsm. If we assume that the total osmolality within the cell equals that of the serum, which has a mean value in our studies of 285.3 mOsm., then the osmotic activity of intracellular solutes other than hemoglobin would be: 285.3 − 18.7 = 266.6 mOsm. With hemolysis, the hemoglobin would be diluted to a concentration of 15.4 Gm. per 100 ml. solution. At this concentration the hemoglobin would have, by Adair's data, an osmotic activity of 3.7 mOsm.

The average contents of water measured in 10 samples of blood (together with packed cell volume) and five samples of serum were 0.3145 ml. H_2O in the red cell per ml. blood and 0.5092 ml. H_2O in the serum per ml. blood.

A prediction of the total osmotic activity of the hemolyzed sample can be made with the above data by the following calculations, if one assumes that the activity coefficients of other solutes in red cell and serum will change relatively little when the two solutions are mixed:

$$\text{Predicted osmotic activity after hemolysis}$$

$$= \frac{\left(\begin{matrix}\text{H}_2\text{O content}\\ \text{of serum}\end{matrix} \times \begin{matrix}\text{osmotic activity}\\ \text{of serum}\end{matrix}\right) + \left(\begin{matrix}\text{H}_2\text{O content}\\ \text{of cells}\end{matrix} \times \begin{matrix}\text{osmotic activity of cells}\\ \text{other than hemoglobin}\end{matrix}\right)}{\text{total H}_2\text{O content of 1 ml. blood}} + 3.7$$

$$= \frac{(0.5092 \times 285.3) + (0.3145 \times 266.6)}{0.8237} + 3.7$$

$$= 281.8 \text{ mOsm.}$$

Therefore, the predicted decrease in osmotic activity of normal whole blood upon hemolysis is: 285.3 − 281.8 = 3.5 mOsm. This compares well with the average values found in the present studies of a decrease of 3.7 mOsm. in samples handled under oil, or 4.5 mOsm. without oil.

Thus the change in osmotic activity which occurs with hemolysis of red cells in whole blood *could* be accounted for by the change in the activity coefficient of the hemoglobin alone. Similar calculations have not been made for the series of samples of blood-plus-saline before and after hemolysis at different concentrations because of the unreliable nature of measurements of packed cell

volume of red cells exposed to hypotonic or hypertonic media (13). A reliable value for packed cell volume is required for the calculation of the concentration of hemoglobin in the unhemolyzed cells, from which its osmotic activity may be determined from Adair's data. However, the differences in osmotic activity seen in the various blood-saline mixtures, before and after hemolysis, are in the direction which is to be expected from Adair's observations.

It may be noted that the apparent loss of osmotic activity found by Appelboom, Brodsky, Tuttle and Diamond (14) for boiled whole blood might also be accounted for by the change in the activity coefficient for hemoglobin introduced by dilution of their samples.

The fact that the changing activity coefficient of hemoglobin can account for the change in osmotic activity with hemolysis does not, of course, exclude other possible explanations of the observed change. A more direct comparison of the osmotic activity of the pure contents of the red cells with that of their surroundings is highly desirable. In the present studies attempts to determine directly, with the Fiske osmometer, the freezing point of pure hemolyzed red cells did not give satisfactory results because of the great and irregular degree of supercooling. Previous writers have recognized supercooling as a problem (15, 16). The wide range of values obtained in our attempts, similar to the range reported by Brodsky and co-workers (4), for crushed red cells from normal dogs, could not be considered satisfactory direct measures of the osmotic activity of ruptured red cells.

Attempts to compare the osmotic activity of hemolyzed red cells with that of their surroundings by a comparative vapor pressure technique [the isopiestic technique (17)] were also unsatisfactory. Details of the problems encountered will be furnished upon request.

A third method of comparing the colligative properties of these solutions, the use of melting points, gave more satisfactory results, as follows.

5. *Melting points of hemolyzed red cells and their former surrounding fluid*

Three types of samples were used: *1*) whole blood mixed one-to-one with a sodium chloride solution, 5.0 Gm. per L., and then separated into cells and supernatant; *2*) whole blood mixed one-to-one with sodium chloride solution, *22.22* Gm. per L., and then separated into cells and supernatant; and *3*) whole blood separated into cells and serum. Separation of cells, hemolysis of the red cells and measurement of melting points on microscopic samples were done as described in Methods.

Tables VI, VII and VIII give the results. By an analysis of variance of these data one can place the limits of confidence given in the tables about the mean differences between melting points of hemolyzed red cells and the surrounding fluid to which they had been exposed prior to hemolysis. Thus, as shown in Table VI for red cells exposed to a hypotonic solution, the probability is 0.95 that the narrow range, +0.0062 to −0.0017° C. (+3.32 to −0.91 mOsm.), includes the true value for the mean difference between surrounding solutions and red cell contents. This range includes zero difference; *i.e.*, the mean difference in melting point is not significantly different from zero. The same type of statement can be made from Table VII, for red cells exposed to a hypertonic solution, where the probability is 0.95 that the range, +0.0054 to −0.013° C. (+2.90 to −6.81 mOsm.), includes the true value for the mean difference, and from Table VIII for red cells of normal whole blood, where the probability is 0.95 that the range, +0.0004 to −0.0067° C. (+0.19 to −3.57 mOsm.), includes the true value for the mean

TABLE VI

Melting points of components of samples of whole blood mixed 1:1 with NaCl solution, 5.0 Gm. per L.

Sample	1 Melting point of supernatant	2 Melting point of hemolyzed red cells
	°C.	°C.
NH	−0.401, 0.400	−0.411, 0.408
AM	−0.401, 0.403	−0.400, 0.404
BW	−0.410, 0.404	−0.410, 0.409
MS	−0.406, 0.405	−0.405, 0.402
WT	−0.410, 0.410	−0.410, 0.412
WT	−0.410, 0.410	−0.413, 0.413

Mean difference, melting point of supernatant minus melting point of hemolyzed red cells = +0.0023° C. (+1.23 mOsm.); 95% confidence limits about the mean difference = +0.0062° C. to −0.0017° C. or +3.32 mOsm. to −0.91 mOsm.

TABLE VII

Melting points of components of whole blood mixed 1:1 with NaCl solution, 22.2 Gm. per L.

Sample	1 Melting point of supernatant	2 Melting point of hemolyzed red cells
	°C.	°C.
MT	−1.010, 1.002, −1.002, 0.998, 1.000	−0.980, 0.995, 0.985, −0.980, 0.988, 0.995
AL	−1.020, 1.022, −1.030, 1.032	−1.040, 1.050, 1.035
EO	−1.018, 1.018	−1.030, 1.040, 1.020
BW	−1.020, 1.025	−1.015, 1.025, 1.030
CM	−1.010, 1.010	−1.012, 1.012
LH	−1.015, 1.014	−1.002, 0.995, 0.995
DK	−1.012, 1.008	−1.008, 1.005
AB	−1.005, 0.998	−0.998, 1.003
TF	−1.040, 1.040	−1.020, 1.030
TD	−1.026, 1.030	−1.022, 1.020

Mean difference, melting point of supernatant minus melting point of hemolyzed red cells = −0.0034° C. (−1.82 mOsm.); 95% confidence limits about the mean difference = +0.0054° C. to −0.013° C. or +2.90 mOsm. to −6.81 mOsm.

difference between hemolyzed red cells and serum.

The studies on whole blood (Table VIII) are presented in two parts. Series A includes the first samples studied by this method. Statistical analysis of the first 10 samples indicated a difference in melting point between serum and cells, that of the cells being slightly higher (*i.e.*, less solute concentration). However, it was noted that this difference was contributed almost entirely by the differences in the first three samples that had been measured. Accordingly, another series of whole blood samples was studied, with two additional precautions: The samples were centrifuged in a temperature controlled centrifuge at 22° C., and the order in which the two readings on serum and two readings on red cells were done was randomized for the total of four readings on each sample. The control of temperature of centrifugation was instituted because it had been noted that in a noncooled centrifuge the samples became quite warm by the end of centrifugation.

The results of measurements on whole blood with these added precautions and at a time when technical handling of the whole procedure was improved by practice are given in Series B of Table VIII. Variability within samples was decreased and there is clearly no significant difference in this series in the melting point of hemolyzed red cells and serum from the same samples. The 95 per cent confidence limits on the mean difference of + 0.00042° C. are from −0.00712 to + 0.00795° C. (+ 4.26 to − 3.82 mOsm.), distributed almost equally about zero difference. As already noted, if all determinations on normal whole blood are included in one analysis, the mean difference is also not significantly different from zero.

Thus there appears to be no significant difference between the melting points of hemolyzed red cells and the fluid to which the cells had been exposed, prior to separation and hemolysis, at the three levels of osmotic activity studied.

DISCUSSION

In the few previous studies of osmotic properties of red cells which have utilized direct measure-

TABLE VIII

Melting points of components of whole blood

Sample	Melting point of serum	Melting point of hemolyzed red cells
	°C.	°C.
A. First series		
HO	−0.547, 0.547	−0.530, 0.535, 0.540
CM	−0.540, 0.542	−0.531, 0.530, 0.528
LH	−0.540, 0.540	−0.533, 0.525
DK	−0.535, 0.542	−0.540, 0.538
AB	−0.535, 0.535	−0.530, 0.531
TF	−0.530, 0.530	−0.528, 0.533
TD	−0.540, 0.545	−0.532, 0.532
NH	−0.517, 0.523	−0.518, 0.525
AM	−0.530, 0.531	−0.530, 0.528
BW	−0.540, 0.539	−0.537, 0.533

Mean difference, melting point of serum minus melting point of hemolyzed red cells = −0.0053° C. (−2.84 mOsm.); 95% confidence limits about the mean difference = −0.0014° C. to −0.0092° C. or −0.73 mOsm. to −4.95 mOsm.

B. Second series		
SF	−0.567, 0.561	−0.572, 0.576
JM	−0.530, 0.530	−0.530, 0.533
TF	−0.540, 0.535	−0.522, 0.532
CG	−0.530, 0.530	−0.536, 0.535
NC	−0.519, 0.520	−0.520, 0.519
RS	−0.529, 0.529	−0.527, 0.523

Mean difference, melting point of serum minus melting point of hemolyzed red cells = +0.00042° C. (0.23 mOsm.); 95% confidence limits about the mean difference = +0.0080° C. to −0.0071° C. or +4.26 mOsm. to −3.82 mOsm.

For Table VIII treated as a unit, the mean difference = −0.0032° C. (−1.72 mOsm.); 95% confidence limits about the mean difference = +0.00036° C. to −0.0067° C. or +0.19 mOsm. to −3.57 mOsm.

ment of a colligative property of the red cell contents, the data have been in general agreement with the hypothesis that the red cell is in osmotic equilibrium with its surroundings. Hill (18) employed a thermo-electric method for the determination of vapor pressures and obtained similar values for the serum and hemolyzed red cells from one sample of slaughterhouse blood. Roepke and Baldes (19) also used a thermo-electric method for measuring vapor pressures, and found the values for lysed human red cells to be very nearly equal to those of the intact cells (*i.e.*, the trapped extracellular fluid). When human cells were washed with hypertonic solutions of NaCl or sucrose, they found a decrease in vapor pressure after hemolysis, but this they attributed to dilution by trapped extracellular fluid. They recognized that such dilution would change the osmotic coefficient of hemoglobin.

The present experiments with melting point determinations demonstrate, with considerable statistical confidence, that the osmotic activity of the contents of the red cell is not significantly different from that of the extracellular phase to which the cell was exposed prior to rupture. This is true not only of cells previously in normal serum, but of cells which had been exposed to extracellular mixtures of serum and saline at concentrations from 210 to 520 mOsm. The red cell therefore appears to act as a "perfect osmometer" over the range of concentration studied here, gaining or losing sufficient water to remain in osmotic equilibrium with its surroundings. Stated in other terms, the activity of water inside the red cell is the same as that outside over a wide range of activities. Two untested assumptions underlie this statement: *1*) The actual process of rupture of the red cell membrane by freezing and thawing does not significantly alter the solute concentration in the red cell contents; and *2*) measurements made at -0.3 to $-1.0°$ C. accurately reflect the comparative colligative properties of red cells and surroundings at room temperature or body temperature. This assumption is supported by the finding of Roepke and Baldes (19) that there is no significant effect of temperature, from 0 to 29.4° C., on the comparative vapor pressures of red cells and reference solutions of serum, sodium chloride or sucrose.

In contrast to the above results and interpretation of studies of colligative properties, the studies of the osmotic behavior of the red cell using measurement of changes of cell volume have been generally interpreted as indicating that the cell is not a perfect osmometer [see Ponder (20, 21) for reference to the early literature and for discussion of these findings and of the sources of error in measuring cell volumes]. In most such experiments the changes in cell volume when the osmotic activity of the extracellular fluid is changed have not been as great as would be predicted for a simple osmometer. Even though a few studies, such as that by Hendry (13), give opposite results, the general consistency of direction of change in the data obtained by various methods of measuring cell volume makes it necessary to seek some explanation for the less than expected changes in volume with changes in external osmotic activity.

Four explanations have been proposed: *1*) The structure of the membrane resists change in volume. This view would allow the red cell to be in osmotic equilibrium with its surroundings in isotonic media but not in hypotonic or hypertonic media, which is not consistent with the findings of the present study.

2) The red cell may gain or lose solutes as the composition of the surrounding media is changed. The present studies have no direct bearing upon such possible movements. However, the results obtained with the addition of sucrose solutions (Table IV) and urea (Table V) indicate that the osmotic behavior of the red cell is related to the concentrations of solutes which do *not* cross the red cell membrane.

3) Some of the water within the red cell is "bound", *i.e.*, not available to participate in changes in the red cell content of water in response to changes in osmotic activity outside the cell.

4) The changes in cell volume are what would be expected on the basis of Adair's evidence (11) for the changing osmotic coefficient of hemoglobin, and with allowance for this property of hemoglobin the red cell does behave as a perfect osmometer.

The acceptability of the "bound" water concept would seem to depend upon the sense in which this term is used. It can be taken to mean the same thing as is meant by the changing osmotic

coefficient of hemoglobin. That is, when one states that the osmotic coefficient of hemoglobin increases with increasing concentration of hemoglobin in a solution, an equivalent statement would be that the activity coefficient (and therefore total activity) of water decreases under these circumstances. If "bound" water is equated with "decreased activity coefficient" of water, then explanations 3 and 4 are equivalent and are consistent with available evidence including the present study.

Another approach to the question of "bound" water has been to add known amounts of solutes which would be expected to distribute in all available water which could act as "solvent" water and then to measure the concentration of the solute in the system or its effect on the colligative properties of the system. Earlier studies indicated the presence of a significant proportion of "nonsolvent" water in the red cell (*22*, *23*). Hill (18) found less than 5 per cent of red cell water to be nonsolvent water. Roepke and Baldes (19) found small but consistent amounts of "nonsolvent" water which increased as the nondiffusible solute concentration of the extracellular phase was raised. They believed that the changing osmotic coefficient of hemoglobin described by Adair (11) could account for most of the "anomalous osmotic behavior" of red cells, but that some of the water of cells was not solvent for added crystalloids. However, Hutchinson (24), using $C^{14}H_3OH$, C^{14}-n-butyl alcohol and H_2O^{18}, found that these molecules reached rapid equilibrium with the contents of human red cells and that all of the water of the red cells acted as "free" water with respect to these substances. This is probably the most refined technique that has been applied to the measurement of "free" water. MacLeod and Ponder (*25*) have pointed out that "nonsolvent" water and "bound" water may not be identical.

Dick and Lowenstein (*26*) recently measured the change in red cell volume in the hypotonic range by a technique of immersion refractometry. Their data, which differed from that of the earlier literature in that they were so gathered that statistical treatment was possible, were consistent with the hypothesis that the red cell is a "perfect osmometer" if due allowance is made for Adair's evidence on the physicochemical properties of hemoglobin. The same interpretation fits the present data on the decrease in osmotic activity of whole blood, blood-plus-saline and blood-plus-sucrose solution. Thus it may be said that evidence from virtually all studies of the osmotic behavior of the red cell, when considered in the light of the known properties of hemoglobin, is consistent with the view that the red cell is in osmotic equilibrium with its surroundings over a wide range of concentrations.

Two observations about the method of conducting studies of the colligative properties of biological fluids have emerged from these experiments. First, examination of colligative properties of other types of cells will probably require a technique by which the cells may be effectively separated from the extracellular fluid prior to rupture of the cell membranes. It appears likely that the type of relationship which exists between the osmotic coefficient of hemoglobin and its concentration also applies to the proteins of other cells. Dick (*27*) comments on the probable importance of the changing osmotic coefficients of intracellular proteins in his study of the osmotic properties of fibroblasts.

Second, the problem of supercooling of fluids containing a high proportion of nonaqueous substances, which prevented accurate determination of the freezing point of hemolyzed red cells in this study, would likely be true of any other intracellular fluid. Others have reported a great variability among determinations of this type (4). Other ways of measuring colligative properties, such as the melting point technique used in this study or that described by Maffly and Leaf (*28*), would probably be a sounder approach.

SUMMARY

The osmotic behavior of the normal human red cell has been studied by two techniques which measure colligative properties. In all studies the number of samples was large enough to allow a statistical statement about the limits of confidence of the results.

The freezing point depressions of whole blood and serum from the same samples were found to be identical; this was also true when saline solutions of various concentrations were mixed with the whole blood. The freezing point depression of whole blood, whole blood-plus-saline or whole blood-plus-sucrose solutions, was found to be less

after hemolysis than before hemolysis. Such a difference was present over a wide range of osmotic concentrations and varied directly with the concentration in a highly predictable fashion. These data are consistent with Adair's observation (11) that the osmotic coefficient of hemoglobin varies in a curvilinear fashion with its concentration in solution. These findings are therefore compatible with the hypothesis that the red cell remains in osmotic equilibrium with its surrounding fluid. Most previous studies which bear on this point may be similarly interpreted.

Attempts to measure the freezing point depression of hemolyzed red cells alone were not satisfactory because of the problem of supercooling. Attempts to measure the relative vapor pressures of hemolyzed red cells and serum by the isopiestic technique were also not successful.

By use of a technique for measurement of melting points of microscopic samples, it proved possible to determine this property of hemolyzed red cells and the extracellular fluid to which they had been exposed before hemolysis. Over a range of concentrations from 210 to 520 milliosmoles per Kg. H_2O, the melting points of the intracellular and extracellular phases were found to be not significantly different.

It is concluded that the normal human red cell is in osmotic equilibrium with its surrounding fluid and that this equilibrium is maintained over a wide range of concentrations.

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